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UNIVERSITY OF ALBERTA

**STUDIES ON THE MYXOMA VIRUS
ANTI-INFLAMMATORY SERPIN, SERP-1**

BY

PIERS DAVID NASH



A Thesis

Submitted to the Faculty of Graduate Studies and Research
In partial fulfillment of the requirements for the degree of
Doctor of Philosophy

Department of Biochemistry

Edmonton, Alberta

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~~Piers Nash~~

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MAY 31, 1999

Date

The true method of knowledge is experiment.

- *William Blake* -

**That which we are, we are;
One equal temper of heroic hearts,
Made weak by time and fate, but strong in will
To strive, to seek, to find, and not to yield.**

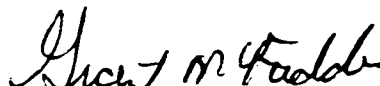
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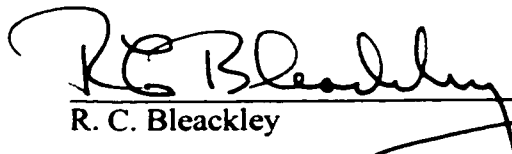
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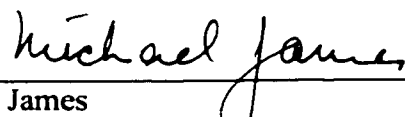
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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled *Studies on the Myxoma Virus Anti-Inflammatory Serpin, SERP-1* by Piers David Nash in partial fulfillment of the requirements for the degree of Doctor of Philosophy.


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ABSTRACT

SERP-1 is a secreted serpin encoded by myxoma virus, a poxvirus pathogen of rabbits. SERP-1 is required for myxoma virus virulence, and the purified protein has been shown to possess anti-inflammatory activity in animal models of restenosis and antigen-induced arthritis. SERP-1 forms SDS-stable complexes with uPA, tPA, plasmin, thrombin and factor Xa, and is exclusively cleaved by elastase and cathepsin G. Peptide sequencing confirmed Arg₃₁₉-Asn₃₂₀ to be the P1-P1' residues and replacing these with Ala-Ala abolished all inhibitory activity. Kinetic analysis of SERP-1 produced second-order rate constants that vary over 3 logs, from $k_{inh.} = 3 \times 10^5 \text{ M}^{-1}\text{s}^{-1}$ for reaction with thrombin to $\approx 600 \text{ M}^{-1}\text{s}^{-1}$ for C1s, and steady-state inhibition constants that vary over 4 logs, from $K_i = 10 \text{ pM}$ for thrombin to $\approx 100 \text{ nM}$ for C1s. Measured stoichiometries of inhibition ranged from 1.4 ± 0.1 for uPA to 13 ± 3 for thrombin. The observed variations in inhibition kinetics were analyzed to explore which steps in the reaction mechanism contribute to the inhibitory specificity of SERP-1. At serpin concentrations comparable to those of the target proteinase and to K_i , as appears likely for SERP-1 *in vivo*, its inhibitory specificity becomes less dominated by $k_{inh.}$ and increasingly dependent on the partitioning ratio within the branched kinetic mechanism and on the half-life of the inhibited complex.

Using the extensive set of data for the inhibition kinetics of SERP-1, we observed that a linear free energy relationship exists between the $\log k_{inh.}$ and $\log K_i$. This is

interpreted in terms of free energy relationships and provides thermodynamic evidence that the serpin-proteinase complex may be reversible.

Mutation of the SERP-1 reactive centre loop (RCL) allowed the construction of chimeras of variant RCLs within the SERP-1 scaffold. Analysis of these chimeras for the ability to interact with a panel of eight proteinases indicated that while the inhibitory profile could be altered, that the chimeras were universally poorer inhibitors than wild-type SERP-1.

Examination of the role of SERP-1 glycosylation showed the N-linked glycosylation of Asn₁₇₂ was essential for SERP-1 to avoid degradation and efficiently traffic from the ER of infected cells. Elimination of Asn₉₉ had a similar but less severe effect upon SERP-1 stability and secretion. Furthermore, SERP-1 is modified by the myxoma virus encoded MST3N sialyltransferase, and is the first reported viral protein to be modified by a virally encoded glycosyltransferase. Sialylation of SERP-1 by the MST3N gene product creates a distinct charged species of SERP-1, but this does not effect *in vitro* proteinase inhibition. This represents a detailed dissection of SERP-1 inhibitory properties and post-translational modifications that brings us closer to understanding how SERP-1 functions on a molecular level.

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LIST OF ABBREVIATIONS

| | |
|----------|--|
| 4-MU | 4-methylumbelliferone |
| BGMK | baby green monkey kidney epithelial cell line |
| bp | base pair |
| BUdR | Bromodeoxyuridine |
| CAPS | 3-cyclohexylamino-1-propanesulfonic acid |
| CrmA,B,C | cytokine response modifier A, B, or C |
| DMEM | Dulbeccos modified Eagles medium |
| DNA | deoxyribonucleic acid |
| E. coli | Escherichia coli |
| EDTA | ethylene diamine tetraacetic acid |
| EGF | epidermal growth factor |
| ER | endoplasmic reticulum |
| FBS | fetal bovine serum |
| FPLC | fast protein liquid chromatography |
| hNE | human neutrophil elastase |
| HPLC | high pressure liquid chromatography |
| ICE | interleukin 1- β converting enzyme |
| IFN | interferon |
| IL- | interleukin |
| Kb | kilobases |
| mAb | monoclonal antibody |
| MGF | myxoma growth factor |
| MHC | major histocompatibility complex |
| moi | multiplicity of infection |
| MUGB | 4-methylumbelliferyl <i>p</i> -guanidinobenzoate |
| NCS | newborn calf serum |
| NK | natural killer |
| ORF | open reading frame |

| | |
|------------------|--|
| p.i. | post-infection |
| PBS | phosphate buffered saline |
| PCR | polymerase chain reaction |
| pfu | plaque forming units |
| RCL | reactive centre loop |
| RNA | ribonucleic acid |
| RPV | rabbitpox virus |
| SDS | sodium dodecyl sulfate |
| PAGE | polyacrylamide gel electrophoresis |
| Serpin | serine proteinase inhibitor (refers to proteins belonging to this family) |
| SFV | Shope fibroma virus |
| SI | stoichiometry of inhibition |
| Spi-1,2,3 | serpins 1,2,3 (orthopoxvirus encoded) |
| TBS | Tris buffered saline |
| TIR | terminal inverted repeats |
| TK | thymidine kinase |
| TNF | tumour necrosis factor |
| tPA | tissue-type plasminogen activator |
| uPA | urokinase-type plasminogen activator |
| X-gal | 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside |

CHAPTER I

GENERAL INTRODUCTION¹

There is a single light of science, and to brighten it anywhere is to brighten it everywhere.

-Isaac Asimov

¹ Portions of the material contained herein has been published: Nash, P., Barrett, J., Cao, J., Hota-Mitchell, S., Lalani, A., Everett, H., Xu, X., Robichaud, J., Hnatiuk, S., Ainslie, C., Seet, B., and McFadden, G. (1999) *Immunological Reviews* 168: 103-120; Nash, P. D., Lucas, A., and McFadden, G. (1997) in "The SERPINS", Plenum Press, New York.

Immunomodulation by viruses

For countless eons, viruses have engaged and co-evolved with their vertebrate hosts. This dynamic interplay of pathogen and host has been an essential determinant for the development of the complex vertebrate immune system. It has also resulted in the natural adaptation of specific viral strategies to evade or subvert aspects of the immune and inflammatory system. So while the conventional view is that viruses are simply the infectious and destructive causative agents of disease, our laboratory studies viruses as tools to assist in the unraveling of secrets of the immune system (Barry and McFadden, 1998; Ploegh, 1998). When confronted with a viral challenge, vertebrate hosts deploy varied and diverse aspects of a complex immune system in a multi-pronged attack aimed at eliminating the virus. Against this extensive array of immune modalities, viruses have successfully co-evolved distinct strategies to thwart these host immune responses. The success of a virus depends largely on its ability either to evade or actively to dismantle those portions of the host immune repertoire that are functionally antagonistic to viral replication. Studying these very strategies that a successful viral pathogen uses to affect its survival allows us to learn more not only about the virus but also about the host's immune system itself.

The specific strategies used by particular viruses vary dramatically according to their encoding capacity (Ploegh, 1998). Viruses with small genomes often ensure their survival by exploiting weaknesses or gaps in the host immune repertoire to avoid detection or replicate very rapidly, effectively out-pacing the immune

response. The larger DNA viruses are of particular interest because they use their larger genetic encoding capacity to produce a range of viral proteins aimed at disabling the host immune response (Barry and McFadden, 1998; Gooding, 1992; Moss, 1996; Smith, 1994; Spriggs, 1996). Poxviruses are among the largest eukaryotic DNA viruses and have the unusual capacity to replicate autonomously in the cytoplasm of infected cells (Fenner et al., 1989; Moss, 1996). Many poxvirus proteins have been defined as virulence factors on the basis that, when present, they confer increased pathogenicity and improve viral replication within immunocompetent hosts. When the genes that encode these virulence factor proteins are deleted, the resulting virus strain generally exhibits an attenuated or altered disease phenotype (Buller and Palumbo, 1991; McFadden, 1995; Smith, 1993; Turner and Moyer, 1990). Our work has focused on myxoma virus, a poxvirus that exclusively infects rabbits, and how this virus interacts with the host immune system.

Viral immunomodulatory proteins can be grouped into several categories based on their targets and mechanisms of action. Two general mechanistic classes can be defined based on their sites of activity. Extracellular “viromimetics” are either secreted or surface bound virus encoded proteins which actively disrupt the host immune response by mimicry of host immune factors, while intracellular “virostealth” strategies prevent the infected cell from being recognized by the immune system or from entering into programmed cell death as a result of virus infection. These broad classes can be further subdivided based on the mode of action of the viral proteins involved (Figure I-1). Viromimetics can be divided into

viroceptors and virokines, while virostealth proteins can be divided into inhibitors of apoptosis and downregulation mediators of surface receptors (McFadden, 1995). *Viroceptors* are secreted or cell surface viral glycoproteins homologous to cellular receptors that generally act by competitively binding cytokine ligands thereby short-circuiting an immune or inflammatory signal. *Virokines* refer to secreted viral proteins that mimic host cytokines or inhibitors in order to promote growth, to act as inhibitors of the inflammatory cascades, or to favor an immune response that is beneficial to the virus. *Viromitigators* are intracellular viral proteins aimed at disrupting specific signal transduction cascades. The most recently studied group of these are the viral proteins involved in mitigating apoptosis of infected cells. *Virostealth* refers to the general strategy by which the virus seeks to hide its presence from the immune system by specifically downregulating specific cell surface markers such as class I MHC molecules that act as the window for the immune system to detect and target virus infected cells.

The origin of the various viral immunomodulatory proteins is not always obvious. While some are clearly derived from ancestral cellular homologs that were hijacked from the infected host during the process of co-evolution, others have no known mammalian homolog and are to date found only in viruses.

In this decade, a paradigm shift has occurred in our thinking of viruses as merely vehicles of plague and pestilence to regarding them as investigational tools for studying the innermost workings of the immune system. Thus, viruses which have co-evolved with animal hosts for countless millions of years have much to teach us

and have great potential as tools for understanding and potentially manipulating the immune system for therapeutic applications (Barinaga, 1992).

Myxoma virus and myxomatosis

Myxoma virus is a poxvirus pathogen of rabbits that was first identified as the causative agent of myxomatosis, a lethal disease of European rabbits (*Oryctolagus cuniculus*) first described at the end of the 19th century (Fenner and Meyers, 1978; Fenner and Ratcliffe, 1965). Myxomatosis is transmitted by arthropod vectors, particularly mosquitoes, and is so remarkably lethal to European rabbits that less than 1% of susceptible animals survive infection. The disease of myxomatosis is characterized by extensive fulminating internal and external lesions and severe immunosuppression accompanied by supervening Gram-negative bacterial infections of the respiratory tract (DiGiacomo and Mare, 1994; Fenner and Ratcliffe, 1965; Strayer, 1989). Myxoma virus evolved in populations of the *Silvalagus* species of North and South American rabbit. In these indigenous hosts, myxoma virus causes a persistent infection with minimal cellular immune recognition and only minor symptoms. It appears that the virus and its natural host rabbits (*Silvalagus bachmani* and *Silvalagus brasiliensis*) have evolved a benign and relatively non-pathogenic relationship. When myxoma virus made the species leap into the related but distinct European rabbit the infection suddenly manifested as the pathogenic syndrome referred to as myxomatosis. This drastic change in pathogenic phenotype

between two related host groups emphasizes the unpredictable behavior of viruses that cross host-species boundaries.

The profound virulence of myxoma virus led to its exploitation in the early 1950's as a means of biological population control. The virus was introduced into Southern Australia in an attempt to reduce the feral rabbit population that had overrun large areas of the Australian countryside (Fenner and Ratcliffe, 1965). Although there was initially a tremendous reduction in rabbit populations this strategy of biological warfare did not prove to be a long-term solution. Not only did resistant rabbits soon emerge and repopulate the infected areas, but the dominant field strains of the virus became progressively attenuated (Fenner and Meyers, 1978; Fenner and Ratcliffe, 1965; O'Neill, 1994).

The nature of the molecular mechanisms by which myxoma virus is able to exert such profound effects on the immune system of the European rabbit has been the subject of intensive experimental scrutiny over the past decade. A number of the virulence genes encoded by myxoma virus have been identified and studied at the molecular level (see Figure I-2). Among these are genes that express proteins which interact with known components of the host's immune system or interfere with intracellular signal transduction cascades.

Viroceptors: windows on the immune system

The phenomenon of molecular piracy of cytokine receptors by poxviruses has been extensively reviewed (Alcami and Smith, 1995; McFadden, 1995; Pickup,

1994). The term *viroceptor* was originally coined to describe secreted soluble virus-encoded cytokine receptors that function by binding host cytokines, thereby preventing subsequent interactions with their true cellular receptors (Upton et al., 1991). This definition has been expanded to include the growing number of virus encoded transmembrane proteins with significant homology to cellular cytokine receptors. Examples of viroceptors are found throughout the large DNA viruses, but most especially in members of the poxvirus family (Table I-1). Three viroceptors have been identified from myxoma virus, among which are the soluble TNF receptor (M-T2), the high affinity soluble chemokine receptor (M-T1), and the prototypic viral IFN- γ receptor (M-T7).

M-T2: A TNF receptor homolog

Although viroceptors now constitute a large and growing family of virus encoded immunomodulatory proteins (McFadden, 1995), the first members were identified less than a decade ago. The first viroceptor to be identified was the S-T2 gene from Shope fibroma virus (SFV) (Upton et al., 1987). Based on sequence analysis, S-T2 was recognized in 1990 as a member of the tumor necrosis factor receptor (TNF-R) superfamily (Smith et al., 1990). This receptor family is characterized by the presence of multiple N-terminal cysteine-rich repeat domains (CRDs) that mediate interaction with the TNF family of ligands. TNF was initially identified because of its anti-tumor activity, however this molecule has also been shown to have anti-viral activities that result in cytolysis of infected cells, induction

of apoptosis and inhibition of viral replication (Wong et al., 1992). TNF-R homologs have been identified in a variety of poxviruses, including cowpox virus and variola, but exist only as non-functional copies in vaccinia virus (Howard et al., 1991; Hu et al., 1994; Shchelkunov et al., 1993). The myxoma virus-encoded homolog (M-T2) occurs in two copies and was so named because it is the second open reading frame (ORF) from the terminal ends of the genome (Figure I-2) (Upton et al., 1991).

Detailed sequence characterization of M-T2 indicated extensive sequence identity and similarity with the ligand binding domains of the TNF-Rs and established the presence of a classic signal sequence and four cysteine rich domains (CRDs) towards the N-terminus (Smith et al., 1990; Upton et al., 1991). The CRDs are conserved protein folding domains that define membership to the TNF-R superfamily. A single CRD is roughly 40 amino acids in length and contains a series of conserved cysteines that form unique sets of disulfide bonds that define the folded tertiary structure of the receptors. The presence of a signal sequence and lack of a transmembrane domain in any of the T2 family members suggested that they are secreted viroceptors, capable of binding and inhibiting TNF (Smith et al., 1990). In this way the virus is thought to interact with the TNF ligands prior to engagement with surface receptors that would normally trigger an anti-viral response.

M-T2 has been shown to bind and inhibit rabbit TNF in a species specific fashion (Schreiber and McFadden, 1994). The overexpressed M-T2 protein was tested for the ability to inhibit cytolysis of TNF sensitive L929-8 cells. M-T2 was able to inhibit rabbit TNF mediated lysis cells but could not protect the cells from

murine or human TNF lysis. Scatchard analysis of M-T2 binding to the rabbit TNF ligand demonstrated sufficiently high affinity binding ($K_d=170-295$ pM) such that it is predicted to effectively compete with soluble mammalian cellular TNF receptors (Schreiber et al., 1996). Binding affinity of M-T2 to murine TNF was 10 fold lower ($K_d=1.7$ nM) and could not inhibit the cytolytic activity of TNF on mouse cells (Schreiber et al., 1996). No binding at all was observed with the human TNF. Purified M-T2 is secreted in both the monomeric and dimeric forms, and although both forms bind with comparable affinities, the M-T2 dimer is a far more potent inhibitor of TNF cytolytic activity than is the M-T2 monomer (Schreiber et al., 1996). This is probably a reflection of the ability of the dimer to more effectively prevent the oligomerization of TNF receptors by the TNF trimer, which is required for signalling. In order to delineate the region of M-T2 necessary for binding of rabbit TNF, a series of deletion mutants were constructed and expressed using a recombinant vaccinia virus system. All mutations within the unique carboxy terminus resulted in poor protein secretion suggesting that the C-terminus of M-T2 plays a role in the intracellular trafficking or correct folding of the protein (Schreiber and McFadden, 1996). Nevertheless, the C-terminus deletions did not affect the ability of M-T2 to bind or inhibit TNF. In contrast, deletions within the N-terminus that removed any one of the first three CRDs resulted in the inability of the mutant to bind to, or inhibit, rabbit TNF. This confirmed the prediction that the CRDs are essential for TNF binding (Schreiber et al., 1997).

The N-terminal region of M-T2 was also shown to inhibit apoptosis of myxoma infected lymphocytes (Schreiber et al., 1997). This intracellular anti-apoptotic feature is distinct from extracellular TNF inhibition and demonstrated that M-T2 has at least two distinct anti-viral functions. Thus far, this is a feature unique to M-T2 and has not been demonstrated for any other viral TNF-Rs. This additional feature of M-T2 is described below in greater detail later.

M-T1: A soluble scavenger of CC-chemokines

Chemokines are small pro-inflammatory proteins that act in the host defense against virus infection (Baggiolini, 1998; Luster, 1998). The β - or CC-subfamily of chemokines are important in activating and mobilizing monocytes/macrophages, T-lymphocytes, natural killer cells and dendritic cells to sites of infection, inflammation or tissue damage. Not surprisingly, certain large DNA viruses have evolved strategies for neutralizing the biological activity of chemokines. Myxoma virus encodes a secreted glycoprotein, M-T1, that binds CC-chemokines with high affinity, preventing them from engaging their cellular receptors (Graham et al., 1997; Lalani et al., 1998).

M-T1 is the gene product of the open reading frame found at the end of the terminal inverted repeats of the myxoma virus genome (Fig. I-2) (Graham et al., 1997). M-T1 is expressed as an early gene product and is secreted as early as two to four hours post-infection. The protein is relatively acidic (predicted *pI* ~4.5) and is post-translationally modified with N-linked oligosaccharides (Graham et al., 1997).

M-T1 belongs to a larger family of related 35-40 kDa secreted poxvirus proteins which are collectively known as the T1/35kDa family of poxvirus CC-chemokine inhibitors (Alcami et al., 1998; Graham et al., 1997; Smith et al., 1997). The vaccinia virus (VV) and rabbitpox virus (RPV) homologs of M-T1 are known as the "major secreted 35kDa" proteins (Martinez-Pomares et al., 1995; Patel et al., 1990). Members of this family have variable sequence similarity to one another but all exhibit the strict conservation of eight cysteine residues (Alcami et al., 1998; Graham et al., 1997; Smith et al., 1997). Interestingly, members of the T1/35kDa family do not share notable sequence identity to host chemokine receptors or any other known cellular proteins.

In vitro, T1/35kDa proteins have been shown to bind to CC-chemokines in a non-species specific fashion (Alcami et al., 1998; Lalani et al., 1998; Smith et al., 1997). The high-affinity interactions between T1/35kDa and CC-chemokines effectively prevent these ligands from binding their cognate CC-chemokine receptors (Alcami et al., 1998; Smith et al., 1997). Purified M-T1 or RPV 35kDa have been shown to block the CC-chemokines, MIP-1 α and MCP-1, from eliciting intracellular calcium signalling in target monocytes with an inhibition constant (K_i) ranging from ~ 0.1 -1 nM (Lalani et al., 1998). Moreover, M-T1 potently abrogates CC-chemokine-mediated migration of human monocytes *in vitro* (Lalani et al., 1998). T1/35kDa block neither CXC-chemokine-receptor binding nor the chemotactic activities of CXC-chemokines, suggesting that the T1/35kDa viral proteins are

specific inhibitors for the CC-subfamily of chemokines (Alcami et al., 1998; Lalani et al., 1998; Smith et al., 1997)

In vivo, M-T1 retards chemotaxis of inflammatory leukocytes into virus-infected tissues. European rabbits infected with a recombinant myxoma virus containing a targeted disruption of the M-T1 gene display an increase in infiltrating monocytes/macrophages into the tissue lesions surrounding the primary infection site during the early stages of infection compared to wild type myxoma virus-infected lesions (Lalani et al., 1999). Despite this, deletion of M-T1 from myxoma virus or the 35kDa gene from rabbitpox virus has no effect on the mortality of their infected hosts (Lalani et al., 1999; Martinez-Pomares et al., 1995). Thus, although M-T1 functions to block the influx of infiltrating leukocytes into tissues during myxoma virus infection (see Figure I-3), deletion of M-T1 alone has no effect in ameliorating pathogenesis in infected rabbits.

The M-T1 glycoprotein shares only ~40% amino acid identity to its vaccinia and rabbitpox virus 35kDa counterparts. Despite this, their functional properties *in vitro* appear to be equivalent (Lalani et al., 1998). This has been taken to suggest that the CC-chemokine binding and inhibitory properties of the T1/35kDa family members may reside within limited regions of conservation or that these viral proteins adopt a unique, yet to be defined structural conformation (Lalani and McFadden, 1997). Recent work using nested deletions of the M-T1 gene suggest that disruption of any of the conserved cysteines abrogates CC-chemokine binding (Robichaud, 1999). Since even very small disruptions in M-T1 protein structure

cause loss of function, it appears that it is the overall protein fold, rather than limited areas of conservation that are important for M-T1 function.

M-T7: IFN- γ Receptor Homolog

One of the best characterized anti-viral host responses to virus infection is mediated by the interferons (IFNs). IFN receptor triggering leads to activation of the JAK/STAT signal transduction pathway which mediates upregulation of many genes involved in establishing, among other things, an anti-viral state. IFN- γ is a lymphocyte-derived cytokine known to induce the expression of many such genes. Although originally identified as a soluble mediator with direct antiviral activity, IFN- γ regulates many diverse aspects of immune responses including bactericidal activity of phagocytes, stimulation of antigen presentation through class I and class II major histocompatibility complex (MHC) molecules, cell proliferation and apoptosis (Boehm et al., 1997). Myxoma virus, among its array of immunomodulators, produces an IFN γ -receptor homolog to subvert the cellular responses to IFN- γ (Upton et al., 1992).

M-T7 is a 37 kilodalton (kDa) N-linked glycoprotein that is the major secreted protein produced during myxoma virus infection (Mossman et al., 1995; Upton et al., 1992). This protein was first identified as a soluble IFN- γ receptor homolog, sharing significant sequence similarity in its N-terminal region to the ligand-binding domain of mammalian IFN- γ receptors (Upton et al., 1992). Viral homologs of the IFN- γ receptor have been identified in other poxviruses including variola

(Massung et al., 1994), ectromelia virus (Mossman et al., 1995a), vaccinia, cowpox and camelpox viruses (Alcami and Smith, 1995). However, the major biological difference amongst these homologs has been the unique species specificity of M-T7 (Mossman et al., 1995). M-T7 binds to and nullifies the anti-viral effects of rabbit IFN- γ but cannot bind or block human or murine IFN- γ (Mossman et al., 1995). In contrast, the vaccinia encoded IFN- γ receptor is less restricted in its ability to bind IFN- γ from different species (Alcami and Smith, 1995; McFadden et al., 1998; Mossman et al., 1995a).

M-T7 is capable of high affinity binding to rabbit IFN- γ ($K_d=1.2$ nM) and has been shown to inhibit the binding of IFN- γ to its cellular receptor thereby abolishing all of its anti-viral activities (Mossman et al., 1995). M-T7 is the most abundantly secreted myxoma protein ($>5 \times 10^7$ molecules/cell/hour), is synthesized at early times post-infection and is stable in the supernatants of infected cells until late times post-infection (Mossman et al., 1995). Rabbits infected with a recombinant M-T7-knockout myxoma virus can effectively recover from infection whereas rabbits infected with wild type myxoma virus exhibit greater than 99% mortality (Mossman et al., 1996). Purified M-T7 was reported by chemical cross-linking assays to interact with members of the CXC, CC and C chemokine subfamilies (Lalani et al., 1997), though the physiological significance of this has yet to be elucidated. Thus, M-T7 represents a major virulence factor during myxoma virus infection.

Virokines

The second strategy of immune evasion by viruses is the capture and expression of host cytokines and growth factors. This strategy is employed by a number of viruses, all of which are restricted to the herpesvirus and poxvirus families (McFadden et al., 1998). The piracy of host cytokines has given rise to a class of molecules termed *virokines* (Table I-2) (Kotwal and Moss, 1988). Over time, the definition of virokine has expanded to encompass cytokine analogs and agonists, growth factors, and inhibitors such as the secreted serine proteinase inhibitors of the poxvirus family (McFadden, 1995). While the expression of these virokines has been shown to be important for virus survival in immunocompetent hosts, the rationale for this particular strategy remains imperfectly understood. Two members of this family have been identified from the study of myxoma virus: the myxoma growth factor (MGF), and SERP-1, the only known virus-encoded serine proteinase inhibitor that is secreted from infected cells (Nash et al., 1997; Upton et al., 1990).

Myxoma Growth Factor

Myxoma growth factor is an 85-residue polypeptide, which shares 80% identity with the Shope fibroma virus epidermal growth factor (EGF) homolog, SFGF (Chang et al., 1987; Upton et al., 1987). All poxviral EGF homologs, including vaccinia growth factor (VGF), MGF and SFGF, are predicted to have similar secondary and tertiary structure as their cellular counter parts, as indicated by the

conserved six cysteine residues that form three intramolecular disulfide bonds (for a review, see (McFadden et al., 1995)).

Both MGF and SFGF are expressed as early gene products (Chang et al., 1990). The virus genes are present as a single copy near the TIR at the left end of the virus genome (Chang et al., 1987; Upton et al., 1987). Immunoprecipitation of the SFV infected cell lysate, in the presence and absence of tunicamycin, demonstrated that the SFGV is heavily glycosylated (Chang et al., 1990). It is predicted that the MGF would be post-translationally modified in the same way. Unlike VGF and cellular EGF and TGF α , both MGF and SFGF lack the hydrophobic sequence near their C termini, which has been shown to be a membrane anchor sequence. VGF is secreted as a truncated form from the extracellular portion of larger membrane-bound glycosylated protein precursors (Chang et al., 1988; Stroobant et al., 1985), but the precise processing pathway of SFGF or MGF remains to be defined.

EGF activates tyrosine kinase on erbB receptors (EGF receptor) via auto-phosphorylation, and initiates mitogenesis in the responsive cells. Although native MGF and SFGF are heavily glycosylated proteins, it seems that the glycosylation is not essential for the protein's biochemical function. Non-glycosylated synthetic peptides spanning residues 30 to 83 of MGF and 26 to 80 of SFGF have been shown to have the receptor binding activity (Lin et al., 1988; Lin et al., 1991). MGF is non-essential for myxoma virus replication in tissue cultures, but when tested *in vivo*, the myxoma virus MGF deletion mutant demonstrated a significant degree of attenuated clinical manifestations in European rabbits in comparison to the wild type parental

strain (Opgenorth et al., 1992; Opgenorth et al., 1993). The histopathological examination showed that the MGF deletion mutant virus caused substantially less proliferation of the epithelial cell layers overlying myxoma lesions in the conjunctiva and respiratory tract (Opgenorth et al., 1992). More interestingly, replacing the MGF gene with SFGF, VGF or TGF α could fully restore the virus virulence. The disease course produced by the restored myxoma MGF deletion mutant virus was indistinguishable at both clinical and histopathological level (Opgenorth et al., 1993). Thus, poxvirus EGF homologs (VGF, MGF, and SFGF) have interchangeable biological functions with the cellular counterparts. Transgenic mice expressing MGF showed proliferation and arborization of breast ducts and ductules, and also gastric epithelial hyperplasia (Strayer et al., 1993). Therefore, based on above evidence, MGF possesses genuine EGF function, i.e. stimulating mitogenesis, proliferation and differentiation of responsive cells.

Several hypotheses have been proposed regarding the biological significance of poxvirus encoded EGF homologs (for a review, see (McFadden et al., 1995)). Based on the evidence outlined above, the poxvirus encoded EGF homologs appear to stimulate biosynthesis in both infected and uninfected (those adjacent to the infected cells) cells and in doing so, provide a more favorable environment for the virus replication. Interestingly, MGF binds to the erbB-family of receptors with lower affinity than the cellular ligands, but is a more potent signalling ligand because it does not induce receptor downregulation (Tzahar et al., 1998).

Viromitigators: Multiple apoptosis inhibitors

In addition to the combined actions of the host immune system, invading viruses must deal with the innate ability of certain somatic cells to undergo apoptosis or programmed cell death in response to virus infection. While apoptosis can be used by some viruses to promote virus dissemination and secondary infection of phagocytic cells, apoptosis can also be highly detrimental if it occurs prior to virus maturation and disrupts the viral replicative strategy. Given the critical importance of preventing premature apoptosis of infected cells, it is not surprising that viruses have adapted a wide range of strategies to inhibit apoptosis (McFadden and Barry, 1998; O'Brien, 1998). Figure I-4 illustrates the Fas/TNF apoptosis pathway with several of the known poxvirus inhibitors indicated. Myxoma virus encodes at least five proteins that have been implicated in the inhibition of apoptosis (see Table I-3). These include the caspase inhibitor SERP-2, the intracellular membrane associated protein M11L, the ER resident soluble M-T4 protein, the cytoplasmic ankyrin repeat protein M-T5, as well as the TNF receptor homolog M-T2.

Caspase Inhibition by SERP-2

The mechanisms by which individual viromitigators abrogate apoptosis in response to viral infection is not always clear. This is because the viral proteins are not yet extensively characterized, and because of the complex and varied nature of the cellular pathways leading to apoptosis. Among the key components of the apoptotic machinery are a class of proteinases known as the caspases (Nicholson and

Thornberry, 1997; Thornberry and Lazebnik, 1998). Not surprisingly, viruses utilize specific inhibitors of caspases as one strategy to block apoptosis of infected cells. Myxoma virus encodes a caspase inhibitor known as SERP-2, that is a second member of the serpin superfamily identified from the myxoma virus genome (Petit et al., 1996). SERP-2 is closely related to two previously identified poxvirus serpins, namely crmA of cowpox virus and Spi-2 of vaccinia virus (Pickup et al., 1986; Smith et al., 1989). Although crmA/Spi-2 and SERP-2 conform to the serpin homology, they are, in fact, cross-class proteinase inhibitors capable of inhibiting the caspase family of intracellular cysteine proteinases. CrmA targets caspase 1 (IL-1 β converting enzyme or ICE) (Ray et al., 1992), caspase 8 (FLICE) (Srinivasula et al., 1996) and the serine proteinase granzyme B (Quan et al., 1995) and thus is implicated in both inflammation and apoptosis (Enari et al., 1995; Gagliardini et al., 1994; Miura et al., 1993; Ray et al., 1992; Tewari and Dixit, 1995; Tewari et al., 1995).

SERP-2 is a 333 amino acid product of an early gene and can be detected as early as 2h after infection and remains present at late times (>15h) (Petit et al., 1996). The SERP-2 protein can be detected in monomeric and dimeric forms and remains intracellular throughout the infection cycle. Although its reactive center is at present undefined, SERP-2 has been shown to inhibit human ICE/caspase 1 *in vitro* and prevent the processing of pro-IL-1 β to its mature form (Petit et al., 1996). The inhibitory activity of SERP-2 on ICE/caspase 1 is reportedly less than that of crmA (Turner and Moyer, 1998). It is unclear whether this is the result of species

specificity incurred by the use of a human enzyme to test the inhibitory activity or whether this indicates that caspase 1/ICE is not the primary target of SERP-2 *in vivo*.

The disease symptoms of myxomatosis elicited by the SERP-2 deletion mutant are markedly less severe than those of the wild type virus. Notably, the targeted disruption of SERP-2 results in milder disease symptoms, minor secondary lesions and considerably lower mortality (Messud-Petit et al., 1998). Histological analysis of lesions indicated an accelerated inflammatory response as would be expected if the virus failed to inhibit IL-1 β processing in infected macrophages. Furthermore, cells within the draining lymph nodes of rabbits infected with the SERP-2 knock-out virus showed elevated levels of apoptosis, indicating that the SERP-2 deletion mutant fails to interrupt the apoptotic response triggered by infection of immune cells. This may contribute to the observation that this virus has a less severe disease phenotype than the parental virus (Messud-Petit et al., 1998).

M11L and Immune Modulation

The M11L gene encodes another determinant of myxoma virus virulence. Disruption of the M11L coding sequence in the viral genome considerably attenuates the virus (Oppenorth et al., 1992). Infection of rabbits with the M11L-knockout virus failed to result in mortality, indicating a disease process that was clearly attenuated compared to the normal outcome of myxomatosis. In addition, the primary lesions resulting from infections by the M11L mutant displayed decreased

levels of cellular proliferation and hemorrhage, but increased evidence of edema and leukocyte infiltration when compared to the lesions produced by myxoma virus. Interestingly, despite its attenuated disease phenotype, the deletion mutant did result in numerous secondary lesions and the recovered animals were resistant to subsequent challenge with wild type myxoma virus (Opgenorth et al., 1992). Overall, this suggests that the host is able to mount a more effective immune response to myxoma virus when M11L is not expressed.

Characterization of the M11L sequence (Graham et al., 1992) has revealed no database homologs, apart from genes encoded by the related poxviruses, Shope fibroma virus and swinepox virus. The M11L gene product is 166 amino acid in length and has no distinct structural motifs, apart from an 18 residue domain near the C-terminus with distinct hydrophobic character.

Infection of a rabbit T-lymphocyte cell line (RL-5) with the M11L knock-out virus indicated that M11L plays a role in preventing apoptosis of infected cells (Macen et al., 1996). Unlike parental myxoma virus, infection by the M11L deletion mutant triggered apoptosis with extensive DNA fragmentation observed within 6 hours post-infection, implying that M11L is required during infection of immune cells to prevent initiation of an apoptotic response. These findings correlate with an earlier observation that replication of the M11L deletion mutant in primary rabbit splenocytes is impaired (Opgenorth et al., 1992).

M-T2 is an intracellular apoptotic inhibitor

M-T2 was initially identified as a viral TNF receptor homolog (see above) but it has also been shown to be involved in blocking programmed cell death. In contrast to the wild type myxoma virus, the M-T2 knockout virus induced virus infected RL5 cells to undergo apoptosis (Macen et al., 1996). In rabbit peripheral blood mononuclear cells, M-T2-knockout virus infection specifically caused apoptosis of non-adherent leukocytes but not adherent monocytes. This result is consistent with the earlier observation that both wild type and M-T2-knockout virus can efficiently replicate in rabbit fibroblasts while the latter is unable to productively infect T lymphocytes (Upton et al., 1991).

To define its anti-apoptotic role, purified M-T2 protein was tested for the ability to prevent the apoptosis of M-T2-knockout virus infected RL5 cells. Interestingly, the exogenous M-T2 failed to prevent the infected cells from undergoing apoptosis, although M-T2 efficiently blocked extracellular TNF induced cytolysis of sensitive cells (Macen et al., 1996) (Macen et al., 1996; Schreiber and McFadden, 1994; Schreiber et al., 1996). This suggests that M-T2 might not function extracellularly to block apoptosis. Several lines of evidence now support the notion that M-T2 is poorly secreted, and a fraction of the protein accumulates in an intracellular compartment (Schreiber et al., 1997). In order to characterize its anti-apoptotic effects, a series of C-terminal truncated M-T2 myxoma viruses were tested in the apoptosis assay. Surprisingly, the anti-apoptotic activity was observed in M-T2 constructs that contained only the first two CRDs, despite the previous finding

that the three N-terminal CRDs are required for TNF binding and inhibition (Schreiber and McFadden, 1996; Schreiber et al., 1997). It thus appears that M-T2 possesses dual function, and that its anti-apoptotic role differs from its TNF binding function in terms of both localization and distinct functional domains of the protein.

Currently, two models can be suggested to account for the possible mechanism(s) M-T2 uses to inhibit apoptosis. First, on the basis of structure of human TNF-receptors and the similarity of TNF-receptor to M-T2, it is feasible that M-T2 could associate with a cellular pro-apoptotic superfamily member to form a dominant-negative receptor complex which could then block otherwise normal cellular receptor oligomerization and the subsequent signalling (Figure I-5). Second, the presence of functional intracellular M-T2 suggests that M-T2 could interact with downstream signalling molecules and therefore block a relevant apoptotic pathway. Further work on the localization of M-T2 and the interaction of M-T2 with other cellular molecules are required to clarify its anti-apoptotic role.

M-T5: An ankyrin repeat protein

The myxoma M-T5 gene maps in the TIRs (Figure I-2), and the absence of easily identifiable signal sequences suggests it to be located in the cytoplasmic compartment. The M-T5 483 amino acid polypeptide is predicted to have a molecular weight of 55 kDa.

There are very few characteristic features detected upon analysis of the primary protein sequence of M-T5. The protein does not appear to have any

significant regions of hydrophobicity but possesses three distinct ankyrin repeat domains (ARDs). The ankyrin protein from red blood cells contains 23 such ARDs, which are proposed to be involved in protein folding and/or protein-protein interactions (Lambert et al., 1990). From the erythrocyte ankyrin domains, a very loose consensus of 33 amino acids has been defined. This ankyrin consensus and the three ARDs of M-T5 are shown in Figure I-6.

A myxoma virus has been constructed in which both copies of the M-T5 open reading frame have been interrupted, generating an M-T5 knockout virus (Mossman et al., 1996). Studies revealed that the lack of M-T5 gene product in rabbit T lymphocytes resulted in rapid cessation of host and virus protein synthesis and subsequent apoptotic death of these cells (Mossman et al., 1996). This phenomenon was not observed in infected rabbit fibroblast cells. When the M-T5-knockout virus was used to infect susceptible European rabbits, all recovered completely from infection, contrasting with the 100% mortality in the control group of wild type myxoma infected rabbits (Mossman et al., 1996). In addition, the M-T5 minus infected rabbits demonstrated an almost complete absence of secondary lesion formation and no secondary Gram-negative bacterial infection, all of which were detected in all wild type infected animals (Mossman et al., 1996). Histological analysis revealed that the primary site of inoculation of the M-T5 knockout virus in the rabbits showed very little edema and no hemorrhage, contrary to the edematous and hemorrhagic lesions observed in wild type infected rabbits (Mossman et al.,

1996). Thus, these cell culture and pathogenesis studies demonstrated that M-T5 is a potent virulence factor responsible for host range determination.

The extreme virulence attributed to M-T5 through the pathogenesis studies and the ability of M-T5 to inhibit apoptosis are elements shared with two other myxoma virus proteins described in this section, M-T4 and M11L. Where M-T5 is different from these two other virulence factors is in its cytoplasmic location and ARDs. ARDs have been found in a large number of proteins having very diverse functions, from nematodes to humans. For example, the human 2-5A dependent RNase L is an IFN-inducible ribonuclease containing ankyrin repeats; this enzyme is responsible for implementing the anti-viral response in the 2-5A pathway of interferon activity (Dedhar and Hannigan, 1996).

The I κ B family of proteins also contains ARDs. The I κ B proteins are responsible for controlling the levels of NF- κ B, a nuclear transcription factor which activates transcription of many inflammatory cytokines and growth factors (Baldwin, 1996). A recent study on one of the cellular I κ B proteins demonstrated that mutation of certain key residues in the second ARD resulted in a loss of the ability to localize to the nucleus where it normally binds NF- κ B to cause re-localization back out to the cytoplasm (Sachdev et al., 1998). It is noteworthy to mention that the key residues in the proposed nuclear localization signal of I κ B are fairly conserved in M-T5 (see Figure I-6), thus suggesting that such a nuclear localization signal may also exist in M-T5. A further line of evidence supports the importance of ARD-containing elements such as I κ B to viral immune evasion

strategies. A homolog of I κ B containing ARDs has been recently described in African swine fever virus. This protein, designated A238L, was shown to bind and inhibit NF- κ B function in cultured mammalian cells (Revilla et al., 1997) including macrophages, where the mechanism of inhibition was determined to be via binding to calcineurin (Miskin et al., 1998).

M-T5 may be a myxoma virus homolog of one of the above ankyrin domain containing molecules. ARD-containing host-range genes like M-T5 have been found in other orthopoxviruses. Two such proteins that show limited amino acid similarities to M-T5 are the cowpox virus CP77 and the vaccinia virus K1L (Mossman et al., 1996). Like M-T5, both CP77 and K1L are responsible for preventing rapid global cell protein shut-off early after infection. Currently, not much is known about the actual mechanism of action of these ARD-containing gene products.

M-T4: The ER Connection

In addition to cytoplasmic proteins aimed at blocking discrete signal transduction pathways, myxoma virus encodes a novel endoplasmic reticulum (ER)-retained protein, M-T4, that has been as a viromitigator which prevents apoptosis of infected leukocytes (Barry et al., 1997). M-T4 has no known cellular homologs, but related proteins are expressed from several poxviruses. The M-T4 protein is 30kDa with an N-terminal signal sequence and a C-terminal RDEL motif. The RDEL motif has been shown to retain proteins within the endoplasmic reticulum via ER

retrograde transport mediated by the KDEL (Pelham, 1989; Pelham, 1990). This has been confirmed in M-T4 by confocal fluorescence microscopy and immunoprecipitation studies that localize M-T4 to the ER, indicating that the M-T4 RDEL motif is functioning as a *bona fide* ER retention signal (Barry et al., 1997). *In vivo* myxomatosis studies suggest that M-T4 is a host-range type virulence factor. M-T4 deletion mutant virus (M-T4KO) infected rabbits exhibit profound disease attenuation characterized by an almost complete lack of secondary lesions and a 100% survival rate. This suggests that the M-T4KO virus is unable to disseminate *in vivo*. Furthermore, infection of a CD4⁺ rabbit lymphocyte cell line or rabbit primary rabbit peripheral blood lymphocytes with the recombinant M-T4KO virus resulted in apoptosis of the infected cells (Barry et al., 1997).

Virostealth: Hiding from immunosurveillance

The final strategy employed by viruses to evade the immune system is the downregulation of surface molecules that allow an infected cell to convey its abnormal state to the immune system. This strategy seeks to reduce the visibility of the infected cell and prevent the recognition and destruction of virus-infected cells by cytotoxic T and NK cells. This strategy, referred to as virostealth, is employed by both small and large viruses. For example, HIV encodes the Nef protein that is capable of downregulating MHC class I and CD4 molecules through a poorly delineated series of events that involve a novel thioesterase (Aiken et al., 1994; Liu et al., 1997; Willey et al., 1992). Myxoma virus has also been shown to utilize MHC

class I and CD4 downregulation, a phenomenon with significant implications for viral immune-evasion.

MHC I downregulation

Cell mediated acquired immunity is responsible for the identification and destruction of intracellular pathogens, and thus represents a formidable long-term anti-viral strategy. The process of antigen presentation by professional antigen presenting cells (APCs) to T lymphocytes is an initial and critical step in initiating a cellular immune response, making this process a prime target for viral interference. Not surprisingly, a number of viruses have been shown to prevent effective antigen presentation. Myxoma virus has been found to downregulate the cell surface expression of class I major histocompatibility complex (MHC), though the viral proteins responsible for this effect are unknown (Boshkov et al., 1992). The downregulation of class I MHC and CD4 by other viruses is well documented and uses a variety of mechanisms. For example, the E3/19K gene product of adenovirus type 2 binds to class I MHC and inhibits its transport to the cell surface (Wold and Gooding, 1991; Wold and Gooding, 1989; Wold et al., 1994).

In myxoma virus the decrease in class I MHC expression in virally infected cells was shown to be far greater than that seen in cells treated to completely inhibit protein synthesis, ruling out the possibility that the observed downregulation was due simply to host protein synthesis shutdown (Boshkov et al., 1992). It has been suggested that myxoma virus protein(s) directly interact with the class I MHC to

either (i) physically prevent surface expression, or (ii) produce a change in class I MHC conformation (Boshkov et al., 1992).

CD4 is targeted for degradation

Using flow cytometry and immunoprecipitation techniques, Barry et al. (Barry et al., 1995) demonstrated that myxoma infection rapidly leads to a decrease in the level of cell surface cellular CD4 in a rabbit T cell line (RL-5). This phenomenon was shown to be a function of an early gene product and was not due to a generalized loss of cell surface molecules in infected cells, as the levels of three other cell surface proteins (CD18, CD43, and CD45) were not significantly altered during the course of infection. Furthermore, CD4 appears to be actively targeted to the lysosomes for degradation during myxoma virus infection.

Activation of the protein kinase C (PKC) pathway via treatment of cells with phorbol 12-myristate-13-acetate (PMA), is also known to cause a decrease in cell surface expression and degradation of CD4, albeit with different kinetics than those observed in myxoma virus infection. Myxoma virus downregulation and degradation of CD4 was shown not to depend on the activation of PKC, as the use of the PKC inhibitor, staurosporine, did not affect the virus induced loss of cell surface CD4, while the PMA-induced downregulation was sensitive to staurosporine (Barry et al., 1995)

The fate of the tyrosine kinase which associates with the cytoplasmic domain of CD4, p56lck, has also been examined. It was found that the association of p56lck

with CD4 decreased in a similar manner to that of total cellular level of CD4, however, the total cellular level of p56lck was unchanged. Because the total cellular level of p56lck remained the same before and during infection, unlike CD4, dissociation of p56lck must precede degradation of CD4 in the lysosome (Barry et al., 1995)

Insights from the study of viral immunomodulation

There are a number of parallel developments ongoing in the field of immunosubversive viral proteins that further broaden the avenues for potential investigation. Sequencing of the genomes of poxviruses has yielded new potential immunomodulatory proteins. For example, the complete sequence of *Molluscum contagiosum* led to the rapid characterization of a series of novel immunomodulatory proteins, including the discovery of a class of viral death domain antagonist proteins (Bertin et al., 1997), and a viral strategy to deal with the harmful effects of free radicals produced by ultraviolet light or the respiratory burst of certain immune cells (Shisler et al., 1998). The complete sequencing of myxoma virus should yield an abundance of new immunomodulatory proteins as well.

Another intriguing recent development in this field is the observation that some virus encoded proteins appear to have multiple anti-immune functions. This is not a new concept for the small viruses which have limited encoding capacity, but for the larger DNA viruses, however, this realization opens up numerous possibilities. Several examples of proteins with multiple immunomodulatory functions have

recently come from poxviruses. For example, Tanapox virus expresses a major secreted protein that is capable of inhibiting multiple cytokines including IL-2, IL-5, and IFN- γ (Essani et al., 1994). The myxoma virus M-T7 protein has been shown to bind IFN- γ with high affinity, and heparin binding proteins such as chemokines with lower affinity (Lalani et al., 1997), while the M-T2 protein exhibits both TNF binding and acts to block apoptosis of myxoma virus infected lymphocytes (Macen et al., 1996; Schreiber et al., 1997). These examples support the notion that many more virus proteins may exhibit more than one function.

Finally there is the possibility that some of these viral gene products have potential therapeutic applications. The success of purified SERP-1 as an anti-inflammatory agent (Lucas et al., 1996; Maksymowych et al., 1996) may be a glimpse of a future in which the proteins that viruses produced to disable the host immune system are re-assigned to be used as beneficial therapeutic agents.

Viruses that are susceptible to attack by multiple arms of the immune system must develop countermeasures to nullify the systems that are antagonistic to virus replication if the virus is to be an effective long-term pathogen. It is not surprising, therefore, that recent works have led to a proliferation of reported viral immunomodulatory proteins. What is perhaps surprising is the scope and degree to which the host immune repertoire can be subverted by specific viral gene products. The large DNA viruses have the luxury of encoding a wide range of proteins that are not required directly for viral replication alone. Myxoma virus propagates in tissues such as skin, respiratory tract and lymph nodes that are readily accessible to a

variety of the effector elements of the immune system. Like all poxviruses, myxoma has adapted to this inhospitable immune environment by expressing a wide range of proteins to systematically block each threatening aspect of the immune system. The first portion of this chapter has presented an overview of a number of the proteins that have been characterized out of myxoma virus (Figure I-7), but this is still almost certainly an incomplete picture. The cataloging of the myxoma virus genes is an ongoing project, but a related poxvirus, vaccinia virus, has been shown to have at least 55 open reading frames that are dispensable for propagation in tissue culture (Perkus et al., 1991). Myxoma virus expresses dozens of secreted proteins, of which we have so far characterized only five. In addition we have already described phenomena such as MHC I and CD4 downregulations with which we have yet to associate viral gene products. Clearly this remains an area of rich potential.

The SERPINS

Serpins are used in many eukaryotic tissue systems to regulate complex proteinase dependent pathways in order to maintain homeostasis (for review see (Potempa et al., 1994)). Examples of this include the activation of cytokines in the inflammatory network (Forsyth et al., 1994; Komiyama et al., 1994; Matsuda et al., 1994), the complement pathway, fibrinolysis (Lijnen and Collen, 1990), thrombotic cascades (Olds et al., 1994; Olson and Bjork, 1994), tissue remodeling (Smirnova et al., 1994), apoptosis (Houenou et al., 1995; Sarin et al., 1993), and a number of signalling pathways (Altieri, 1995; Coughlin, 1994; Nystedt et al., 1994; Strickland et

al., 1995). In most cases a few key proteinases can be modulated to regulate an entire cascade. It is not surprising, then, that certain pathogens might employ serpins in order to interrupt proteinase dependent host processes that are antagonistic to the invading organism. For example, nematodes of the *Brugia* family of filarial parasites have been reported to express serpins (Blanton et al., 1994; Ghendler et al., 1994; Yenbutr and Scott, 1995). Of interest to virologists is that several serpins have been identified within the genomes of members of the poxvirus family of large DNA viruses. The fact that serpins are expressed even by viruses, the simplest of all organisms, is intriguing, and testifies to the importance of proteinases in the immune response to pathogens. Among the serpins produced by members of the poxvirus family is a unique myxoma virus protein designated SERP-1, on which this thesis will focus.

The Biochemistry of SERPINS

The serpins are a family of structurally related protein inhibitors of serine proteinases. The term serpin is itself, an abbreviation of serine proteinase inhibitor, although not all serpins have inhibitory activity (Potempa et al., 1994). Serpins play a major regulatory role in a host of biological processes that involve proteinase activity, and serpin mutations have been associated with a host of human diseases relating to thrombosis, angioedema, hemorrhage and emphysema (Stein and Carrell, 1995). They are involved in the regulation of such diverse processes as coagulation, fibrinolysis, complement activation, ovulation, embryonic development,

neuromuscular patterning, angiogenesis, inflammation, apoptosis, neoplasia/malignancy and viral pathogenesis (Lawrence, 1997; Rubin, 1996). The mechanism by which serpins act to inhibit their specific target serine proteinase(s) is relatively well understood at both the molecular and biochemical level.

The conserved serpin structure takes the form of a compact globular protein composed of β -sheets connected by α -helices. A single exposed loop known as the reactive centre loop (RCL) forms an arc above one pole of the protein (Figure I-8). The RCL is the business end of the serpin with which it interacts with the target proteinase at a scissile P1-P1' bond. Amino acids in the RCL are referred to according to their position relative to the P1-P1' bond, with residues to the C-terminus assigned the designations of P1'-P2'-P3'...etc., and N-terminal residues referred to as P1-P2-P3-P4...etc. (Figure I-9). In this form the serpin is in a strained or high-energy meta-stable conformation, very much like a set mouse-trap (Whisstock et al., 1998). The current model for the mechanism by which serpins act to inhibit their target proteinase(s) is relatively well defined. The interaction of enzyme and serpin takes place as a series of steps that serve as an elegant example of the molecular dance that is the basis of life. First, the serpin binds to its target proteinase to form a reversible complex analogous to a Michaelis complex between an enzyme and a substrate. Next, the proteinase cleaves the P1-P1' reactive centre peptide bond resulting the formation of a covalent acyl-enzyme intermediate (Lawrence et al., 1994; Wilczynska et al., 1995). This cleavage releases the serpin from its strained state, releasing the "spring" and allowing a rapid insertion of the

reactive centre loop into β -sheet A up to at least the P9 position. Since the enzyme and serpin are covalently bound, this results in a large-scale movement of the enzyme which is effectively dragged across the face of the serpin (Stratikos and Gettins, 1997; Wilczynska et al., 1997). The bound proteinase prevents full insertion of the RCL and the complex becomes locked. This resulting strain is transferred to the enzyme, distorting the active site and preventing deacylation of the acyl-enzyme intermediate, thus trapping the complex. This complex is extremely stable in solution with a half-life in the order of hours to days. Biologically this is ample time for the trapped enzyme to be degraded or removed from circulation.

Conformation of the SERPINS

Serpins undergo dramatic conformational changes when cleaved by the proteinases they inhibit. This was first recognized in 1983 when interpretation of the electron-density map of α 1-antitrypsin cleaved at the scissile bond revealed that the P1 and P1' residues were 65 Å apart (Loebermann et al., 1984). The region N-terminal to the scissile bond of the RCL formed a strand within a large β -sheet. This implied that the mechanism of serpin inhibition differed substantially from other inhibitors. The standard mechanism inhibitors refer collectively to a number of families of small proteins, with the Kunitz and Kazal families being the best known (Laskowski and Kato, 1980). Like serpins, the standard mechanism inhibitors of serine proteinases use a surface loop or reactive site loop to bind to the active site of their target serine proteinase (Stone et al., 1997). The reactive site loop acts as a

pseudo-substrate that docks into the active site. Structurally, the reactive site loop does not change upon binding to the proteinase, and different members of this group exhibit the same structure despite sequence differences. This conical structure is complementary to the active site of the enzyme. Unlike serpins, the entire inhibition mechanism is reversible (Bode and Huber, 1992).

The first structural view of an intact serpin came with crystallization of the non-inhibitory serpin, ovalbumin, in which the region corresponding to the RCL forms an α -helix (Stein et al., 1991). Subsequently, inhibitory serpin structures confirmed that the RCL of inhibitory serpins also exists as a loop structure (Schreuder et al., 1994). Serpins contains three β -sheets and nine α -helices. The main β -sheet, into which the RCL can insert is shown on the front face of the structure in Figure I-8 and labeled as β -sheet A. The reactive centre loop contains the residues that interact directly with the cognate target proteinase. In Figure I-8, the RCL appears as an exposed loop at the top of the serpin structure.

The conformational change that occurs upon cleavage of inhibitory serpins at the RCL is accompanied by an increase in thermostability (Carrell and Boswell, 1986). This is referred to as the S \rightarrow R ('stressed' to 'relaxed') transition (Stein and Chothia, 1991). In addition to the active uncleaved form, a second uncleaved form exists that is referred to as the latent state. In the latent state, the reactive centre loop is inserted into the main β -sheet as in the cleaved form. This state is considered to be an alternative R state, and occurs only in certain serpins. The first direct view of the latent state was seen in the crystal structure of plasminogen activator inhibitor-1 (PAI-1) (Mottonen et

al., 1992). Normal antithrombin does not form the latent conformation spontaneously, although a natural mutant Rouen VI (187Asn → Asp) converts slowly to the latent state (Whisstock et al., 1998). The spontaneous formation of the latent state by PAI-1 is unusual among serpins, suggesting that latency may be part of the control mechanism that allows PAI-1 to circulate in an inactive form, becoming active only at particular sites or tissues through interaction with the glycoprotein vitronectin (Lawrence, 1997). Under certain conditions, serpins can form dimers or even polymers. The Z-variant of α_1 -antitrypsin (Glu342 → Lys) results from a conformational transition and a linkage between the RCL of one molecule and a β -sheet of another (Dafforn et al., 1999). Polymerization is concentration and temperature dependent and occurs primarily in mutant versions of serpins.

Poxviruses are the only viruses that have been shown to encode serpins

The poxviruses comprise a family of large, complex DNA viruses that are capable of autonomous replication within the cytoplasm of infected cells. This sets them apart from the vast majority of DNA viruses that rely on the ability to parasitize the host cell replication machinery, and, consequently, require localization in the nucleus of infected cells. The poxvirus family is divided into two subfamilies, the chordopoxviridae (vertebrate poxviruses), and the entomopoxviridae (insect poxviruses) (Moss, 1990). The chordopoxviridae are further divided into seven genera, of which four, the orthopoxviruses, leporipoxviruses, avipoxviruses, and suipoxviruses have been found by sequencing studies to encode serpins. The

remainder of the poxviruses may well also encode serpins, but sequencing of the genomes of many members of this family is still at a rudimentary stage. Together the poxviruses constitute the etiologic agents of a large number of diseases ranging from benign and localized (e.g. vaccinia virus and Shope fibroma virus) to systemic and highly lethal (smallpox and myxomatosis). All poxviruses have a genome composed of a single linear double stranded DNA with cross-linked termini, which ranges in size from 130 to 375 kbp (for reviews see (Buller and Palumbo, 1991; Fenner et al., 1989; Turner and Moyer, 1990)). The central region of the genome is highly conserved, not only between strains, but even to some extent among all poxviruses. In comparison, the 20-50 kb regions of the DNA closest to the termini are subject to considerable variation among natural poxvirus isolates. The most variable region is the terminal inverted repeat (TIR) region which is duplicated at each end of the genome, and accounts for the only genes that are present in two copies. The degree to which virus genes are conserved between different poxviruses can be correlated to their function. The conserved core of the genome codes primarily for the so-called "housekeeping" genes that are required for the basic survival of the virus and are required for virus replication in cultured cells. The highly variable genes which map outside of the central core of the genome are generally termed non-essential because they are not required for virus propagation in tissue culture. However, many of these genes encode for what are referred to as "virulence factors" which are so named because they confer an increased capacity for propagation within immunocompetent vertebrate hosts and thereby contribute to virus spread and pathogenesis. Such

virulence factors function to allow virus infected cells to survive in complex tissues in the face of challenge by the consolidated actions of the immune and inflammatory systems of the host.

Encoded within the non-essential regions of at least four poxvirus genera are a number of proteins which conform to criteria of the serpin superfamily (Table I-4). The poxvirus from which the viral serpin considered here was identified, namely myxoma virus, is a rabbit-specific pathogen of the leporipoxvirus family, that causes a systemic and lethal disease known as myxomatosis (Fenner and Meyers, 1978). Myxoma virus encodes for many known virulence factors, including the secreted serpin, designated SERP-1. SERP-1 has been characterized in terms of serine proteinase inhibitor function *in vitro* and effects on virus virulence *in vivo*, and is to date the only poxviral serpin known to be secreted from virus infected cells. Three other virus serpins (Spi-1, 2, 3) have been found to be encoded by diverse members of the orthopoxvirus genus, but all of these are believed to remain associated with the virus infected cell throughout the course of infection. SERP-1 is also the only viral serpin encoded within the TIR region of a poxvirus, and is thus present in two copies in the myxoma genome. Although other large DNA viruses, including the herpesviruses and adenoviruses, also express multiple proteins that interfere with host defenses, it is interesting to note that only poxviruses are so far known to encode serpins. The origin of poxviral serpins is still uncertain. All poxvirus genes, including the serpins, are, effectively, cDNAs and do not contain introns. This suggests that if these serpin genes are derived from host cells, they may have

originally been acquired from processed, cytoplasmic mRNA transcripts, rather than directly from the host genome. This is in keeping with the entirely cytoplasmic life cycle of the virus, but it should be noted that not all poxvirus genes have obvious host counterparts and thus the origin of the SERP-1 gene remains speculative.

Other poxviral serpins

The first poxvirus open reading frame with serpin-homology to be discovered was the cowpox virus 38K gene (Pickup et al., 1986). This was later renamed crmA, for cytokine response modifier A (Ray et al., 1992), and, in vaccinia virus, is designated as Spi-2 (Kotwal and Moss, 1989). Under the proposed nomenclature for poxviral serpins, the Spi-2 name is gaining acceptance as the generic name for this serpin (Turner et al., 1995). Spi-2 homologs have been found in most of the orthopoxviruses, including cowpox virus (CPV), vaccinia virus (VV) strain WR, rabbitpox virus (RPV) (Ali et al., 1994), and variola virus (VAR) the causative agent of smallpox (Massung et al., 1993). Unlike myxoma SERP-1, and most mammalian serpins, Spi-2 is not secreted. It is synthesized early during infection, and is present as a cytosolic protein (Kettle et al., 1995; Pickup et al., 1986). In the avian chorioallantoic membrane model of inflammation, Spi-2 expression dramatically reduced heterophil, lymphocyte, and macrophage influx into the infected area. The mechanism of this anti-inflammatory action appears to be the ability of Spi-2 to inhibit interleukin-1 beta converting enzyme (ICE) *in vitro* (Ray et al., 1992). This inhibition was shown to be both rapid and specific with an association rate constant

of $1.7 \times 10^7 \text{ M}^{-1}\text{s}^{-1}$, and an equilibrium constant (K_i) of less than $4 \times 10^{-12} \text{ M}$ (Komiya et al., 1994). The P1 residue of Spi-2, the amino acid after which the serpin is cleaved, is an aspartate for the inhibition of ICE. Interestingly, ICE is a cysteine proteinase, making Spi-2 the first example of a cross-class inhibitor (Komiya et al., 1994). Furthermore, ICE has been implicated in triggering apoptosis (Miura et al., 1993), and Spi-2/crmA is capable of preventing apoptosis by a variety of inducers (e.g. (Gagliardini et al., 1994)). Other large DNA viruses such as baculovirus, adenovirus, and herpesvirus are also known to block apoptosis of infected cells (for extensive review see (White, 1996)), though not through the use of serpins. Spi-2, while conforming to the serpin homology, appears to be a cytosolic cysteine proteinase inhibitor directed against homologs of the ced-3 family of ICE-like proteinases in order to prevent apoptosis of virally infected cells.

Spi-1 is approximately 45% identical to Spi-2, although it differs at the predicted P1-P1' residues, which are Phe-Ser (Kotwal and Moss, 1989). Spi-1 is found at the right end of the genome in the orthopoxviruses CPV, RPV, VV, and VAR, and has been shown to increase host range by preventing virus induced apoptosis in some cell lines (Turner et al., 1995). As is the case with Spi-2, and unlike SERP-1 or mammalian serpins, Spi-1 is neither secreted nor glycosylated. Recently, Spi-1 has been shown to inhibit cathepsin G (R. Moyer, P. Turner, personal communication), though the role of this *in vivo* remains uncertain.

A third serpin, termed Spi-3 is found in most of the orthopoxviruses examined, including VV, RPV, CPV, VAR, and the more divergent raccoonpox virus

(for review see (Turner et al., 1995)). Spi-3 exhibits only low sequence similarity to Spi-1 and Spi-2, and is more similar to mammalian serpins in that it is N-glycosylated. Spi-3 does not, however, appear to be secreted into the medium of virus infected cells, but remains bound to the plasma membrane via a transmembrane domain (R. Moyer, P. Turner, personal communication). It has a putative P1 arginine residue, but no virulence or anti-inflammatory phenotypes have been associated with Spi-3. In addition to serpin activity, Spi-3 functions in the prevention of cell-cell fusion following infection (Law and Smith, 1992; Turner and Moyer, 1992; Zhou et al., 1992). The targets of Spi-3 action appear to be similar to SERP-1 (R. Moyer, P. Turner, personal communication), though mutation of the postulated P1-P1' site does not abrogate the ability of host cells to promote fusion (Turner and Moyer, 1995).

Of the remaining orthopoxviral serpins, none has been well characterized at the biochemical level. Spi-5 and Spi-6 from fowlpox appear to be truncated serpins and are unlikely to be functional inhibitors (Turner et al., 1995). The swinepox Spi-7 protein is another cytosolic relative of Spi-1, Spi-2 and Spi-3, and may serve a similar function (Massung, Jayarama, and Moyer, 1993).

SERP-1 is a virulence factor

SERP-1 (also called Spi-4) has been found to be present in the genomes of only two poxviruses: myxoma virus (myx), and malignant rabbit fibroma virus (MRV), a recombinant between Shope fibroma virus and myxoma virus (Upton et al., 1990). In myxoma virus, the SERP-1 gene lies within the TIR sequences, and is thus

present as two copies, while in MRV, the identical SERP-1 gene maps within the unique sequences adjacent to the TIR, and hence is present in a single copy only (Upton et al., 1990).

When a targeted gene disruption of the single SERP-1 gene was constructed in MRV, the resultant virus exhibited normal growth characteristics in cultured cells, but was severely attenuated in infected rabbits (Upton et al., 1990). Wild type MRV-infected rabbits exhibited large tumors at the primary inoculation site; secondary tumors appeared in the ears by day 7; and progressive bacterial infections of the nasal and conjunctival mucosa were so severe that all rabbits were sacrificed on or before Day 14. The survival rate for European rabbits infected with wild type MRV is less than 1% but, in contrast, rabbits infected with the MRV-S1 deletion mutant showed a marked decrease in the severity of symptoms and 60% survived (Upton et al., 1990). Similarly, the targeted gene disruption of both copies of the SERP-1 gene in myxoma virus had a comparable attenuating effect upon viral virulence, with survival rates following infection with the SERP-1 knock-out myxoma virus also exceeding 60% (Macen et al., 1993). Furthermore, histological sections taken from the lesions of infected animals indicated that in the absence of SERP-1, a more effective inflammatory response occurs, allowing a more rapid resolution of the infection (Macen et al., 1993). This suggests that SERP-1 contributes to viral pathogenesis by interacting with host proteins associated with virus-infected lesions that regulate the early inflammatory response to the virus infection.

SERP-1 is secreted as a stable N-glycosylated protein

Transcriptional analysis of the SERP-1 gene indicates that it is expressed as a late gene, meaning that it appears late in the lytic cycle of infection following virus DNA replication (Macen et al., 1993). This is in keeping with the known SERP-1 promoter sequence which is similar to the consensus late promoter described for other poxviruses, particularly vaccinia virus (Moss, 1990). SERP-1 mRNA first appears at 6 hours post-infection, and remains at high levels throughout the lytic cycle. Polyclonal antisera raised against SERP-1 detect a heterogeneous 55-60 kDa species which appears in extracellular culture supernatants starting at 8 hours post-infection, and remains at high levels as a stable soluble protein (Macen et al., 1993). A recombinant vaccinia virus containing the SERP-1 open reading frame under the control of a strong, synthetic late promoter also expresses and secretes SERP-1 into the culture supernatant, and does so at levels 10 times greater than myxoma. This overexpression has allowed SERP-1 to be purified to homogeneity from supernatants of virus infected cells (Nash et al., 1998). On silver stained SDS-PAGE gels, purified SERP-1 protein migrates as a diffuse 55-60 kDa band, indicating possible glycosylation. Indeed, when SERP-1 produced from the recombinant vaccinia virus vector was subjected to N-glycosidase F, the mobility of SERP-1 was reduced to the predicted size of the polypeptide (42 kDa), consistent with the size predicted from the DNA sequence of the open reading frame. Thus, SERP-1 is expressed as a

secreted, soluble, N-glycosylated protein from cells infected with myxoma, MRV, or VV-SERP-1, but not from any other virus yet described.

SERP-1 is anti-inflammatory in models of restenosis and arthritis

Following the discovery that SERP-1 had potential anti-inflammatory properties (Macen et al., 1993), purified SERP-1 protein has also been tested in non-viral model systems of inflammation relevant to human disease. SERP-1 is a good candidate as a novel anti-inflammatory reagent in medical applications for several reasons. The still unidentified SERP-1 target proteinase appears to be intimately involved in some early stage of inflammation, and the SERP-1 protein itself is active at picogram levels in complex subdermal tissues that are normally highly susceptible to the elements of the inflammatory response. Furthermore, the native SERP-1 protein is believed to be poorly antigenic since no circulating antibodies directed against it can be detected in animals which survive infection by attenuated variants of myxoma virus (unpublished data). The first successful application of SERP-1 as a targeted anti-inflammatory protein was made in a model of coronary restenosis following primary balloon angioplasty in which SERP-1 was employed to reduce plaque development (Lucas et al., 1996). Atherosclerotic plaque growth is one of the leading causes of morbidity and mortality in North America, and has been linked to an excessive inflammatory and thrombotic response to arterial injury. Since both the thrombotic and inflammatory cascades are regulated by serine proteinases, and as such may be regulated by serpins, the effects of SERP-1 infusion at sites of

angioplasty balloon injury in a rabbit model of atherosclerosis was systematically examined. A total of 74 rabbits had either focal infusions of picogram levels of purified SERP-1 protein or systemic infusion of nanogram levels of SERP-1. The reactive centre Ala-Ala mutant of SERP-1 was used as a control. There was a dramatic reduction of atherosclerotic plaque growth 4 weeks after a single SERP-1 infusion at sites of arterial damage but not after infusion of the Ala-Ala mutant. In this rabbit model the reduction in plaque development was associated with a parallel reduction in macrophage infiltration into the balloon-damaged vascular wall that could be detected within 24 hours after SERP-1 infusion (Lucas et al., 1996). These studies indicate that SERP-1 functions through its serine proteinase inhibitory activity both to reduce the level of acute macrophage influx into balloon damaged vasculature, and to diminish subsequent plaque growth at the damaged arterial sites.

Purified SERP-1 protein has also been used successfully in preliminary experiments to reduce arthritis-like inflammation in a rabbit model of rheumatoid arthritis. In an antigen-induced arthritis model of chronic inflammation, purified SERP-1 protein was administered by intra-articular injection after joint inflammation had been induced by systemic antigen stimulation. The synovia were analyzed several weeks after SERP-1 treatment, and SERP-1 was seen to reduce cellular hyperplasia, chronic inflammatory infiltration and cartilage erosion (Maksymowych et al., 1996). As in the restenosis model, the dosage of SERP-1 required for effect was in the picogram to nanogram range.

While the mechanism by which SERP-1 is acting in these systems remains to be elucidated, it is nonetheless important to note the potential of using serpins or small molecule inhibitors of proteinases in such small doses to achieve dramatic clinical results.

Thesis Objectives

The overall objective of the work presented in this thesis was to increase our understanding of the biochemical properties of SERP-1. This was achieved through the detailed analysis of SERP-1 as a proteinase inhibitor and as a glycoprotein. Specifically, this thesis addresses four key areas:

1) Detailed analysis of the interaction of SERP-1 with known proteinases.

We wished to examine the effectiveness of SERP-1 as a serpin and to gain insight into the nature and characteristics of its target proteinases. To achieve this, we identified six proteinases that SERP-1 was able to inhibit from a panel of twenty available enzymes. The kinetics of proteinase inhibition by SERP-1 was studied to determine k_{app} , $K_{I(app)}$, and k_{diss} . The stoichiometry of inhibition was measured, allowing us to calculate k_{inh} and K_I . The putative P1-P1' was confirmed by N-terminal sequencing of the cleaved fragments following thrombin digestion, and the P1-P1' residues were mutated to Ala-Ala to confirm the critical importance of these residues. The results of this work are presented in Chapter II.

2) Having developed an unusually complete set of data for the inhibition kinetics of a single serpin with a panel of proteinases, we wished to examine the nature of the serpin mechanism in greater detail. Using linear free energy relationships in conjunction with free energy profiles we probed the mechanism of serpin action. This work is presented in Appendix A.

- 3) Reactive centre loop mutants of SERP-1 were created by site-directed mutagenesis. These mutants were assessed for their inhibitory properties. Certain SERP-1 mutants were tested as anti-inflammatory compounds in a rat model of restenosis. The results of these studies are presented in Chapter III
- 4) SERP-1 is an N-glycosylated, secreted glycoprotein. Since myxoma virus expresses a sialyltransferase, we wished to examine whether SERP-1 was sialylated by the myxoma MST3N gene product. In addition we constructed mutants of the glycosylation sites in SERP-1 to examine the requirement for correct glycosylation in the processing and secretion of SERP-1. The results of these studies are presented in Chapter IV.

TABLE I-1

EXAMPLES OF VIROCEPTORS FROM POXVIRUSES

| <u>Viroceptor</u> | <u>Virus</u> | <u>Host homolog</u> |
|--------------------------------------|----------------|------------------------------|
| <i>Soluble cytokine receptors</i> | | |
| M-T2 | Myxoma virus | TNF receptor |
| CrmB, C, D | Cowpox virus | TNF receptor |
| M-T7 | Myxoma virus | IFN- γ receptor |
| B8R | Vaccinia virus | IFN- γ receptor |
| B15R | Vaccinia virus | IL-1 β receptor |
| B18R | Vaccinia virus | IFN- α/β receptor |
| M-T1 | Myxoma virus | unknown |
| <i>Membrane-associated receptors</i> | | |
| K2R | Swinepox virus | Chemokine receptor |
| Q2/3L | Capripox virus | Chemokine receptor |

TABLE I-2

EXAMPLES OF VIROKINES FROM POXVIRUSES

| Virokine | Virus | Host homolog |
|------------------------------|-----------------------------|---------------------|
| <i>Growth Factors</i> | | |
| VGF | Vaccinia virus ^a | EGF/TGF α |
| MGF | Myxoma virus ^a | EGF/TGF α |
| A2R | ORF virus | VEGF |
| <i>Cytokines</i> | | |
| vIL-10 | ORF virus | IL-10 |
| MC148R | Molluscum contagiosum | MIP-1 β |
| v-semaphorin | vaccinia | c-semaphorins |
| <i>Proteinase inhibitors</i> | | |
| SERP-1 | Myxoma virus | serpin family |
| <i>Complement</i> | | |
| VCP | vaccinia | C4B-BP |

^a Other poxviruses express VGF/MGF homologs

TABLE I-3

POXVIRUS VIROMITIGATORS OF APOPTOSIS

| Viromitigator | Virus |
|---|---------------------------|
| <i>Caspase Inhibitors^a</i> | |
| SPI-2/crmA | Cowpox virus |
| SERP-2 | Myxoma virus |
| <i>Death Domain Effectors</i> | |
| MC159/160 | Molluscum contagiosum |
| <i>bcl-2/ced-9 homologs</i> | |
| A179L (HMW5-HL) | African swine fever virus |
| <i>Fas/TNF pathway blockade^a</i> | |
| M-T2 | Myxoma virus |
| A224L (4CL) | African swine fever virus |
| <i>PKR Inhibitor</i> | |
| E3L | Vaccinia Virus |
| <i>Unknown Mechanism</i> | |
| M11L | Myxoma virus |
| M-T4 | Myxoma virus |
| M-T5 | Myxoma virus |

^a Caspase inhibitors may also act to block FAS/TNF mediated apoptosis by interfering with the downstream caspases (see Figure I-4)

TABLE I-4

POXVIRUS ENCODED SERPINS

| Serpin | Virus | Inhibits | Comments |
|-------------------------------|-----------------|--|--|
| <i>Secreted Extracellular</i> | | | |
| SERP-1 | Myxoma virus | uPA, tPA, plasmin, thrombin, Xa ^a | Virulence factor, anti-inflammatory |
| <i>Membrane Bound</i> | | | |
| Spi-3 | Orthopoxviruses | uPA, tPA, plasmin, thrombin, Xa ^b | Similar activity to SERP-1, inhibits cell fusion |
| <i>Intracellular</i> | | | |
| Spi-2/crmA | Orthopoxviruses | multiple caspases, granzyme B ^c | Anti-inflammatory; inhibits apoptosis |
| SERP-2 | Myxoma virus | caspase 1 ^c | Virulence factor |
| Spi-1 | Orthopoxviruses | Cathepsin G ^b | Host range |
| Spi-7 | Orthopoxviruses | No known inhibition | |
| Spi-5 | Avipoxviruses | | |
| Spi-6 | Avipoxviruses | | |

^a uPA, urokinase; tPA, tissue type plasminogen activator, Xa, coagulation factor Xa.

^b R. Moyer, personal communication.

^c ICE, Interleukin 1 β -converting enzyme is also called caspase 1

Figure I-1: Characterization of viral immunomodulatory proteins. The viral immunomodulatory proteins produced by myxoma virus are categorized based on their localization (intracellular versus secreted) and mode of action. Viroceptors are virus-encoded receptors that act by competitively binding cytokine ligands while virokines mimic host cytokines or inhibitors. Intracellular immunomodulation is accomplished by virus encoded viromitigators of apoptosis, or virostealth strategies designed to specifically downregulate cell surface markers that act as the window for the immune system to recognize virus-infected cells.

Myxoma Virus Strategies

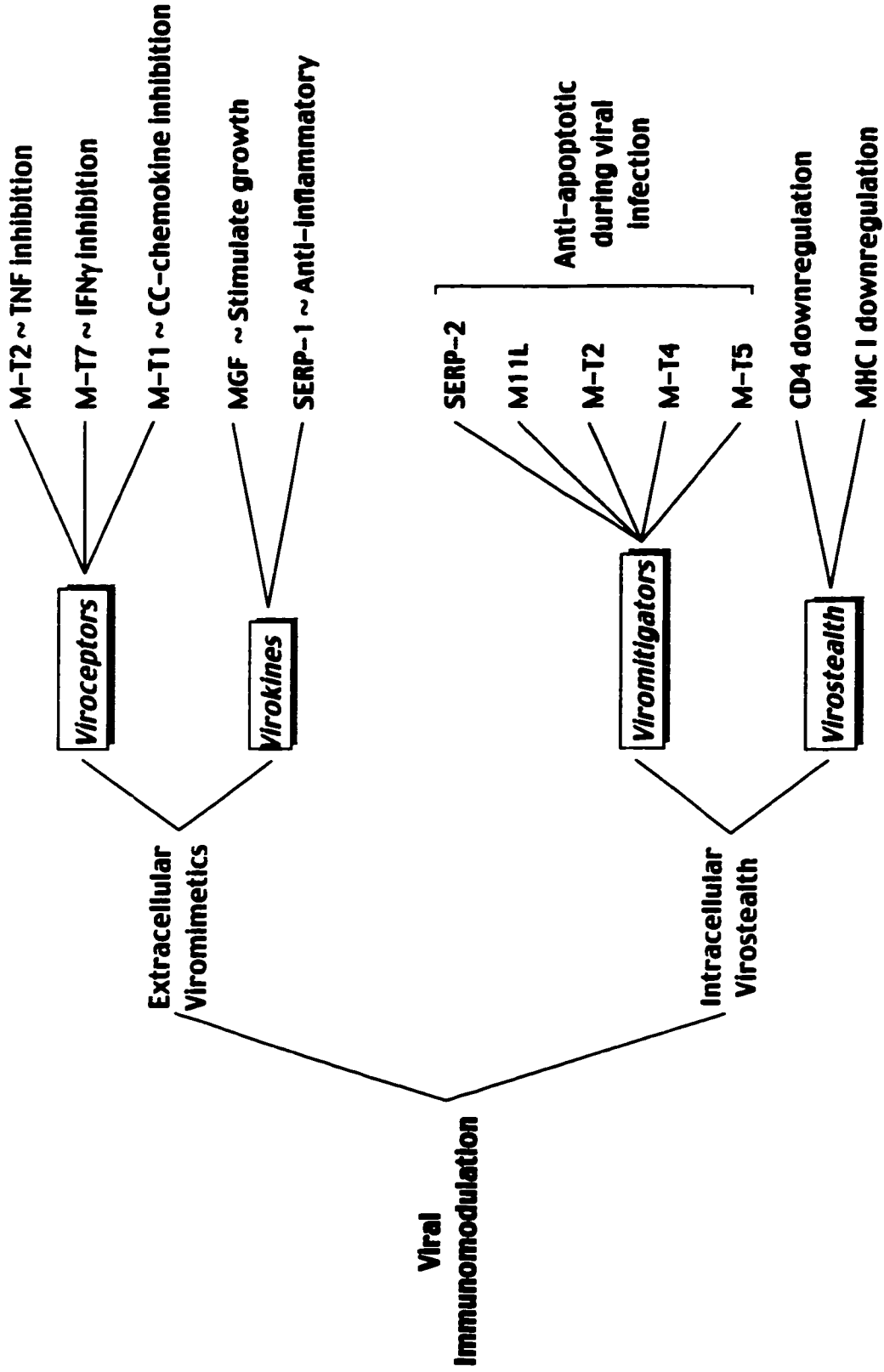


Figure I-2: Diagram of the Myxoma virus genome. Schematic representation of the myxoma virus linear, double stranded DNA genome. The genome is mapped according to BamHI fragments. The left and right hand ends are indicated, and the terminal inverted repeats (TIR) are expanded indicating the virulence factors that we discuss.

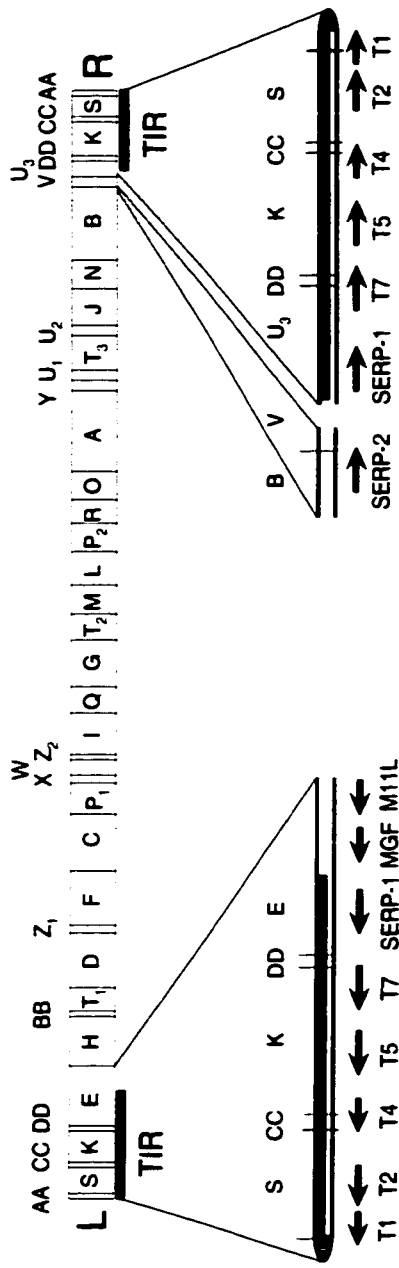
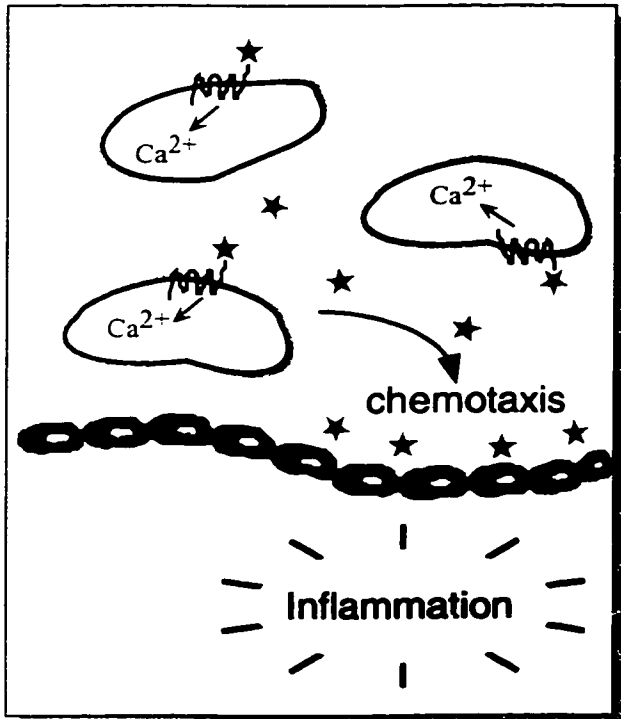


Figure I-3: Inhibition of chemokines by a viral chemokine binding protein. Secreted viral chemokine binding proteins can inhibit cellular chemokines from binding and signaling through their cognate cellular serpentine receptors. A, in a normal inflammatory response cellular chemokines (stars) recruit leukocytes to the sites of tissue damage or infection via a chemokine gradient. B, the viral chemokine binding protein, M-T1 (ovals), binds to host chemokines, preventing them from interacting with their cellular receptors. This effectively blocks chemotaxis and prevents intracellular calcium signaling of pro-inflammatory leukocytes.

A. Cellular chemokine and chemokine receptors interact during a normal inflammatory response.



B. M-T1 inhibits chemokine/chemokine receptor interaction.

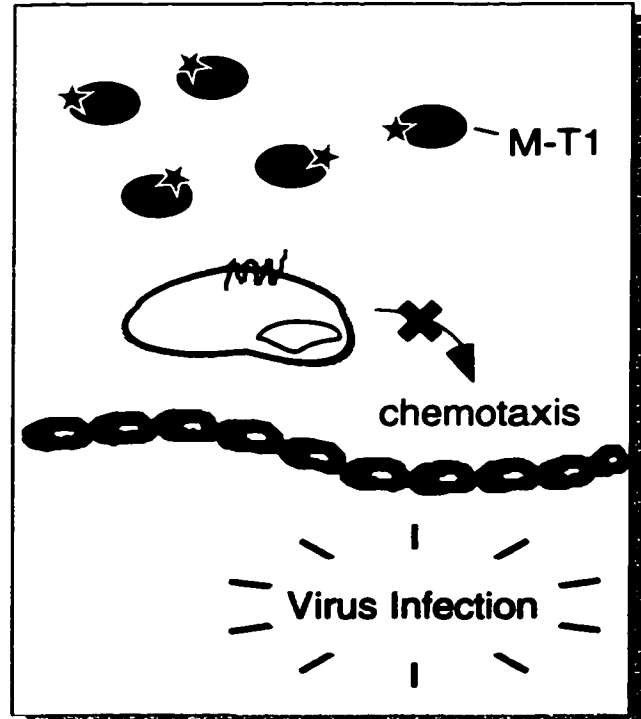


Figure I-4: Inhibition of Fas or TNF mediated apoptosis. Inhibition of apoptosis triggered by activation of the Fas or TNF receptors can be blocked at a variety of points by poxvirus encoded proteins. M-T2 binds to soluble TNF preventing signalling through the TNF-receptor. Ligand interaction with the Fas or TNF-receptor initiates recruitment of adaptor molecules such as TRADD and FADD, which in turn recruit and promote the activation of group 3 caspases such as FLICE/caspase 8. This step can be blocked by v-FLIPs such as MC159. Group 3 caspases act as the first step in a proteinase cascade and activate group 2 effector caspases by cleaving the pro-caspase to produce the active form of the enzyme. Inhibition of group 3 caspases by the intracellular poxvirus serpins of the Spi-2/crma family can block apoptosis. Activated group 3 caspases are shown as heterodimers of two regulatory (white) and two catalytic (gray) domains. Group 2 caspases cleave apoptotic target proteins causing many of the phenotypic changes observed in apoptosis.

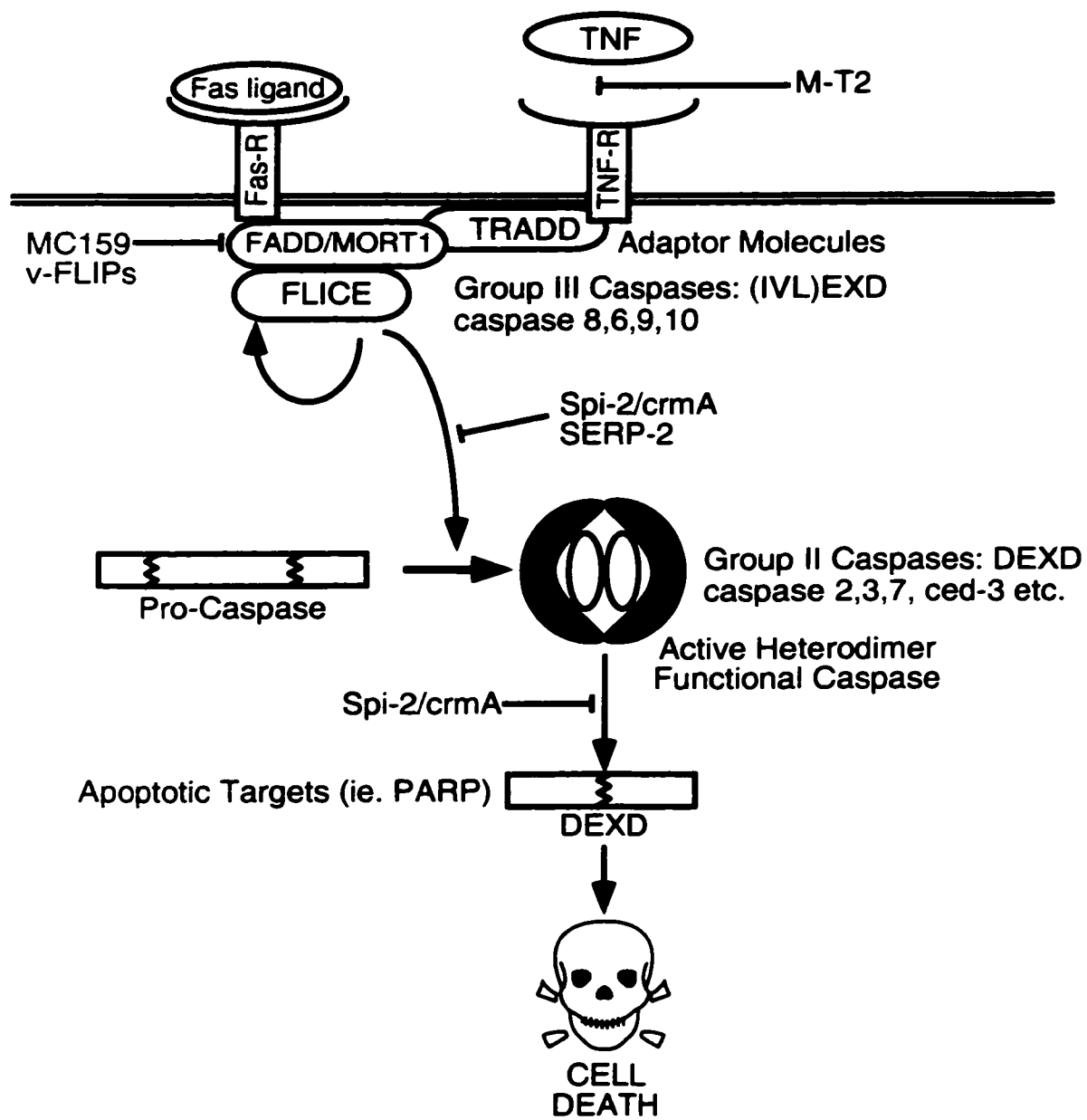
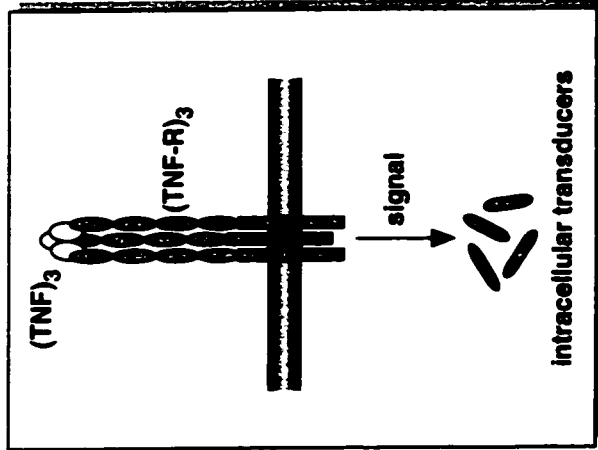
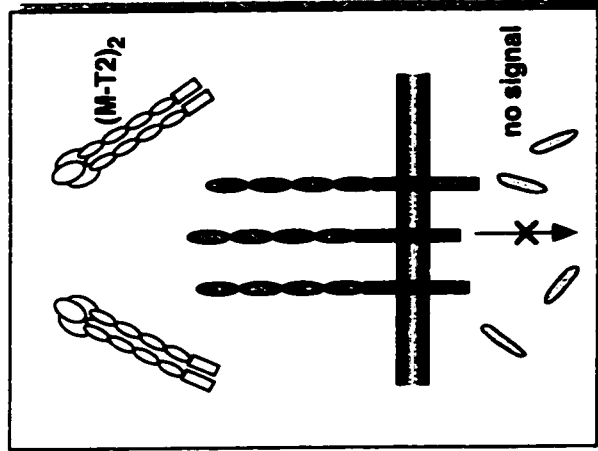


Figure I-5: Inhibition of TNF and TNF-receptor signaling by a poxvirus TNF-receptor homolog. A, Signaling through the TNF-receptor requires trimeric TNF ligand to oligomerize the receptor. B, the myxoma virus protein M-T2 binds to TNF ligand preventing signaling through the TNF receptor. M-T2 is secreted as both a monomer and a dimer, but only the dimer can effectively inhibit signaling by the TNF trimer. C, a hypothetical interaction between M-T2 and a TNF receptor monomer acts in a dominant negative manner to block receptor oligomerization and signaling. In theory, M-T2 could target other members of the TNF-receptor superfamily in this fashion.

A. Signalling through the TNF receptor



B. M-T2 binds TNF preventing signalling through the TNF-R



C. M-T2/TNF-R oligomerization blocks signalling

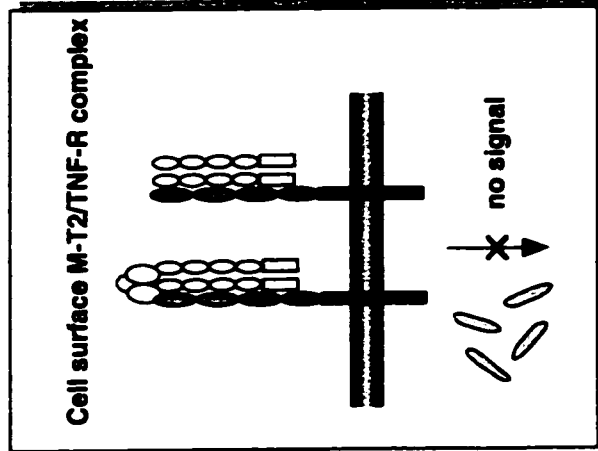
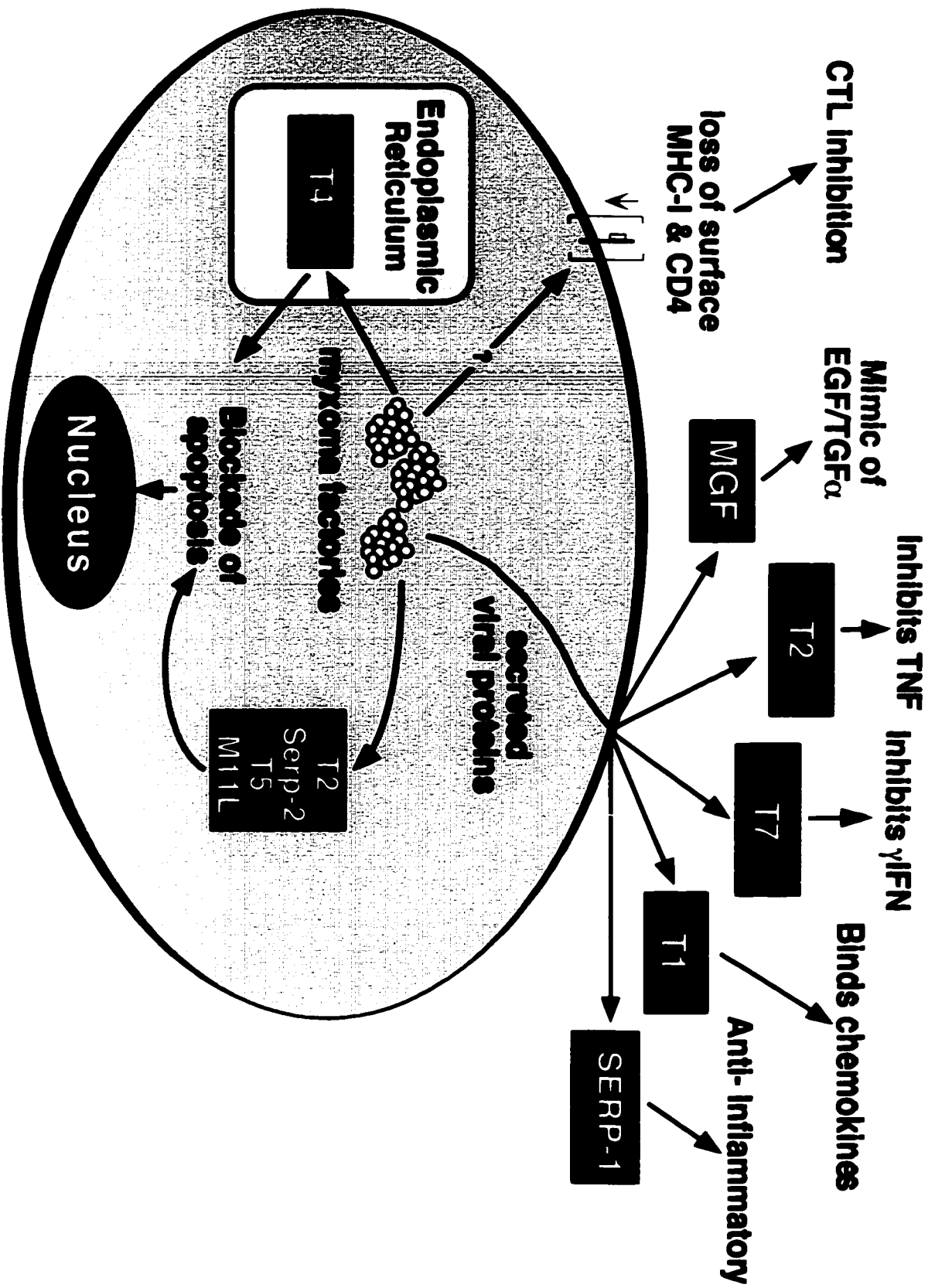


Figure I-6: Secondary structure prediction of M-T5. The ankyrin repeat domains (ARD1, ARD2, and ARD3) of M-T5 were subjected to secondary structure analysis using PREDATOR (EMBL, Heidelberg, Germany). Regions predicted to have beta (β) sheet characteristics and alpha helix (α) characteristics are indicated below each M-T5 ARD. The black diamonds above selected amino acid residues indicate the residues believed to be important in $\text{I}\kappa\text{B}$ nuclear localization. The ankyrin consensus sequence is indicated below the aligned ankyrin repeat domain of M-T5.

DGRYPLLCLLENDRINTARFVRYMIDRGTSVYVRGT **ARD1**
~~~~~~  
LDFTPINYCVI<sup>↓</sup>HND<sup>↓</sup>RRT<sup>↓</sup>FDYLLER<sup>↓</sup>GADPNVVF    **ARD2**  
~~~~~~  
LGNSCLDLAVLNGNKYMVHRLLRKTITPDAYTR **ARD3**
~~~~~~  
  G  T P L H  A A  G H  V V  L L L  G A  N  T

**Figure I-7: Schematic view of Myxoma virus virulence factors.** A schematic representation of the virulence factors encoded by myxoma virus indicating their localization and general mode of action. The cytosolic myxoma factories are indicated in yellow as the production center for viral proteins. Secreted viroceptors and virokines include MGF, SERP-1, M-T1, M-T2, M-T7. The viromitigators SERP-2, M-T2, and M-T5 are shown as intracellular, though not necessarily cytoplasmic proteins, while the M-T4 viromitigator is localized to the endoplasmic reticulum. The downregulation of CD4 and class I MHC is indicated, though the exact mechanism remains elusive.



**CTL Inhibition**

loss of surface  
MHC-I & CD4

Mimic of  
EGF/TGF $\alpha$

**MGF**

**Inhibits TNF**    **Inhibits  $\gamma$ IFN**

**T2**

**T7**

**Binds chemokines**

**T1**

**Anti-Inflammatory**

**SERP-1**

**Endoplasmic Reticulum**

**T4**

**Myxoma factories**

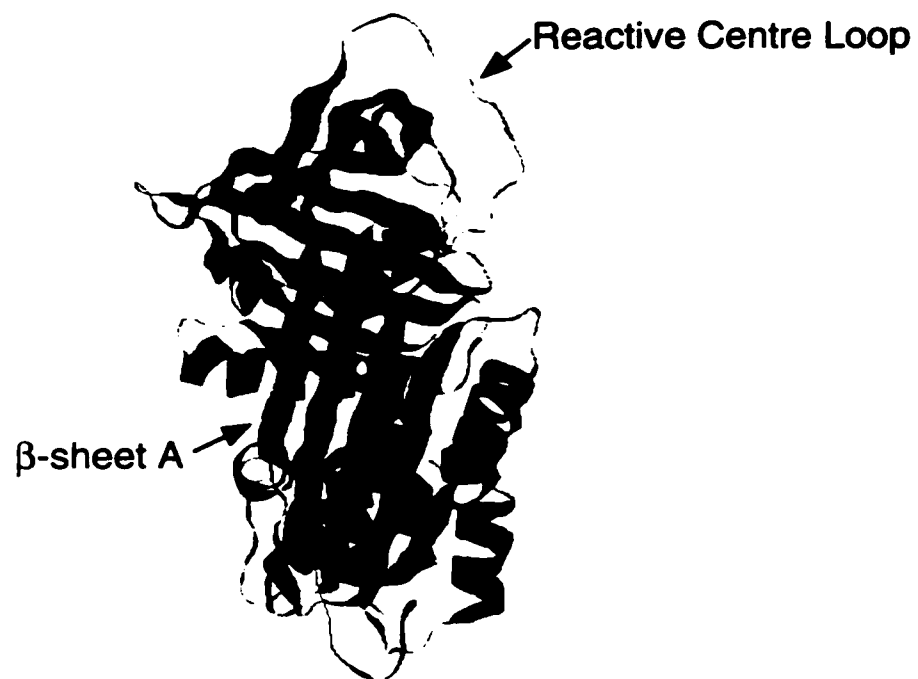
**secreted viral proteins**

**Blockade of apoptosis**

**T2**  
**Serp-2**  
**T5**  
**M11L**

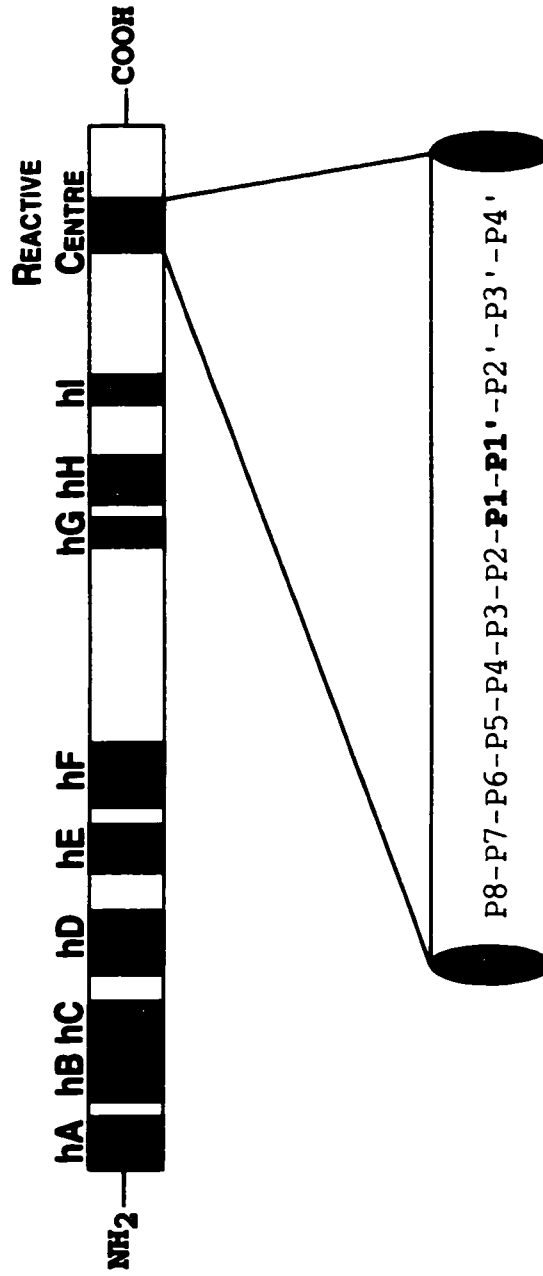
**Nucleus**

**Figure I-8: SERPIN structure.** A ribbon diagram of  $\alpha$ 1-antitrypsin showing the surface exposed reactive centre loop and the  $\beta$ -sheet A which together form the serpin "mousetrap" mechanism for serine proteinase inhibition. Based on the structure by Elliott et al. (1998) produced using RasMol for Macintosh.



**Figure I-9: A schematic representation of a SERPIN with RCL.** General schematic view of serpin structure indicating helixes A through I (light gray) and the reactive center loop (dark gray) expanded below. Comparison of the amino acid sequence of the reactive center loop regions of wild type SERP-1 (SERP-1); SERP-1 mutant SERP-1R319A/N320A (SAA); the Interleukin 1 $\beta$ -converting enzyme inhibitor from cowpox virus (Spi-2/crmA); plasminogen activator inhibitors 1 and 2 (PAI-1, PAI-2); antiplasmin; C1 inhibitor; and antichymotrypsin (Act). The P1-P1' residues surrounding the scissile bond are indicated in bold.

## SERP-1 is a Member of the SERPIN Superfamily



SERP-1  
 SAA  
 Spi-2/crma  
 PAI-1  
 PAI-2  
 Antiplasmin  
 C1 inhibitor  
 Act

T A I T L I P I L T T A T T  
 T A I C L G S I I K  
 A T A V V I I A V  
 P P A A G S A L  
 I I V S T M V T  
 P P A A G S A L  
**R A D R R R R L**  
**N A C M T M T S**  
 A A A A G S L A  
 L L S P H L L L  
 T T T E G S V V



**References**

- Aiken, C., Konner, J., Landau, N. R., Lenburg, M. E., and Trono, D. (1994). Nef induces CD4 endocytosis: Requirement for a critical dileucine motif in the membrane-proximal CD4 cytoplasmic domain. *Cell* **76**: 853-864.
- Alcami, A., and Smith, G. L. (1995). Cytokine receptors encoded by poxviruses: A lesson in cytokine biology. *Immunology Today* **16**: 474-478.
- Alcami, A., and Smith, G. L. (1995). Vaccinia, cowpox and camelpox viruses encode soluble gamma interferon receptors with novel broad species specificity. *J. Virol.* **69**: 4633-4639.
- Alcami, A., Symons, J. A., Collins, P. D., Williams, T. J., and Smith, G. L. (1998). Blockade of chemokine activity by a soluble chemokine binding protein from vaccinia virus. *J. Immunol.* **160**: 624-633.
- Ali, A. N., Turner, P. C., Brooks, M. A., and Moyer, R. W. (1994). The SPI-1 gene of rabbitpox virus determines host range and is required for hemorrhagic pox formation. *Virology* **201**: 305-314.
- Altieri, D. C. (1995). Xa receptor EPR-1. *FASEB J.* **9**: 860-865.
- Baggiolini, M. (1998). Chemokines and leukocyte traffic. *Nature* **392**: 565-568.
- Baldwin, A. S. J. (1996). The NF- $\kappa$ B and I $\kappa$ B proteins: New discoveries and insights. *Annu. Rev. Immunol.* **14**: 641-681.
- Barinaga, M. (1992). Viruses launch their own "Star Wars". *Science* **258**: 1730-1731.

- Barry, M., Hnatiuk, S., Mossman, K., Lee, S.-F., Boshkov, L., and McFadden, G. (1997). The myxoma virus M-T4 gene encodes a novel RDEL-containing protein that is retained within the endoplasmic reticulum and is important for the productive infection of lymphocytes. *Virology* **239**: 360-377.
- Barry, M., Lee, S. F., Boshkov, L., and McFadden, G. (1995). Myxoma virus induces extensive CD4 downregulation and dissociation of p56<sup>lck</sup> in infected rabbit CD4<sup>+</sup> T lymphocytes. *J. Virol.* **69**: 5243-5251.
- Barry, M., and McFadden, G. (1998). Virus encoded cytokines and cytokine receptors. *Parasitology* **115**: S89-S100.
- Bertin, J., Armstrong, R. C., Otilie, S., Martin, D. A., Wang, Y., Banks, S., Wang, G.-H., Senkevich, T. G., Alnemri, E. M., Moss, B., Lenardo, M. J., Tomaselli, K. J., and Cohen, J. I. (1997). Death effector domain-containing herpesvirus and poxvirus proteins inhibit both Fas- and TNFR1-induced apoptosis. *Proc. Natl. Acad. Sci. USA* **94**: 1772-1776.
- Blanton, R. E., Licate, L. S., and Aman, R. A. (1994). Characterization of a native and recombinant *Schistosoma haematobium* serine protease inhibitor gene product. *Mol. Biochem. Parasitol.* **63**: 1-11.
- Bode, W., and Huber, R. (1992). Natural protein proteinase-inhibitors and their interaction with proteinases. *Eur. J. Biochem.* **204**: 433-451.
- Boehm, U., Klamp, T., Groot, M., and Howard, J. C. (1997). Cellular responses to interferon- $\gamma$ . *Annu. Rev. Immunol.* **15**: 749-795.

- Boshkov, L. K., Macen, J. L., and McFadden, G. (1992). Virus-induced loss of class I MHC antigens from the surface of cells infected with myxoma virus and malignant rabbit fibroma virus. *J. Immunol.* **148**: 881-7.
- Buller, R. L. M., and Palumbo, G. J. (1991). Poxvirus pathogenesis. *Microbiol. Rev.* **55**: 80-122.
- Carrell, R. W., and Boswell, D. R. (1986). Serpins: A family of serine protease inhibitors. In *Protease Inhibitors*, A. Barrett and G. Salvesen, eds. (Amsterdam: Elsevier), pp. 403.
- Chang, W., Lim, J. G., Hellström, I., and Gentry, L. E. (1988). Characterization of vaccinia virus growth factor biosynthetic pathway with an antipeptide antiserum. *J. Virol.* **62**: 1080-1083.
- Chang, W., Macaulay, C., Hu, S. L., Tam, J. P., and McFadden, G. (1990). Tumorigenic poxviruses: characterization of the expression of an epidermal growth factor related gene in Shope fibroma virus. *Virology* **179**: 926-30.
- Chang, W., Upton, C., Hu, S. L., Purchio, A. F., and McFadden, G. (1987). The genome of Shope fibroma virus, a tumorigenic poxvirus, contains a growth factor gene with sequence similarity to those encoding epidermal growth factor and transforming growth factor  $\alpha$ . *Molecular and Cellular Biology* **7**: 535-540.
- Coughlin, S. R. (1994). Protease-activated receptors start a family. *Proc. Natl. Acad. Sci. USA* **91**: 9200-9202.

- Dafforn, T. R., Mahadeva, R., Elliott, P. R., Sivasothy, P., and Lomas, D. A. (1999). A kinetic mechanism for the polymerization of  $\alpha$ 1-antitrypsin. *J. Biol. Chem.* **274**: 9548-9555.
- Dedhar, S., and Hannigan, G. E. (1996). Integrin cytoplasmic interactions and bidirectional transmembrane signalling. *Current Opinion in Cell Biology* **8**: 657-659.
- DiGiacomo, R. F., and Mare, C. J. (1994). Viral Diseases. In *The Biology of the Laboratory Rabbit*, P. J. Manning, D. H. Ringler and C. E. Newcomer, eds. (New York and London: Academic Press), pp. 171-204.
- Elliott, P. R., Abrahams, J. P., and Lomas, D. A. (1998). Wild-type alpha 1-antitrypsin is in the canonical inhibitory conformation. *J. Mol. Biol.* **275**: 419-25.
- Enari, M., Hug, H., and Nagata, S. (1995). Involvement of an ICE-like protease in Fas-mediated apoptosis. *Nature* **375**: 78-81.
- Essani, K., Chalasani, S., Eversole, R., Beuving, L., and Birmingham, L. (1994). Multiple anti-cytokine activities secreted from tanapox virus-infected cells. *Microbial Pathogenesis* **17**: 347-353.
- Fenner, F., and Meyers, K. (1978). Myxoma virus and myxomatosis in retrospect: The first quarter century of a new disease. In *Viruses and the Environment*, E. Kurstok and K. Maramorosch, eds. (New York: Academic Press), pp. 539-570.
- Fenner, F., and Ratcliffe, F. N. (1965). *Myxomatosis* (London: Cambridge University Press).

- Fenner, F., Wittek, R., and Dumbell, K. R. (1989). The orthopoxviruses (New York: Academic).
- Forsyth, K. D., Talbot, V., and Beckman, I. (1994). Endothelial serpins--protectors of the vasculature? *Clinical & Experimental Immunology* **95**: 277-82.
- Gagliardini, V., Fernandez, P.-A., Lee, R. K. K., Drexler, H. C. A., Rotello, R. J., Fishman, M. C., and Yuan, J. (1994). Prevention of vertebrate neuronal death by the *crmA* gene. *Science* **263**: 826-828.
- Ghendler, Y., Arnon, R., and Fishelson, Z. (1994). Schistosoma mansoni: Isolation and characterization of Smpi56, a novel serine protease inhibitor. *Exp. Parasitol.* **78**: 121-131.
- Gooding, L. R. (1992). Virus proteins that counteract host immune defenses. *Cell* **71**: 5-7.
- Graham, K. A., Lalani, A. S., Macen, J. L., Ness, T. L., Barry, M., Liu, L.-Y., Lucas, A., Clark-Lewis, I., Moyer, R. W., and McFadden, G. (1997). The T1/35kDa family of poxvirus secreted proteins bind chemokines and modulate leukocyte influx into virus infected tissues. *Virology* **229**: 12-24.
- Graham, K. A., Opgenorth, A., Upton, C., and McFadden, G. (1992). Myxoma virus M11L ORF encodes a protein for which cell surface localization is critical in manifestation of viral virulence. *Virology* **191**: 112-124.
- Houenou, L. J., Turner, P. L., Li, L., Oppenheim, R. W., and Festoff, B. W. (1995). A serine protease inhibitor, protease nexin I, rescues motoneurons from

naturally occurring and axotomy-induced cell death. *Proc. Natl. Acad. Sci. USA* **92**: 895-9.

Howard, S., Chan, Y. S., and Smith, G. (1991). Vaccinia virus homologues of the Shope fibroma virus inverted terminal repeat proteins and a discontinuous ORF related to the tumor necrosis factor receptor family. *Virology* **180**: 633-647.

Hu, F.-Q., Smith, C. A., and Pickup, D. J. (1994). Cowpox virus contains two copies of an early gene encoding a soluble secreted form of the type II TNF receptor. *Virology* **204**: 343-356.

Kettle, S., Blake, N. W., Law, K. M., and Smith, G. L. (1995). Vaccinia virus serpins B13R (SPI-2) and B22R (SPI-1) encode M(r) 38.5 and 40K, intracellular polypeptides that do not affect virus virulence in a murine intranasal model. *Virology* **206**: 136-47.

Komiyama, T., Ray, C. A., Pickup, D. J., Howard, A. D., Thornberry, N. A., Peterson, E. P., and Salvesen, G. (1994). Inhibition of interleukin-1 beta converting enzyme by the cowpox virus serpin CrmA. An example of cross-class inhibition. *J. Biol. Chem.* **269**: 19331-7.

Kotwal, G. J., and Moss, B. (1988). Vaccinia virus encodes a secretory polypeptide structurally related to complement control proteins. *Nature* **335**: 176-181.

Kotwal, G. J., and Moss, B. (1989). Vaccinia virus encodes two proteins that are structurally related to members of the plasma serine protease inhibitor superfamily. *J. Virol.* **63**: 600-606.

Lalani, A. S., Graham, K., Mossman, K., Rajarathnam, K., Clark-Lewis, I., Kelvin, D., and McFadden, G. (1997). The purified myxoma virus IFN- $\gamma$  receptor

homolog, M-T7, interacts with the heparin binding domains of chemokines. *J. Virol.* **71**: 4356-4363.

Lalani, A. S., Masters, J. M., Graham, K. A., Liu, L.-Y., Lucas, A., and McFadden, G. (1999). The role of the myxoma virus soluble CC-chemokine inhibitor glycoprotein, M-T1, during myxoma virus pathogenesis. *Virology* **256**: 233-45.

Lalani, A. S., and McFadden, G. (1997). Secreted poxvirus chemokine binding proteins. *J. Leukoc. Biol.* **62**: 570-576.

Lalani, A. S., Ness, T. L., Singh, R., Harrison, J. K., Seet, B. T., Kelvin, D. J., McFadden, G., and Moyer, R. W. (1998). Functional comparisons among members of the poxvirus T1/35kDa family of soluble CC-chemokine inhibitor glycoproteins. *Virology* **250**: 173-184.

Lambert, S., Huilan, Y., Prchal, J. T., Lawler, J., Ruff, P., Speicher, D., Cheung, M. C., Kan, Y. W., and Palek, J. (1990). cDNA sequence for human erythrocyte ankyrin. *Proc. Natl. Acad. Sci. USA* **87**: 1730-1734.

Laskowski, M., and Kato, I. (1980). Protein inhibitors of proteinases. *Annu. Rev. Biochem.* **49**: 593-626.

Law, K. M., and Smith, G. L. (1992). A vaccinia serine protease inhibitor which prevents virus-induced cell fusion. *J. Gen. Virol.* **73**: 549-57.

Lawrence, D. A. (1997). The serpin-proteinase complex revealed. *Nat. Struct. Biol.* **4**: 339-41.

- Lawrence, D. A., Olson, S. T., Palaniappan, S., and Ginsburg, D. (1994). Serpin reactive center loop mobility is required for inhibitor function but not for enzyme recognition. *J. Biol. Chem.* **269**: 27657-62.
- Lijnen, H. R., and Collen, D. (1990). Regulation and control of the fibrinolytic system. In *Serine protease and their serpin inhibitors in the nervous system*, B. W. Festoff, ed. (New York: Plenum Press).
- Lin, Y.-Z., Caporaso, G., Chang, P.-Y., Ke, X.-H., and Tam, J. P. (1988). Synthesis of a biological active tumor growth factor from the predicted DNA sequence of Shope fibroma virus. *Biochemistry* **27**: 5640-5645.
- Lin, Y.-Z., Le, X.-H., and Tam, J. P. (1991). Synthesis and structure-activity study of myxoma virus growth factor. *Biochemistry* **30**: 3310-3314.
- Liu, L. X., Margottin, F., Le Gall, S., Schwartz, O., Selig, L., Benarous, R., and Benichou, S. (1997). Binding of HIV-1 Nef to a novel thioesterase enzyme correlates with Nef-mediated CD4 downregulation. *J. Biol. Chem.* **272**: 13779-13785.
- Loebermann, H., Tokuoka, R., Deisenhofer, J., and Huber, R. (1984). Human  $\alpha$  1-proteinase inhibitor: Crystal structure analysis of two crystal modifications, molecular model and preliminary analysis of the implications for function. *J. Mol. Biol.* **177**: 531-557.
- Lucas, A., Liu, L., Macen, J. L., Nash, P. D., Dai, E., Stewart, M., Yan, W., Graham, K., Etches, W., Boshkov, L., Nation, P. N., Humen, D., Hobman, M., and McFadden, G. (1996). Virus-encoded serine proteinase inhibitor SERP-1



inhibits atherosclerotic plaque development following balloon angioplasty. *Circulation* **94**: 2890-2900.

Luster, A. D. (1998). Chemokines: Chemotactic cytokines that mediate inflammation. *New England Journal of Medicine* **338**: 436-445.

Macen, J. L., Graham, K. A., Lee, S. F., Schreiber, M., Boshkov, L. K., and McFadden, G. (1996). Expression of the myxoma virus tumor necrosis factor receptor homologue (T2) and M11L genes is required to prevent virus-induced apoptosis in infected rabbit T lymphocytes. *Virology* **218**: 232-237.

Macen, J. L., Graham, K. A., Lee, S. F., Schreiber, M., Boshkov, L. K., and McFadden, G. (1996). Expression of the myxoma virus tumor necrosis factor receptor homologue and M11L genes is required to prevent virus-induced apoptosis in infected rabbit T lymphocytes. *Virology* **218**: 232-237.

Macen, J. L., Upton, C., Nation, N., and McFadden, G. (1993). SERP1, a serine proteinase inhibitor encoded by myxoma virus, is a secreted glycoprotein that interferes with inflammation. *Virology* **195**: 348-63.

Maksymowych, W. P., Nation, N., Nash, P. D., Macen, J., Lucas, A., McFadden, G., and Russell, A. S. (1996). Amelioration of antigen-induced arthritis in rabbits treated with a secreted viral serine proteinase inhibitor. *J. Rheumatol.* **23**: 878-882.

Martinez-Pomares, L., Thompson, J. P., and Moyer, R. W. (1995). Mapping and investigation of the role in pathogenesis of the major unique secreted 35-kDa protein of rabbitpox Virus. *Virology* **206**: 591-600.

- Massung, R. F., Esposito, J. J., Liu, L. I., Qi, J., Utterback, T. R., Knight, J. C., Aubin, L., Yuran, T. E., Parsons, J. M., Loparev, V. N., Selivanov, N. A., Cavallaro, K. F., Kerlavage, A. R., Mahy, B. W. J., and Venter, J. C. (1993). Potential virulence determinants in terminal regions of variola smallpox virus genome. *Nature* **366**: 748-751.
- Massung, R. F., Liu, L. I., Qi, J., Knight, J. C., Yuran, T. E., Kerlavage, A. R., Parsons, J. M., Venter, J. C., and Esposito, J. J. (1994). Analysis of the complete genome of smallpox variola major virus strain Bangladesh-1975. *Virology* **201**: 215-240.
- Matsuda, Y., Kawata, S., Nagase, T., Maeda, Y., Yamasaki, E., Kiso, S., Ishiguro, H., and Matsuzawa, Y. (1994). Interleukin-6 in transcatheter arterial embolization for patients with hepatocellular carcinoma. Effects of serine protease inhibitor. *Cancer* **73**: 53-7.
- McFadden, G. (1995). Viroceptors, virokines and related immune modulators encoded by DNA Viruses (Austin, Texas: R. G. Landes Co.).
- McFadden, G., and Barry, M. (1998). How poxviruses oppose apoptosis. *Semin. Virology* **8**: 429-442.
- McFadden, G., Graham, K., and Opgenorth, A. (1995). Poxvirus growth factors. In Virokines, viroceptors and related modulators encoded by DNA viruses, G. McFadden, ed. (Austin, TX: R. G. Landes Co.).
- McFadden, G., Lalani, A., Everett, H., Nash, P., and Xu, X. (1998). Virus encoded-receptors for cytokines and chemokines. *Seminars in Cell and Developmental Biology* **9**: 359-368.

- Messud-Petit, F., Gelfi, J., Delverdier, M., Amardeilh, M. F., Py, R., Sutter, G., and Bertagnoli, S. (1998). SERP-2, an inhibitor of the interleukin-1 $\beta$ -converting enzyme, is critical in the pathobiology of myxoma virus. *J. Virol.* **72**: 7830-7839.
- Miskin, J. E., Abrams, C. C., Goatley, L. C., and Dixon, L. K. (1998). A viral mechanism for inhibition of the cellular phosphatase calcineurin. *Science* **281**: 562-565.
- Miura, M., Zhu, H., Rotello, R., Hatweig, E. A., and Yuan, J. (1993). Induction of apoptosis in fibroblasts by IL-1 $\beta$ -converting enzyme, a mammalian homolog of the *C. elegans* cell death gene *ced-3*. *Cell* **75**: 653-660.
- Miura, M., Zhu, H., Rotello, R., Hatweig, E. A., and Yuan, J. (1993). Induction of apoptosis in fibroblasts by IL-1 $\beta$ -converting enzyme, a mammalian homolog of the *C. elegans* cell death gene *ced-3*. *Cell* **75**: 653-660.
- Moss, B. (1990). Poxviridae and their replication. In *Virology*, B. N. Fields and D. M. Knipe, eds. (New York: Raven Press, Ltd.), pp. 2079-2111.
- Moss, B. (1996). Replication of poxviruses. In *Virology*, B. N. Fields, D. M. Knipe and P. M. Howley, eds. (New York: Lippincott Raven Press), pp. 2637-2671.
- Mossman, K., Lee, S. F., Barry, M., Boshkov, L., and McFadden, G. (1996). Disruption of M-T5, a novel myxoma virus gene member of the poxvirus host range superfamily, results in dramatic attenuation of myxomatosis in infected European rabbits. *J. Virol.* **70**: 4394-4410.

- Mossman, K., Nation, P., Macen, J., Garbutt, M., Lucas, A., and McFadden, G. (1996). Myxoma virus M-T7, a secreted homolog of the interferon- $\gamma$  receptor, is a critical virulence factor for the development of myxomatosis in European rabbits. *Virology* **215**: 17-30.
- Mossman, K., Upton, C., Buller, R. M. L., and McFadden, G. (1995a). Species specificity of ectromelia virus and vaccinia virus interferon- $\gamma$  binding proteins. *Virology* **208**: 762-769.
- Mossman, K., Upton, C., and McFadden, G. (1995). The myxoma virus-soluble interferon- $\gamma$  receptor homolog, M-T7, inhibits interferon- $\gamma$  in a species-specific manner. *J. Biol. Chem.* **270**: 3031-3038.
- Mottonen, J., Strand, A., Symersky, J., Sweet, R. M., Danley, D. E., Geoghegan, K. F., Gerard, R. D., and Goldsmith, E. J. (1992). Structural basis of latency in plasminogen activator inhibitor-1. *Nature* **355**: 270-3.
- Nash, P., Lucas, A., and McFadden, G. (1997). SERP-1, a poxvirus-encoded serpin, is expressed as a secreted glycoprotein that inhibits the inflammatory response to myxoma virus infection. In *Chemistry and Biology of Serpins*, F. C. Church, D. D. Cunningham, D. Ginsburg, M. Hoffman, S. R. Stone and D. M. Tollefsen, eds. (New York: Oxford University Press), pp. 195-205.
- Nash, P., Whitty, A., Handwerker, J., Macen, J., and McFadden, G. (1998). Inhibitory specificity of the anti-inflammatory myxoma virus serpin, Serp-1. *J. Biol. Chem.* **273**: 20982-20991.
- Nicholson, D. W., and Thornberry, N. A. (1997). Caspases: Killer proteases. *Trends in Biochemical Sciences* **22**: 299-306.

- Nystedt, S., Emilsson, K., Wahlestedt, C., and Sundelin, J. (1994). Molecular cloning of a potential proteinase activated receptor. *Proc. Natl. Acad. Sci. USA* **91**: 9208-9212.
- O'Brien, V. (1998). Viruses and apoptosis. *J. Gen. Virol.* **79**: 1833-1845.
- O'Neill, G. (1994). Australia's most wanted. *Time* **14**: 48-53.
- Olds, R. J., Lane, D. A., Mille, B., Chowdhury, V., and Thein, S. L. (1994). Antithrombin: the principle inhibitor of thrombin. *Seminars in Thrombosis and Hemostasis* **20**: 353-372.
- Olson, S. T., and Bjork, I. (1994). Regulation of thrombin activity by antithrombin and heparin. *Seminars in Thrombosis and Hemostasis* **20**: 373-409.
- Opgenorth, A., Graham, K., Nation, N., Strayer, D., and McFadden, G. (1992). Deletion analysis of two tandemly arranged virulence genes in myxoma virus, M11L and myxoma growth factor. *J. Virol.* **66**: 4720-4731.
- Opgenorth, A., Nation, N., Graham, K., and McFadden, G. (1993). Transforming growth factor  $\alpha$ , Shope fibroma growth factor, and vaccinia growth factor can replace myxoma growth factor in the induction of myxomatosis in rabbits. *Virology* **192**: 701-709.
- Patel, A. H., Gaffney, D. F., Subak-Sharpe, J. H., and Stow, N. D. (1990). DNA sequence of the gene encoding a major secreted protein of vaccinia virus, strain Lister. *J. Gen. Virol.* **71**: 2013-2021.

- Pelham, H. R. B. (1989). Control of protein exit from the endoplasmic reticulum. *Annual Review of Cell Biology* **5**: 1-23.
- Pelham, H. R. B. (1990). The retention signal for soluble proteins of the endoplasmic reticulum. *Trends Biochem. Sci.* **15**: 483-486.
- Perkus, M. E., Goebel, S. J., Davis, S. W., Johnson, G. P., Norton, E. K., and Paoletti, E. (1991). Deletion of 55 open reading frames from the termini of vaccinia virus. *Virology* **180**: 406-410.
- Petit, F., Bertagnoli, S., Gelfi, J., Fassy, F., Boucraut-Baralon, C., and Milon, A. (1996). Characterization of a myxoma virus-encoded serpin-like protein with activity against interleukin-1 $\beta$  converting enzyme. *J. Virol.* **70**: 5860-5866.
- Pickup, D. J. (1994). Poxviral modifiers of cytokine responses to infection (New York: Raven Press, Ltd.), pp. 116-127.
- Pickup, D. J., Ink, B. S., Hu, W., Ray, C. A., and Joklik, W. K. (1986). Hemorrhage in lesions caused by cowpox virus is induced by a viral protein that is related to plasma protein inhibitors of serine protease. *Proc. Natl. Acad. Sci. USA* **83**: 7698-7702.
- Ploegh, H. L. (1998). Viral strategies of immune evasion. *Science* **280**: 248-253.
- Potempa, J., Korzus, E., and Travis, J. (1994). The serpin superfamily of proteinase inhibitors: structure, function, and regulation. [Review]. *J. Biol. Chem.* **269**: 15957-60.

- Quan, L. T., Caputo, A., Bleackley, R. C., Pickup, D. J., and Salvesen, G. S. (1995). Granzyme B is inhibited by the cowpox virus serpin cytokine response modifier A. *J. Biol. Chem.* **270**: 10377-10379.
- Ray, C. A., Black, R. A., Kronheim, S. R., Greenstreet, T. A., Sleath, P. R., Salvesen, G. S., and Pickup, D. J. (1992). Viral inhibition of inflammation: Cowpox virus encodes an inhibitor of the interleukin-1 $\beta$  converting enzyme. *Cell* **69**: 597-604.
- Revilla, Y., Cebrián, A., Baixeras, E., Martínez, C., Viñuela, E., and Salas, M. L. (1997). Inhibition of apoptosis by the African swine fever virus bcl-2 homologue: Role of the BH1 domain. *Virology* **228**: 400-404.
- Robichaud, J. (1999). Mutational analysis of the myxoma virus T1 protein reveals essential domains necessary for M-T1 function. Thesis in Microbiology and Immunology at The University of Western Ontario, London.
- Rubin, H. (1996). Serine protease inhibitors (SERPINS): where mechanism meets medicine. *Nature Medicine* **2**: 632-633.
- Sachdev, S., Hoffman, A., and Hannink, M. (1998). Nuclear localization of I $\kappa$ B is mediated by the second ankyrin repeat: The I $\kappa$ B $\alpha$  ankyrin repeats define a novel class of *cis*-acting nuclear import sequences. *Mol. Cell. Biol.* **18**: 2524-2534.
- Sarin, A., Adams, D. H., and Henkart, P. A. (1993). Protease inhibitors selectively block T cell receptor-triggered programmed cell death in a murine T cell hybridoma and activated peripheral T cells. *J. Exp. Med.* **178**: 1693-700.

- Schreiber, M., and McFadden, G. (1996). Mutational analysis of the ligand binding domain of M-T2 protein, the tumor necrosis factor receptor homologue of myxoma virus. *J. Immunol.* **157**: 4486-4495.
- Schreiber, M., and McFadden, G. (1994). The myxoma virus TNF-receptor homologue inhibits TNF $\alpha$  in a species specific fashion. *Virology* **204**: 692-705.
- Schreiber, M., Rajarathnam, K., and McFadden, G. (1996). Myxoma virus T2 protein, a tumor necrosis factor (TNF) receptor homolog, is secreted as a monomer and dimer that each bind rabbit TNF $\alpha$ , but the dimer is a more potent TNF inhibitor. *J. Biol. Chem.* **271**: 13333-13341.
- Schreiber, M., Sedger, L., and McFadden, G. (1997). Distinct domains of M-T2, the myxoma virus TNF receptor homolog, mediate extracellular TNF binding and intracellular apoptosis inhibition. *J. Virol.* **71**: 2171-2181.
- Schreuder, H. A., de Boer, B., Dijkema, R., Mulders, J., Theunissen, H. J. M., Grootenhuis, P. D. J., and Hol, W. G. J. (1994). The intact and cleaved human antithrombin III complex as a model for serpin-proteinase interactions. *Nat. Struct. Biol.* **1**: 48-54.
- Shchelkunov, S. N., Blinov, V. M., and Sandakhchiev, L. S. (1993). Genes of variola and vaccinia viruses necessary to overcome the host protective mechanisms. *FEBS Lett.* **319**: 80-3.
- Shisler, J. L., Senkevich, T. G., Berry, M. J., and Moss, B. (1998). Ultraviolet-induced cell death blocked by a selenoprotein from a human dermatotropic poxvirus. *Science* **279**: 102-105.



- Smirnova, I. V., Ho, G. J., Fenton, J. W. n., and Festoff, B. W. (1994). Extravascular proteolysis and the nervous system: serine protease/serpin balance. [Review]. *Seminars in Thrombosis & Hemostasis* **20**: 426-32.
- Smith, C. A., Davis, T., Anderson, D., Solam, L., Beckmann, M. P., Jerzy, R., Dower, S. K., Cosman, D., and Goodwin, R. G. (1990). A receptor for tumor necrosis factor defines an unusual family of cellular and viral proteins. *Science* **248**: 1019-1023.
- Smith, C. A., Smith, T. D., Smolak, P. J., Friend, D., Hagen, H., Gerhart, M., Park, L., Pickup, D. J., Torrance, D., Mohler, K., Schooley, K., and Goodwin, R. G. (1997). Poxvirus genomes encode a secreted soluble protein that preferentially inhibits  $\beta$  chemokine activity yet lacks sequence homology to known chemokine receptors. *Virology* **236**: 316-327.
- Smith, G. L. (1993). Vaccinia virus glycoproteins and immune evasion. *J. Gen. Virol.* **74**: 1725-1740.
- Smith, G. L. (1994). Virus strategies for evasion of the host response to infection. *Trends Microbiol.* **2**: 81-88.
- Smith, G. L., Howard, S. T., and Chan, Y. S. (1989). Vaccinia virus encodes a family of genes with homology to serine proteinase inhibitors. *J. Gen. Virol.* **70**: 2333-2343.
- Spriggs, M. K. (1996). One step ahead of the game: Viral immunomodulatory molecules. *Annu. Rev. Immunol.* **14**: 101-131.

- Srinivasula, S. M., Ahmad, M., Fernandes-Alnemri, T., Litwack, G., and Alnemri, E. S. (1996). Molecular ordering of the Fas-apoptotic pathway: The Fas/APO-1 protease Mch5 is a CrmA-inhibitable protease that activates multiple Ced-3/ICE-like cysteine proteases. *Proc. Natl. Acad. Sci. USA* **93**: 14486-14491.
- Stein, P., and Chothia, C. (1991). Serpin tertiary structure transformation. [Review]. *J. Mol. Biol.* **221**: 615-21.
- Stein, P. E., and Carrell, R. W. (1995). What do dysfunctional serpins tell us about molecular mobility and disease?. [Review]. *Nat. Struct. Biol.* **2**: 96-113.
- Stein, P. E., Leslie, A. G., Finch, J. T., and Carrell, R. W. (1991). Crystal structure of uncleaved ovalbumin at 1.95 Å resolution. *J. Mol. Biol.* **221**: 941-59.
- Stone, S. R., Whisstock, J. C., Bottomley, S. P., and Hopkins, P. C. (1997). SERPINS: a mechanistic class of their own. *Adv Exp Med Biol* **425**: 5-15.
- Stratikos, E., and Gettins, P. G. (1997). Major proteinase movement upon stable serpin-proteinase complex formation. *Proc. Natl. Acad. Sci. USA* **94**: 453-458.
- Strayer, D. (1989). Poxviruses. In *Virus-induced Immunosuppression*, S. Specter, M. Bendinelli and H. Friedman, eds. (New York: Plenum), pp. 173-192.
- Strayer, D. S., Yang, S., and Schwartz, M. S. (1993). Epidermal growth factor-like growth factors. I. Breast malignancies and other epithelial proliferations in transgenic mice. *Laboratory Investigation* **69**: 660-673.
- Strickland, D. K., Kounnas, M. Z., and Argraves, S. (1995). LDL receptor-related protein: A multiligand receptor for lipoprotein and proteinase catabolism. *FASEB Journal* **9**: 890-898.

- Stroobant, P., Rice, A. P., Gullick, J. W., Cheng, D. J., Kerr, I. M., and Waterfield, M. D. (1985). Purification and characterization of vaccinia virus growth factor. *Cell* **42**: 383-393.
- Tewari, M., and Dixit, V. M. (1995). Fas- and tumor necrosis factor-induced apoptosis is inhibited by the poxvirus crmA gene product. *J. Biol. Chem.* **270**: 3255-60.
- Tewari, M., Tlford, W. G., Miller, R. A., and Dixit, V. M. (1995). CrmA, a poxvirus-encoded serpin, inhibits cytotoxic T-lymphocyte mediated apoptosis. *J. Biol. Chem.* **270**: 22705-22708.
- Thornberry, N. A., and Lazebnik, Y. (1998). Caspases: Enemies within. *Science* **281**: 1312-1316.
- Turner, P. C., and Moyer, R. W. (1998). The control of apoptosis by poxviruses. *Semin. Virology* **8**: 453-469.
- Turner, P. C., and Moyer, R. W. (1990). The molecular pathogenesis of poxviruses. In *Poxviruses*, R. W. Moyer and P. C. Turner, eds. (Berlin: Springer-Verlag). pp. 125-151.
- Turner, P. C., and Moyer, R. W. (1995). Orthopoxvirus fusion inhibitor glycoprotein SPI-3 (open reading frame K2L) contains motifs characteristic of serine proteinase inhibitors that are not required for control of cell fusion. *J. Virol.* **69**: 5978-87.
- Turner, P. C., and Moyer, R. W. (1992). An orthopoxvirus serpinlike gene controls the ability of infected cells to fuse. *J. Virol.* **66**: 2076-85.

- Turner, P. C., Musy, P. W., and Moyer, R. W. (1995). Poxvirus serpins. In *In Viroceptors, Virokines and Related Immune Modulators Encoded By DNA Viruses*, G. McFadden, ed. (Austin, TX: R.G. Landes Company), pp. 67-88.
- Tzahar, E., Moyer, J. D., Waterman, H., Barbacci, E. G., Levkowitz, G., Shelly, M., Strano, S., Pinkas-Kramarski, R., Pierce, J. H., Andrews, G. C., and Yarden, Y. (1998). Pathogenic poxviruses reveal viral strategies to exploit the ErbB signaling network. *EMBO Journal* **17**: 5948-5963.
- Upton, C., DeLange, A. M., and McFadden, G. (1987). Tumorigenic poxviruses: genomic organization and DNA sequence of the telomeric region of the Shope fibroma virus genome. *Virology* **160**: 20-30.
- Upton, C., Macen, J. L., and McFadden, G. (1987). Mapping and sequencing of a gene from myxoma virus that is related to those encoding epidermal growth factor and transforming growth factor alpha. *J. Virol.* **61**: 1271-5.
- Upton, C., Macen, J. L., Schreiber, M., and McFadden, G. (1991). Myxoma virus expresses a secreted protein with homology to the tumor necrosis factor receptor gene family that contributes to viral virulence. *Virology* **184**: 370-382.
- Upton, C., Macen, J. L., Wishart, D. S., and McFadden, G. (1990). Myxoma virus and malignant rabbit fibroma virus encode a serpin-like protein important for virus virulence. *Virology* **179**: 618-631.
- Upton, C., Mossman, K., and McFadden, G. (1992). Encoding of a homolog of the interferon- $\gamma$  receptor by myxoma virus. *Science* **258**: 1369-1372.

- Whisstock, J., Skinner, R., and Lesk, A. M. (1998). An atlas of serpin conformations. *Trends Biochem. Sci.* **23**: 63-67.
- White, E. (1996). Life, death, and the pursuit of apoptosis. *Genes & Devo.* **10**: 1-15.
- Wilczynska, M., Fa, M., Karolin, J., Ohlsson, P.-I., Johansson, L., and Ny, T. (1997). Structural insights into serpin-protease complexes reveal the inhibitory mechanism of serpins. *Nat. Struct. Biol.* **4**: 354-356.
- Wilczynska, M., Fa, M., Ohlsson, P.-I., and Ny, T. (1995). The inhibition mechanism of serpins. *J. Biol. Chem.* **270**: 29652-29655.
- Willey, R. L., Maldarelli, F., Martin, M. A., and Strebel, K. (1992). Human immunodeficiency virus type 1 Vpu protein induces rapid degradation of CD4. *J. Virol.* **66**: 7193-7200.
- Wold, W. S. M., and Gooding, L. (1991). Region E3 of adenovirus: A cassette of genes involved in host immunosurveillance and virus cell interactions. *Virology* **184**: 1-8.
- Wold, W. S. M., and Gooding, L. R. (1989). Adenovirus region E3 proteins that prevent cytolysis by cytotoxic T cells and tumor necrosis factor. *Molecular Biology and Medicine* **6**: 433-452.
- Wold, W. S. M., Hermiston, T. W., and Tollefson, A. E. (1994). Adenovirus proteins that subvert host defenses. *Trends Microbiol.* **2**: 437-443.
- Wong, G. H. W., Kamb, A., and Goeddel, D. V. (1992). Antiviral properties of TNF. In *Tumor Necrosis Factors: The Molecules and Their Emerging Role in Medicine*, B. Beutler, ed. (New York: Raven Press, Ltd.), pp. 371-381.

Yenbutr, P., and Scott, A. L. (1995). Molecular cloning of a serine proteinase inhibitor from *Brugia malayi*. *Infection & Immunity* **63**: 1745-53.

Zhou, J., Sun, X. Y., Fernando, G. J., and Frazer, I. H. (1992). The vaccinia virus K2L gene encodes a serine protease inhibitor which inhibits cell-cell fusion. *Virology* **189**: 678-86.

## CHAPTER II

### INHIBITORY SPECIFICITY OF THE ANTI INFLAMMATORY MYXOMA VIRUS SERPIN, SERP-1.<sup>1</sup>

There is one thing even more vital to science than intelligent methods;  
and that is, the sincere desire to find out the truth, whatever it may be.

*-Charles Sanders Pierce -*

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## INTRODUCTION

Serpins comprise a conserved superfamily of single-chain proteins that is widely distributed in nature. Almost all serpin family members have activity as inhibitors of serine proteinases, and serpins have been shown to serve in the regulation of a variety of proteinase-mediated processes including extracellular matrix remodeling, modulation of inflammatory responses, fibrinolysis, complement activation and blood coagulation (Rubin, 1996). At least 90 naturally occurring serpin mutations have been associated with human disease (Stein and Carrell, 1995). Given the critical role that proteinases and serpins play in the maintenance of homeostasis, it is not surprising that certain parasitic organisms use serpins to disrupt host processes that would otherwise be antagonistic to the parasite's survival. Several filarial parasites have been reported to express serpins (Blanton et al., 1994; Ghendler et al., 1994; Yenbutr and Scott, 1995), as have members of the poxvirus family of large DNA viruses (Turner et al., 1995). Myxoma virus, a leporipoxvirus, expresses SERP-1, the only known virus-encoded serpin that is secreted from infected cells (Macen et al., 1993; Nash et al., 1997). Infection with myxoma virus produces a rapidly lethal disease state in European rabbits (*Oryctolagus cuniculus*) known as myxomatosis (Fenner and Meyers, 1978; Fenner and Ratcliffe, 1965), a characteristic feature of which is the specific down-regulation of host inflammatory and immune responses (Nash et al., 1999). Myxoma virus causes severe immune dysregulation which disrupts the ability of the infected host to combat the infection; morbidity is



generally the result of supervening Gram-negative infections since the immunocompromised host is unable to counteract even routine bacterial assault. No single viral protein appears sufficient to cause this degree of immunodysfunction; rather, it is through the combined action of an array of secreted and intracellular factors that the virus accomplishes this task (McFadden, 1995). Interest in SERP-1 arose from its identification as the first virus-encoded serpin to be among these virulence factors. The secretion of SERP-1 from infected cells serves to dampen the *in vivo* inflammatory response to myxoma virus infection, and gene knock-out analysis revealed greater inflammation, more rapid resolution of the infection and a consequent attenuation of virulence upon infection of immunocompetent host animals (Macen et al., 1993; Upton et al., 1990). This finding suggests that SERP-1 contributes to viral pathogenesis by interacting with and inhibiting host proteins involved in the regulation of inflammation. Purified SERP-1 expressed from recombinant vaccinia virus has been tested in inflammatory models for restenosis following balloon angioplasty (Lucas et al., 1996) and rheumatoid arthritis (Maksymowych et al., 1996), and in both cases proved to be effective at reducing localized inflammation at very low levels of SERP-1 protein. SERP-1 is the product of a gene located within the terminal inverted repeat region of the myxoma genome (Block et al., 1985) and thus is present in two copies (Macen et al., 1993). The expressed gene product is a secreted, N-glycosylated protein with a predicted molecular weight of 39.9 kDa, though the mature glycoprotein migrates as a diffuse band of 50 - 55 kDa on SDS-PAGE. A preliminary survey of the inhibitory

properties of SERP-1 using unfractionated supernatants from myxoma virus-infected cells has been reported by Lomas *et al.* (Lomas *et al.*, 1993).

Serpins interact with proteinases via a flexible exposed loop, known as the reactive centre loop (RCL)<sup>1</sup>, that is critical to inhibitory function but is also susceptible to cleavage by non-target proteinases (Potempa *et al.*, 1994). The inhibitory specificity of serpins is largely defined by the nature of the residues at the P1-P1' positions (Schechter and Berger, 1967) that flank the site in the RCL at which the serpin becomes cleaved upon reaction with target proteinases (Carrell and Boswell, 1986; Carrell *et al.*, 1987). The branched kinetic mechanism in scheme II-1 has been proposed to account for currently available data on the mechanism of serpin action (Patston *et al.*, 1994; Wright and Scarsdale, 1994). A key feature of this mechanism is that it includes an intermediate, [EI], which can partition to react either through hydrolysis to release free enzyme, E, and cleaved serpin, I', or alternatively by undergoing a major conformational change leading to the formation of a highly stabilized covalent inhibited complex, EI\* (Engh *et al.*, 1995; Wilczynska *et al.*, 1997; Wright and Scarsdale, 1994). The structural rearrangement that produces EI\* is accompanied by insertion of a portion of the cleaved RCL into  $\beta$ -sheet A of the serpin structure (Huntington *et al.*, 1997), and results in a large increase in stability (Patston and Gettins, 1996). This substantial conformational rearrangement has been suggested to distort the geometry of key residues in the active site of the proteinase and to thereby decrease the ability of the enzyme to achieve its release from the serpin through enzyme-catalyzed deacylation (Rubin *et al.*, 1994). Dissociation of

EI\* to give active enzyme plus cleaved serpin is typically extremely slow (O' Malley et al., 1997).

The range of proteinases inhibited under a given set of experimental conditions has been established for many serpins, and in numerous cases the kinetics of interaction with one or more proteinase targets have been investigated. However, relatively little attention has been paid to determining which steps in the mechanism contribute to inhibitory specificity. The relative lack of emphasis on this issue may be because many of the earliest and most thoroughly investigated serpins are those found in the blood. These serpins typically circulate at concentrations which are orders of magnitude above their  $IC_{50}$  for interacting with target proteinases, such that specificity is governed almost entirely by relative on-rates. However, serpins such as SERP-1, or inhibitors of caspases and other intracellular targets, may act at much lower concentrations *in vivo*. Under these conditions, the possibility exists that other steps in the mechanism might influence whether or not a particular proteinase will be subject to effective inhibition by a given serpin.

In this chapter, we describe the purification to homogeneity of wild-type SERP-1, and of a R319A-N320A double mutant in which the predicted P1-P1' residues in the RCL are changed to alanine, expressed using a recombinant vaccinia virus expression system. Both purified wild-type SERP-1 and R319A/N320A were tested against a panel of proteinases for their ability to form SDS-resistant complexes, or to be cleaved at the RCL. We also describe a detailed kinetic study of the reactions of SERP-1 with a panel of six proteinases that were inhibited by the

serpin, using the techniques of slow-binding inhibition kinetics and inhibition titrations. We interpret the results of these studies in terms of the contributions that specific steps in the inhibition mechanism make towards controlling the inhibitory specificity of SERP-1 (Patston et al., 1994).

## **EXPERIMENTAL PROCEDURES**

**Materials:** The human enzymes plasmin, thrombin, trypsin, cathepsin G, C1s, tissue-type plasminogen activator (tPA, two chain form), and urokinase-type plasminogen activator (uPA, low molecular weight form) were obtained from Sigma, as were thermolysin, subtilisin and bovine chymotrypsin. Human neutrophil elastase (hNE), cathepsin G, and C1s were supplied by CalBiochem. Complement factor D was a generous gift of Fred Taylor of Biogen, Inc. The complement proteins factor B and C3, used for the generation of the alternative pathway C3 convertase, were obtained from Quidel (San Diego, CA). Polyacrylamide gels were from Novex (San Diego, CA). Proteinase substrates Chromozym-TRY, Chromozym-TPA, Chromozym-TH, Chromozym-U, and Chromozym-X were purchased from Boehringer-Mannheim, and D-Val-Leu-Lys-pNA was obtained from Sigma.

**Viruses and cell lines:** The complete SERP-1 gene, including the signal sequence, was inserted into the thymidine kinase locus of vaccinia virus strain WR under the

control of a strong, synthetic late promoter by homologous recombination as described (Macen et al., 1993), in order to produce a recombinant virus termed VV-S1. At late times (>8 hours) after infection of BGMK cells with VV-S1 virus, SERP-1 accumulates as a stable glycoprotein in the culture supernatant. Expression was confirmed by Western blot analysis using polyclonal rabbit anti-SERP-1 serum. At 20 hours post-infection, the supernatant was collected and SERP-1 was purified as described below.

**Preparation of a reactive center mutant of SERP-1:** Reactive center mutant SERP-1, designated R319A/N320A, was produced using the U.S.E. mutagenesis system (Pharmacia Biotech). SERP-1 in the p22MAP B/H-8 vector (Macen et al., 1993) was subjected to site directed mutagenesis using the oligonucleotide:

5'-ATCGCCGTGAGGGCGGCCGCGGGGATGAGGGTGAT-3'

which corresponds to a sequence change in the codons responsible for the P1 and P1' residues from Arg<sub>319</sub>-Asn<sub>320</sub> to Ala<sub>319</sub>-Ala<sub>320</sub>. The resulting mutant was confirmed by sequencing, and a 1344bp BamHI/BglII fragment containing the complete ORF was then ligated into the BamHI site of pMJ601 (Davidson and Moss, 1990). This pMJ-S1<sub>AA</sub> construct allowed insertion of the mutant SERP-1 gene into the TK gene of vaccinia virus under the control of a strong, synthetic late promoter using previously described methods (Macen et al., 1993). Expression of the R319A/N320A protein from the recombinant vaccinia virus, designated VV-S1<sub>AA</sub>, was confirmed by immunoblotting using rabbit polyclonal anti-SERP-1 antiserum (Macen et al., 1993).

**Purification of wild type and mutant SERP-1:** BGMK cells ( $2 \times 10^8$  cells) were adsorbed with VV-S1 or VV-S1<sub>AA</sub> at a multiplicity of infection of 1 pfu/cell for 2 hours at 37°C in 10 ml of DMEM containing 10% newborn calf serum. The inoculum was removed, and the cells were washed three times with 50 ml of sterile PBS to remove medium and serum proteins. Serum-free DMEM (15 ml) was added to each bottle and the infection was allowed to proceed for 20 hours at 37°C. The culture supernatant was collected, spun at 5000 x g to pellet cells and cell debris, and stored at -20°C. The medium containing secreted viral proteins was concentrated approximately 50-fold using an Amicon pressure cell equipped with a 10 kDa cut-off membrane, and was then dialyzed against 25 mM Tris, pH 8.0 at 4°C using Spectrapore dialysis tubing with a 30 kDa molecular weight cut-off. The dialyzed samples were spun at 14000 x g to remove precipitates, loaded onto a MonoQ anion exchange column (Pharmacia), and eluted with a linear salt gradient (0 - 300 mM NaCl in 25 mM Tris, pH 8.0). Fractions were analyzed by SDS-PAGE, and those containing SERP-1 were identified by immunoblotting using anti-SERP-1 antiserum (Macen et al., 1993), pooled, and concentrated to 1 ml using Centriprep 10 concentrators (Amicon). This material was further purified on a Superdex 75 gel filtration column (Pharmacia) (150 mM NaCl, 25 mM Tris, pH 8.0). Fractions were collected and analyzed for SERP-1 by SDS-PAGE, visualizing by silver staining and immunoblotting using anti-SERP-1 antiserum (Macen et al., 1993).

**Western blot analysis of the products of reaction of SERP-1 with proteinases:** SERP-1 (180 nM) was incubated with a slight molar excess of each proteinase for the

times indicated in a total reaction volume of 10  $\mu$ l (100 mM NaCl, 2 mM CaCl<sub>2</sub>, 0.005% Triton X-100, 100 mM Tris-HCl, pH 7.5). Control experiments established that both SERP-1 and the proteinases are stable under these conditions. Reactions were quenched by boiling for 5 minutes in SDS-loading buffer containing 100 mM dithiothreitol and 2% SDS. Samples were separated on 4 - 20% linear gradient or 10% Tris-glycine SDS polyacrylamide gels (Novex) using the Laemmli buffer system (Laemmli, 1970). Proteins were transferred to Hybond C-extra (Amersham) nitrocellulose by electroblotting, blots were blocked in TBS (150 mM NaCl, 2.5 mM KCl, 25 mM Tris-HCl, pH 7.4) containing 5% (w/v) skimmed milk powder and 0.1% (v/v) Tween-20 for >2 hours, and SERP-1 was detected by incubating for 1 hour with 0.05% (v/v) polyclonal rabbit anti-SERP-1 anti-serum (Macen et al., 1993) in blocking buffer. After washing with TBS containing 0.1% (v/v) Tween-20, a secondary goat anti-rabbit antibody conjugated to horseradish peroxidase (Biorad) was applied for 1 hour. Immunoreactive SERP-1 species were visualized using ECL detection (Amersham) on Kodak X-ray detection type film. Reactions that showed complete cleavage of SERP-1 by the proteinase, with no evidence of the formation of a stable SERP-1-enzyme complex, were repeated with shorter incubation times in order to see whether reactions proceeded via a transient inhibited complex. Reactions in which the serpin appeared unchanged at the end of the incubation period were repeated at higher enzyme concentrations and for longer incubation periods.

**Cleavage site sequence analysis:** The position at which SERP-1 becomes cleaved upon treatment with thrombin, cathepsin G or human neutrophil elastase, was

determined by N-terminal sequencing of the 5 kDa C-terminal cleavage fragment of SERP-1, according to Hewick *et al.* (Hewick *et al.*, 1981). Briefly, 30 pmol of SERP-1 was digested to completion by thrombin, cathepsin G or hNE. The C-terminal fragment of SERP-1 was separated by SDS-PAGE and transferred to ProBlott membrane (Applied Biosystems) by semi-dry electroblotting (10mM CAPS, 10% Methanol, pH 11). Protein bands were visualized by Coomassie Blue R-250 staining, and the 5 kDa band corresponding to the C-terminal fragment of SERP-1 was excised from the blot, washed in 50% HPLC-grade methanol/water, and air-dried. The excised membrane was loaded onto an Applied Biosystems (ABI) Model 470A gas phase sequencer and the resulting phenthiohydantoin (PTH) amino acids analyzed on-line using an ABI 120A analyzer equipped with a PTH C18 2.1 x 220 mm column. Data was analyzed using ABI 610A software.

**Standardization of enzyme and SERP-1 solutions:** Plasmin, thrombin and uPA were standardized by active site titration according to Method A of Jameson *et al.* (Jameson *et al.*, 1973). Briefly, enzyme was added at a final concentration of 10-60 nM to a stirred quartz cuvette containing 4-methylumbelliferyl *p*-guanidinobenzoate (MUGB, 0.8-2.0  $\mu$ M) in a total volume of 2.5 ml (100 mM Tris-Cl, 100 mM NaCl, 2 mM CaCl<sub>2</sub> and 0.005% Triton X-100, pH 7.5, 25°C). The molar concentration of active enzyme was determined from the magnitude of the burst in 4-methylumbelliferone (4-MU) formation upon addition of the enzyme, converted to molar units by reference to a standard curve for 4-MU fluorescence measured under identical conditions. Control experiments showed that the size of the burst was



directly proportional to the final concentration of each enzyme. Concentrations of active enzyme in each stock solution were determined from the mean of at least 3 measurements. Individual measurements differed from the mean by  $\leq 3\%$  for uPA and plasmin and by  $\leq 8\%$  for thrombin. Factor Xa reacted with MUGB too slowly to allow this method to be used, and so a concentrated incubation method (Method B of Jameson *et al.* (Jameson et al., 1973)) was used for this enzyme. This involved incubating 15, 30 or 40  $\mu\text{l}$  of a factor Xa stock solution ( $\approx 5 \mu\text{M}$ ) with 10  $\mu\text{l}$  aqueous MUGB solution (0.2 mM) in a total reaction volume of 50  $\mu\text{l}$  (final buffer: 80 mM Tris-Cl, 80 mM NaCl, 1.6 mM  $\text{CaCl}_2$  and 0.004% Triton X-100, pH 7.5, 25°C). Aliquots (10  $\mu\text{l}$ ) were removed at 15 minute intervals and diluted to a total volume of 2.5 ml in a quartz fluorescence cuvette (100 mM Tris-Cl, 100 mM NaCl, 2 mM  $\text{CaCl}_2$  and 0.005% Triton X-100, pH 7.5, 25 °C). The concentration of active factor Xa was determined by fluorometric measurement of the concentration of 4-MU released by reaction with the enzyme, extrapolated to zero reaction time and corrected for the background hydrolysis of MUGB by subtraction of the 4-MU fluorescence seen in a background reaction containing 0.2 mM MUGB but no enzyme. We estimate a precision of  $\pm 6\%$  for these measurements. Neither tPA nor C1s reacted suitably with MUGB to allow standardization of these two enzymes with this reagent.

The concentration of purified SERP-1 was determined by absorbance at 280 nm using a molar extinction coefficient of  $32\,700 \text{ M}^{-1}\text{cm}^{-1}$ . This value was measured using mammalian cell derived recombinant SERP-1 of identical amino acid sequence

(Michelle McKay, unpublished data). The experimentally determined extinction coefficient was within 5% of that calculated from the amino acid sequence according to the method of Gill and von Hippel (Gill and von Hippel, 1989).

**$K_M$  determinations for chromogenic proteinase substrates:** Estimates of  $K_M$  for the reaction of each enzyme with the appropriate chromogenic substrate were determined directly under the conditions used for the slow-binding inhibition experiments.  $K_M$  values for the reactions of uPA with chromozym-U, tPA with Chromozym-tPA, C1s with Chromozym-TRY, thrombin with Chromozym TH and factor Xa with Chromozym X were found to be 140  $\mu$ M, 160  $\mu$ M, 1.5 mM, 22  $\mu$ M, and 510  $\mu$ M, respectively.

**Slow binding inhibition kinetics:** Inhibition progress curves were obtained by incubating a limiting concentration of proteinase (5 - 125 pM) with various concentrations of SERP-1 from 0.125 to 100 nM in the presence of the appropriate chromogenic substrate. In all cases, serpin was present at  $\geq 10$ -fold excess over proteinase in order to achieve pseudo-first-order conditions with respect to SERP-1, and concentrations of enzyme and substrate were chosen so that  $\leq 10\%$  of the substrate was hydrolyzed over the entire duration of the assay. Reactions were performed at 37°C in a total volume of 800  $\mu$ l (100 mM Tris-HCl, 100 mM NaCl, 2 mM CaCl<sub>2</sub>, 0.005% Triton X-100, pH 7.5) in plastic cuvettes sealed with Parafilm. The time dependent inhibition of each enzyme by SERP-1 was monitored by following the rate of production of *p*-nitroaniline (pNA) at 405 nm at 1 or 5 minute intervals for a period of 1000 minutes, using a CARY 3E spectrophotometer

equipped with a six-cell cuvette holder. Each set of six reactions comprised five reactions containing various concentrations of SERP-1 and one control reaction containing enzyme and substrate with no SERP-1. In the  $[\text{SERP-1}] = 0$  controls, progress curves were observed to be linear, indicating that the enzyme activity was stable over the course of the reaction. For each enzyme, two to five sets of measurements spanning at least 6 different concentrations of SERP-1 were used to calculate the kinetic constants. The reactions were allowed to proceed until the steady state velocity of pNA formation was attained, and the progress curves for each reaction were then fitted to the integrated rate equation for slow binding inhibition (Equation 1) (Morrison and Walsh, 1988; Stone and Hermans, 1995), in which  $A_t$  is the absorbance at 405 nm at time  $t$ , due to the evolution of *p*-nitroaniline;  $k_{\text{obs}}$  is the apparent first-order rate constant for the inhibition of enzyme by SERP-1;  $v_i$  and  $v_s$  are the initial and steady-state velocities for reaction of the chromogenic substrate; and  $d$  is the initial absorbance at 405 nm.

$$A_t = v_s t + \left( \frac{v_i - v_s}{k_{\text{obs}}} \right) (1 - e^{-k_{\text{obs}} t}) + d \quad \text{Equation 1}$$

Values for  $v_i$ ,  $v_s$ , and  $k_{\text{obs}}$  were obtained for the progress curves measured at each SERP-1 concentration using nonlinear regression analysis (DeltaGraph 3.5). Rate constants for the release of active enzyme from the enzyme-SERP-1 inhibited complex,  $k_{\text{diss}}$ , were measured by pre-incubating proteinase and SERP-1 together at high concentration (10 - 100 nM), and diluting the resulting inhibited complex 50-fold

into a slow-binding inhibition assay containing the appropriate chromogenic substrate. Reactivation of the enzyme was monitored by following the absorbance at 405 nm until the new steady state was reached. Progress curves were fitted to Equation 1, for which  $k_{\text{obs}} = k_{\text{diss}}$ .

**Stoichiometry of inhibition:** The stoichiometry of inhibition (SI) was measured for the reactions of SERP-1 with uPA, plasmin, thrombin and Xa using a method similar to that of Patston *et al.* (Patston *et al.*, 1991). Accurately known, fixed concentrations of each proteinase were incubated with SERP-1 at various molar ratios in a total reaction volume of 25 or 30  $\mu\text{l}$  (100 mM Tris-HCl, 100 mM NaCl, 2 mM  $\text{CaCl}_2$ , 0.005% Triton X-100, pH 7.5, 37 °C). For each proteinase, a control reaction was included that contained no SERP-1. Enzymes were used at the following concentrations: uPA and plasmin, 40 nM; thrombin, 5 nM; Xa, 16.7 nM. Reactions were incubated for 1 hr, except for the slower reaction with Xa for which a 10 hour incubation was required for complete reaction. In all cases, control experiments at longer reaction times were performed to show that reaction was complete, and that incubation under these conditions did not cause any significant loss of activity in the enzyme only control. At the end of the incubation period, residual proteinase activity was determined in activity assays containing an appropriate chromogenic substrate. SI values for reaction with each enzyme were determined by plotting residual proteinase activity against the mole ratio of SERP-1 to proteinase, and extrapolating the linear portion of the inhibition titration curve to zero residual activity to give the minimum number of equivalents of SERP-1 required

to fully inhibit the enzyme. SI values were measured a minimum of three times in at least two independent experiments for each enzyme.

## RESULTS

SERP-1 and the RCL mutant R319A/N320A were cloned into a vaccinia virus expression system, expressed and purified from viral supernatant as described in *Experimental Procedures*. Figure II-1 shows the purification of SERP-1 protein as monitored by silver-stained SDS-PAGE. Lane 1 shows the crude viral supernatant, lane 2 shows the partially purified material after the mono-Q FPLC step, and lane 3 shows SERP-1 purified through the final gel filtration step to an apparent purity of >95%, as judged by overloaded Coomassie-stained and silver-stained gels and by the observation of a single peak on reversed-phase HPLC (data not shown). Both wild-type and mutant SERP-1 migrate on SDS gels as a diffuse band, with an apparent molecular weight of 50-55 kDa due to the presence of N-linked glycosylation (Macen et al., 1993). The yield of SERP-1 after the two column purification was  $\approx 25 \mu\text{g}$  per  $10^8$  BGMK cells infected. A faint Western blot positive band at 110 kDa, corresponding to a dimer, was sometimes observed by SDS-PAGE, as has been seen for other serpins (Lomas et al., 1993; Macen et al., 1993). The presence and intensity of this band varied from gel to gel, even for the same SERP-1 sample. This observation, and the fact that the band could be eliminated under

conditions of high SDS plus reducing agents (data not shown), suggested that this band was due to formation of a small amount of dimer during SDS-PAGE sample preparation. Gel shift experiments, performed as described below, confirmed that all of the SERP-1 protein was able to react with appropriate target proteinases, indicating that no latent or inactive SERP-1 was present.

**Purified SERP-1 forms SDS-stable complexes with plasmin, uPA, tPA, Xa and thrombin:** Inhibition of serine proteinases by serpins is accompanied by the formation of an SDS-stable complex that can be seen as a high molecular weight band on SDS gels (Christensen et al., 1995). Lomas *et al.* (1993) have reported that gel-shifted complexes of this type can be observed upon incubation of SERP-1 from unfractionated viral supernatants with tPA, uPA, plasmin and C1s, but not with thrombin, factor Xa or other proteinases tested. In order to define the inhibitory specificity of SERP-1 better, we have repeated and extended their survey by investigating the reactions of purified SERP-1 with an extensive panel of proteinases. While few, if any, of the enzymes tested are plausible candidates for the target of SERP-1 inhibition *in vivo*, a broad panel of available proteinases was examined in the expectation that the results would help establish what properties *in vivo* targets for SERP-1 inhibition are likely to possess. In an initial screen, purified SERP-1 ( $\approx 180\text{nM}$ ) was incubated for two hours with a slight molar excess of each enzyme (100 mM Tris-HCl, 100 mM NaCl, 2 mM  $\text{CaCl}_2$ , 0.005% Triton X-100, pH 7.5, 37°C), as described in *Experimental Procedures*. The distribution of SERP-1 in the product mixture between native, complexed and cleaved forms was determined from

the migration of the corresponding SERP-1 bands on SDS-PAGE, as detected by Western blotting using a rabbit anti-SERP-1 polyclonal antibody (Figure II-2). In cases where no high molecular weight complex with the proteinase was observed in the initial screen, the experiment was repeated with different incubation times and at different enzyme/SERP-1 ratios to ensure that the existence of a slow-forming or transient complex was not being missed. Figure II-2A shows that purified SERP-1 reacts with plasmin (lane 2), uPA (lane 3), tPA (lane 4) and Xa (lane 6) to form SDS-stable complexes that migrate with apparent molecular weights that are consistent with the expected values for their respective complexes with SERP-1 of about 135, 93, 123 and 105 kDa. Most of the inhibited proteinases contain disulfide linked subunits, which allowed the identity of the higher molecular weight bands to be confirmed by performing electrophoresis under reducing conditions and checking that the apparent molecular weight of the SERP-1-proteinase complexes changed by the expected amounts. In addition to forming an inhibited complex, reaction with these enzymes also produced varying amounts of a species running at about 50 kDa, which is the size expected for SERP-1 that has been cleaved at or near the predicted cleavage site in the RCL. The generation of some amount of cleaved serpin has been frequently found to accompany the inhibition of proteinases by serpins (Christensen et al., 1995; Lawrence et al., 1995), either through the hydrolytic breakdown of the inhibited complex or because of the operation of a branched kinetic mechanism (Patston et al., 1994). The observation of an inhibited complex with Xa was unexpected, as this enzyme had been reported previously to cleave SERP-1 without

forming a stable complex (Lomas et al., 1993). Our result was confirmed by the observation that SERP-1 is able to inhibit Xa in kinetic experiments (see below). Thrombin (lane 5) initially appeared to form exclusively cleaved serpin, migrating at approximately 50 kDa, without the formation of any detectable higher molecular weight complex. However, by reducing the reaction time we were able to observe the transient formation of a small amount of a SERP-1/thrombin complex, as shown in Figure II-2B. This result was confirmed by the observation that thrombin is inhibited by SERP-1 in kinetic experiments. hNE and cathepsin G also appeared to exclusively form cleaved SERP-1 (Figure II-2A, lanes 8 and 9). However, unlike thrombin, additional tests under varied conditions gave no evidence for any stable complex between SERP-1 and either of these enzymes. The inability of SERP-1 to inhibit cathepsin G was confirmed in kinetic experiments. Reaction with C1s also failed to give evidence for the formation of a stable complex (Figure II-2A, lane 10), though the results of Lomas *et al.* suggest that SERP-1 does inhibit this enzyme, and we also observed evidence for weak inhibition of C1s by SERP-1 in kinetic experiments. Reaction with trypsin produced two discrete bands, corresponding to the 50 kDa cleaved form seen with the other enzymes together with an additional smaller form of cleaved SERP-1 (Figure II-2A, lane 7); the two fragments appeared rapidly and were stable once formed. Reaction with chymotrypsin also gave two discrete SERP-1 cleavage fragments, though in this case both observed products were substantially smaller than 50 kDa. (data not shown). The inability of SERP-1 to inhibit chymotrypsin was confirmed in kinetic experiments (data not shown). No



reaction was apparent upon incubation of SERP-1 with kallikrein, Factor D,  $\beta$ XIIa or the alternative pathway C3 convertase (Figure II-2A lanes 11 and 12 and data not shown). Bovine factor  $\beta$ XIIa was tested because of a published report of a bovine serpin, believed to target this enzyme, that has an asparagine at the putative P1' position in the RCL and either lysine or arginine at the P1 position (Muldbjerg et al., 1993). This is the only example, other than SERP-1, of a serpin reported to have a P1' asparagine, and thus raised the possibility that SERP-1 might target  $\beta$ XIIa. Finally, and as expected, proteinases from other structural and mechanistic classes – cathepsin B, cathepsin D, thermolysin and subtilisin – either failed to react with SERP-1 or caused the complete disappearance of the SERP-1 band, presumably due to its cleavage into small fragments that were not detectable by Western blotting (data not shown). Our results, compared with those of Lomas *et al.* (Lomas et al., 1993), are summarized in Table II-1.

**Identity and functional importance of P1-P1' residues in SERP-1:** The location of the P1-P1' cleavage site in the RCL was confirmed by sequencing the N-terminus of the 5 kDa SERP-1 cleavage fragment released upon reaction with thrombin followed by denaturation of the reaction products. A sequence of NALTAIVANK was determined, which corresponds to residues 320-329 of the SERP-1 sequence and thus confirms that reaction with thrombin occurs at the bond between Arg<sub>319</sub> and Asn<sub>320</sub>. In order to explore the functional importance of these residues for SERP-1 inhibitory activity, a mutant SERP-1 was constructed in which arginine-319 and asparagine-320 were changed to alanine residues. Mutation of the P1-P1' site in

other serpins has been shown to abolish inhibitory activity (Patston and Gettins, 1994). The resulting mutant protein, termed R319A/N320A, was tested for its ability to interact with the same panel of proteinases used to test wild-type serpin, as described in *Experimental Procedures*. The results are summarized in Table II-1. Figure II-2C shows that R319A/N320A did not react with plasmin, tPA, uPA, thrombin or Xa (lanes 2-6, respectively). No high molecular weight complexes were observed, nor was there any evidence of the production of any smaller cleaved forms of the molecule. R319A/N320A was tested for inhibition of plasmin, tPA and uPA in activity assays, and showed no effect in either slow binding inhibition assays or by end-point measurements of enzyme activity after prolonged incubation with an excess of the mutant serpin. The abolition of interactions with SERP-1 by the mutations in positions 319 and 320 confirms the importance of the P1-P1' residues for inhibition by SERP-1. Interestingly, R319A/N320A reacted with elastase and cathepsin G to yield a 50 kDa product (Figure II-2C, lanes 7 and 8), identical to the result seen when these enzymes were reacted with wild type SERP-1. This result suggests that these enzymes react at a site in or near the RCL but not at Arg<sub>319</sub>-Asn<sub>320</sub>. This was confirmed by Edman degradation sequencing of the 5 kDa fragment released upon reaction of w.t. SERP-1 with cathepsin G and hNE. The results showed that both of these enzymes cleave after Ile<sub>314</sub> in the RCL of w.t. SERP-1, five residues upstream of Arg<sub>319</sub>. This result shows that cleavage of SERP-1 at a site close to P1-P1' in the RCL does not lead to formation of an inhibited complex (Patston and Gettins, 1994).

**Reaction kinetics for inhibition of plasmin, tPA, uPA, thrombin and Xa by SERP-1:** Of the extensive panel of proteinases tested, only plasmin, tPA, uPA, thrombin and Xa were observed to form SDS-stable complexes with SERP-1, though Lomas *et al.* have shown evidence for the formation of a complex between SERP-1 and C1s (Lomas *et al.*, 1993). We attempted to quantify the effectiveness of SERP-1 at inhibiting these six enzymes by investigating their reactions with SERP-1 using the techniques of slow binding inhibition kinetics (Morgenstern *et al.*, 1994; Stone and Hermans, 1995). Each proteinase was incubated with various concentrations of SERP-1 in the presence of a fixed concentration of an appropriate chromogenic proteinase substrate. The time-dependent inactivation of the enzyme through reaction with SERP-1 was monitored continuously by following the accompanying decrease in the rate of substrate turnover. Experiments were carried out at a low concentration of each enzyme (5-125 pM) so that the reaction could be followed for many hours without significantly depleting the chromogenic substrate. SERP-1 was always present at concentrations that were in >10-fold excess with respect to the proteinase, thus giving rise to pseudo-first-order conditions with respect to SERP-1. Figure II-3 shows a family of inhibition progress curves for the reaction of tPA with 0 - 20 nM SERP-1. The data show that higher concentrations of SERP-1 cause the reactions to approach the steady state more rapidly, and give a lower velocity of substrate turnover at the steady state, indicating that a greater fraction of the enzyme has become inhibited through reaction with SERP-1. The rate of substrate turnover was observed to decrease to a finite steady-state level, and not to zero as would be

expected if inhibition by SERP-1 proceeded to completion, indicating the existence of one or more pathways by which the inhibited complex can break down to release active enzyme (Stone and Hermans, 1995). Inhibition data was measured for each proteinase at  $\geq 6$  different SERP-1 concentrations chosen to span the range from slow or negligible inhibition to rapid and extensive reaction with SERP-1. Progress curves for reaction at each SERP-1 concentration were fitted to Equation 1 (Stone and Hermans, 1995) to obtain values for the rate constant for the approach to the steady state,  $k_{obs}$ , and the velocity of substrate turnover at the steady state,  $v_s$ , as described in *Experimental Procedures*. Figure II-3 (inset) shows the relationship between  $v_s$  and the concentration of SERP-1 for reaction with tPA. The solid line represents the fit to Equation 2, and gives an apparent inhibition constant ( $IC_{50}$ ) of 360 pM for reaction with this enzyme.

$$v_s = v_i / (1 + [SERP-1] / IC_{50}) \quad \text{Equation 2.}$$

The  $IC_{50}$  value determined for the reaction with each enzyme was corrected for the binding of chromogenic substrate in the assay as described in Stone and Hermans (Stone and Hermans, 1995), to give a steady-state inhibition constant,  $K_{I(app)}$ , which represents the concentration of SERP-1 at which the enzyme is distributed equally between free and SERP-1-inhibited (complexed) forms. Values for  $K_{I(app)}$  for all of the enzymes tested are given in Table II-2.

Figure II-4 shows the dependence of  $k_{obs}$  on the concentration of SERP-1 for reaction with uPA, tPA, plasmin, thrombin and factor Xa. These plots are linear, and show no evidence of hyperbolic curvature that would indicate the existence of one or more stable intermediate complexes forming prior to the rate-limiting step for formation of the final inhibited complex. The slopes of the best fits to the data in Figure II-4 give values for the apparent second-order rate constants for reaction of SERP-1 with plasmin, tPA, uPA, thrombin and Xa of  $k_{app} = 4.8 \pm 0.5 \times 10^4$ ,  $4.3 \pm 0.4 \times 10^4$ ,  $5.0 \pm 0.6 \times 10^4$ ,  $2.6 \pm 0.4 \times 10^4$ , and  $1.7 \pm 0.5 \times 10^3 \text{ M}^{-1}\text{s}^{-1}$ , respectively, after correction for competitive binding of the chromogenic substrate present during the reaction (Stone and Hermans, 1995). Values for  $k_{app}$  and  $K_{I(app)}$  for C1s were estimated from measurements made at a single SERP-1 concentration of 100 nM; the weak inhibition observed for this enzyme required amounts of SERP-1 that were too great to allow more detailed investigation of this reaction. The apparent second-order rate constants for these reactions are presented in Table II-2. The kinetic constants for reaction with plasmin, tPA and uPA are in good agreement with those measured by Lomas *et al.* who reported  $k_{app}$  values of  $3.4 \times 10^4$ ,  $3.6 \times 10^4$  and  $4.3 \times 10^4 \text{ M}^{-1}\text{s}^{-1}$ , respectively, using crude viral supernatants (Lomas et al., 1993). Our estimated value for C1s is also in reasonable agreement with their value of  $k_{app} = 1.3 \times 10^3 \text{ M}^{-1}\text{s}^{-1}$ .

The rate at which each inhibited complex breaks down to release active enzyme was measured directly by incubating each enzyme with a small excess of SERP-1 at concentrations well above  $K_{I(app)}$ , and then diluting the resulting enzyme-

serpin complex 50-fold, to a concentration below  $K_{I(app)}$ , into reaction buffer containing chromogenic substrate. The release of inhibition manifests itself as upward curvature in the progress curve, tending to a new steady-state velocity of substrate turnover commensurate with the lower concentration of SERP-1 present in the final reaction mixture. Equation 1 was used to fit the reactivation progress curves by nonlinear regression analysis, such that  $k_{obs} = k_{diss}$ . Measured rate constants for the dissociation of complexes formed between SERP-1 and plasmin, tPA, thrombin and Xa were  $k_{diss} = 3 \pm 0.4 \times 10^{-5}$ ,  $6 \pm 0.5 \times 10^{-6}$ ,  $2 \pm 0.2 \times 10^{-6}$ , and  $8 \pm 0.4 \times 10^{-6} \text{ s}^{-1}$ , respectively. Rate constants for the release of active enzyme from the inhibited complexes can also be calculated from the measured  $k_{app}$  and  $K_{I(app)}$  values using the relationship  $k_{diss} = K_{I(app)}k_{app}$ . Values for  $k_{diss}$  that were calculated in this way are shown in Table II-2, and agree well with the  $k_{diss}$  values that were measured directly.

**Stoichiometries of inhibition:** Figures II-2A and 2B show evidence for the formation of varying amounts of cleaved SERP-1 upon reaction with plasmin, tPA, uPA, thrombin and Xa. The  $k_{diss}$  values shown in Table II-2 suggest that the inhibited complexes formed with these enzymes dissociate too slowly for any significant amount of cleaved serpin to be released during the 2 hr incubation period used for the measurements in Figure II-2A, though the possibility that the complex breaks down more readily during SDS-PAGE sample preparation cannot be discounted. An alternative explanation for the origin of the cleaved SERP-1 is the existence of a branched kinetic mechanism, illustrated in Scheme II-1, such as has been demonstrated for other serpins (Gettins et al., 1993; Gettins et al., 1996; Gils

and Declerck, 1997; Huntington et al., 1997; Patston et al., 1994). If this is the case for SERP-1, then the large variations in the proportion of cleaved serpin formed from reaction with the different enzymes in Figures II-2A and 2B – from very little with uPA to  $\geq 90\%$  with thrombin – suggest the existence of correspondingly large variations in partitioning within the branched pathway, for reaction with different enzymes. In order to determine whether the cleaved serpin seen in Figures II-2A and II-2B arises from a branched mechanism, and to quantify any differences in partitioning within such a mechanism, we measured the stoichiometry of inhibition (SI) for the reactions of SERP-1 with uPA, plasmin, thrombin and Xa. SI values were determined using a SERP-1 stock solution that had been accurately standardized by its absorption at 280 nm using an extinction coefficient determined by amino acid analysis, and reacting it with enzyme solutions that had been accurately standardized by active site titration. Gel shift experiments, such as those described above, established that all of the SERP-1 protein in this standardized solution was active. Figure II-5 shows the titration of a fixed concentration of uPA with varying concentrations of SERP-1 and establishes that  $1.4 \pm 0.1$  equivalents of SERP-1 are required to achieve exactly 100% inhibition, corresponding to an  $SI = 1.4 \pm 0.1$  (Patston et al., 1991). This value was consistent with the distribution of SERP-1 between cleaved and complexed forms that was estimated by Western blot, or measured by quantitative densitometry of Coomassie blue stained SDS polyacrylamide gels (data not shown). Figure II-5 also shows that titrations of plasmin, thrombin, and factor Xa with SERP-1 similarly give rise to linear titration

curves, resulting in the SI values shown in Table II-2. The values range from the relatively low value found for reaction with uPA to a high of  $SI = 13 \pm 3$  for reaction with thrombin. The active site titrant MUGB did not react with tPA in our hands and, lacking an accurate method for the standardization of this enzyme, we were unable to perform a titration against SERP-1 of the type shown in Figure II-5. However, the roughly equal intensities of the bands for cleaved and complexed SERP-1 seen in Western blots suggest that for reaction with tPA,  $SI \approx 2$  (limits: 1.5 - 3).

## DISCUSSION

The gel shift experiments shown in Figure II-2 confirm the observations of Lomas *et al.* (Lomas et al., 1993), made using w.t. SERP-1 from unfractionated viral supernatant, that this secreted viral serpin inhibits tPA, uPA and plasmin. We additionally identify factor Xa and thrombin as *in vitro* targets for SERP-1 inhibition. Both thrombin and Xa were reported by Lomas *et al.* to cleave SERP-1 without forming any detectable inhibited complex. Our subsequent kinetic analysis showed thrombin to be among the most reactive targets of SERP-1 inhibition, however the high SI seen for this enzyme made the observation of an inhibited complex on Western blots quite difficult. Another difference between our results and those of Lomas *et al.* was that in the previous study no reaction was seen between SERP-1



and cathepsin G (Lomas et al., 1993), whereas in the present study reaction at a somewhat higher enzyme:SERP-1 ratio showed that cathepsin G does react with SERP-1, cleaving at a single site in the RCL. A final difference between our results and those of Lomas *et al.* is that in the present study we were unable to detect any stable inhibited complex between SERP-1 and C1s on Western blots, though we did find evidence for weak inhibition of this enzyme by SERP-1 in kinetic experiments. The discrepancies between the two studies illustrate that the ability to observe a stable inhibited enzyme-serpin complex on SDS gels can be quite sensitive to the relative and absolute concentrations of serpin and enzyme used, to the duration and conditions of the reaction, and possibly also to the purity of the serpin preparation.

Thrombin was shown, by sequencing of the cleaved serpin, to react with SERP-1 at the predicted site between Arg<sub>319</sub>-Asn<sub>320</sub>. All of the other enzymes that were found to be inhibited by SERP-1 also have a primary specificity for cleaving after a basic residue, and the fact that mutating Arg<sub>319</sub>-Asn<sub>320</sub> to Ala-Ala abolished the reactivity of SERP-1 towards all of these enzymes argues strongly that they all act at this position in the RCL. The failure of these enzymes to be inhibited by the mutant SERP-1 is unlikely to be due to improper folding or long-range disruption of the serpin structure because of the location of the mutations in a flexible exposed loop (Patston and Gettins, 1994). This assumption is supported by the observation that cathepsin G and elastase, which were shown to act at Ile<sub>314</sub>-Thr<sub>315</sub>, just five residues upstream of Arg<sub>319</sub> in the RCL, appear on Western blots to display the same highly specific single cleavage of w.t. and the mutant SERP-1.

Table II-2 contains rate constants for the formation of the inhibited complex and for its decomposition, together with stoichiometries of inhibition (SI), for reaction with the six proteinases that were found to be inhibited by SERP-1. These results comprise an unusually comprehensive and quantitative data set for direct comparison of the reactivity of a single serpin with a series of target proteinases. In order to properly evaluate the factors that govern the specificity of SERP-1 for the enzymes tested, we must consider the data in Table II-2 in terms of the branched kinetic mechanism shown in Scheme II-1. The minimal mechanism in Scheme II-1 incorporates the basic features of the serpin inhibition mechanism as established for the reactions of other serpin-proteinase pairs (Patston et al., 1994; Wright and Scarsdale, 1994). The applicability of this branched mechanism to the reaction of SERP-1 with the enzymes listed in Table II-2 is demonstrated by the observation that, in every case, reaction leads to the formation of cleaved SERP-1 concomitantly with the formation of the inhibited enzyme-serpin complex, leading to SI values that are in all cases greater than 1 (Patston et al., 1991; Schechter et al., 1997). The precise chemical and structural nature of the partitioning intermediate, [EI], in the serpin inhibition mechanism remains in question. Published suggestions include the possibility that [EI] is an acyl-enzyme intermediate (Lawrence et al., 1995; Wilczynska et al., 1995), a related covalent species trapped at the tetrahedral intermediate stage (Matheson et al., 1991), or possibly a noncovalent intermediate that precedes P1-P1' bond cleavage (O' Malley et al., 1997). Our data provide no evidence to indicate which of these possibilities applies to the reactions of SERP-1.

Despite the undefined nature of the partitioning intermediate, we can nevertheless use SI to calculate the partitioning ratio between the pathway that leads to inhibited complex (EI\*) and the pathway that leads directly to the release of cleaved SERP-1 (I'), using the relation  $SI = 1 + k_3/k_4$  (Patston et al., 1994). Partitioning ratios for the reactions of SERP-1 with plasmin, tPA, uPA, thrombin and Xa are shown in Table II-3. In order for us to interpret the remaining data in Table II-2 in terms of the branched kinetic mechanism shown in Scheme II-1, we must correct the apparent second-order rate constants,  $k_{app}$ , to account for the effect of partitioning, as described by Hood *et al.* (Hood et al., 1994). Multiplying  $k_{app}$  by SI compensates for the fact that for an SI of 10, for example, only 1 out of every 10 reactions between enzyme and serpin leads to the formation of the inhibited complex, EI\*. Because of this effect, the true second-order rate constant for reaction between E and I in such a case is 10 times higher than the apparent rate constant for the formation of EI\* that is given by  $k_{app}$  (Hood et al., 1994). The true second-order rate constants for reaction of SERP-1 with each of the six proteinases for which we have kinetic data, calculated from  $k_{inh.} = k_{app} \times SI$ , are collected in Table II-3. The apparent steady-state  $K_{I(app)}$  values from Table II-2 must also be corrected for the effect of partitioning, in this case by dividing by a factor of SI, if they are to reflect the balance between the true rate constants for the formation and decomposition of EI\* (Hood et al., 1994). The corrected values for  $K_I$  are also given in Table II-3.

The data in Table II-3 show that the rates at which SERP-1 reacts with the six proteinases listed vary from  $3.4 \times 10^5 \text{ M}^{-1}\text{s}^{-1}$  for reaction with thrombin to  $5.4 \times$

$10^3 \text{ M}^{-1}\text{s}^{-1}$  for reaction with Xa, with C1s having an estimated value for  $k_{\text{inh}}$ , that is an order of magnitude lower still. These variations in  $k_{\text{inh}}$ , which span 3 orders of magnitude, account for most of the variations in  $K_{\text{I}}$ . Values for  $K_{\text{I}}$  vary from 10 pM for thrombin to 1.3 nM for Xa, and span  $\approx 4$  orders of magnitude if the estimated value of  $K_{\text{I}} \approx 100 \text{ nM}$  for C1s is included. Interestingly, values for  $k_{\text{diss}}$  vary over a relatively narrow range, indicating that the kinetic barrier for decomposition of  $\text{EI}^*$  is less sensitive to differences in the structure and catalytic properties of the proteinase than are the other kinetic parameters for the reaction. Of the reactions included in Table II-3, only that with thrombin approaches the second-order rate constants seen for reactions between serpins and the proteinases believed to represent their specific targets *in vivo*, which typically exceed  $10^5 \text{ M}^{-1}\text{s}^{-1}$  (Olson et al., 1993; Travis and Salvesen, 1983). Reaction with thrombin, however, is characterized by a high partitioning ratio, indicating that the majority of reactions between SERP-1 and this enzyme are nonproductive and lead to cleavage and inactivation of SERP-1 rather than to inhibition of the enzyme. In general, the partitioning ratios in Table II-3 are higher than are typically seen for the reaction of serpins with their specific target proteinases (Patston et al., 1991). Thus, none of the enzymes in Table II-3 appear to possess all of the characteristics of fast inhibition, low SI and slow dissociation of the inhibited complex that would be anticipated to exist for the reaction of SERP-1 with a true *in vivo* target (Travis and Salvesen, 1983; Zhou et al., 1997).

The data in Tables II-2 and II-3 allow us to draw several conclusions about which steps in the inhibition mechanism are involved in governing the inhibitory

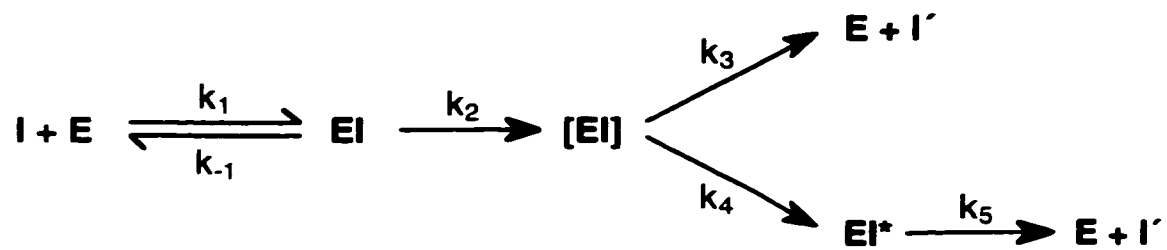
specificity of SERP-1. Under conditions in which the serpin is present at concentrations significantly higher than both  $K_I$  and the concentration of its proteinase target, the specificity of SERP-1 is primarily governed by the rate of its initial reaction with enzyme; even for an enzyme that reacts with a high SI, the amount of serpin lost through non-productive cleavage will be insignificant. However, even under conditions of excess serpin, variations in SI may influence inhibitory specificity. An example of this is provided by thrombin for which, as a consequence of its high SI, inhibition is effectively slower than inhibition of uPA or tPA despite the fact that thrombin undergoes a substantially faster initial reaction with SERP-1. Variations in SI assume a larger role in governing inhibitory specificity under conditions in which the serpin is present at comparable concentration to its target or targets. Under these conditions, inhibition of proteinases for which a high SI is observed will be partial at best, irrespective of how fast the reaction occurs, because most of the serpin will be inactivated through cleavage at the RCL. Moreover, it is possible that the presence *in vivo* of a highly reactive proteinase with a high SI would reduce the likelihood that any other proteinase present will become inhibited, by competing for serpin and irreversibly cleaving it to the inactive form, I'. Finally, at concentrations of serpin that are very low, the dissociation rate of EI\* may also begin to influence inhibitory specificity. For example, plasmin and tPA are both inhibited by SERP-1 at similar rates and have similar values for SI. However, at concentrations of  $\approx 100$  pM, SERP-1 would be expected to inhibit tPA much more effectively than plasmin, if present in excess over both, because at this concentration

it exceeds the  $K_i$  of 70 pM for reaction with tPA but is well under the  $K_i$  of 220 pM for reaction with plasmin. The difference in  $K_i$  values for these two enzymes arises from the 5-fold faster rate at which EI\* decomposes in the case of plasmin. The results in Table II-3 therefore suggest that at high concentrations of SERP-1, inhibitory specificity is predominantly governed by  $k_{app}$ , which depends on  $k_{inh}$  and  $k_3/k_4$ . At concentrations of serpin that are comparable to that of the target proteinase, specificity becomes much more strongly dependent on the magnitude of  $k_3/k_4$ . Finally, at very low serpin concentrations that begin to approach  $K_i$ , specificity can also be influenced by the magnitude of  $k_{diss}$ . Although many serpins, most notably those that circulate in the blood, are present at very high concentrations *in vivo*, it is likely that SERP-1 achieves its anti-inflammatory effect at much lower concentrations, acting at levels comparable to those of its target proteinases (Lucas et al., 1996; Macen et al., 1993; Maksymowych et al., 1996). This may also be true in other biologically important situations, such as the inhibition of intracellular caspases by CrmA (Zhou et al., 1997) or other serpin inhibitors of apoptosis.

Initial interest in SERP-1 was sparked by the discovery that it is a virulence factor of myxoma virus, acting in concert with other secreted and intracellular receptor mimics and inhibitors to cause severe immune dysfunction in the host (McFadden, 1995). Furthermore, SERP-1 was observed to act as an anti-inflammatory agent during myxoma virus infection (Macen et al., 1993), and when administered as a purified protein in two *in vivo* models of inflammation (Lucas et al., 1996; Maksymowych et al., 1996). Therefore, the target proteinases of SERP-1

are likely to be critical to initiating or propagating the early inflammatory signal in response to virus infection. The low doses of SERP-1 required for efficacy in various *in vivo* models (Lucas et al., 1996; Maksymowych et al., 1996) suggest that its target proteinase or proteinases are present at exceedingly low levels at the site of action. Although the results obtained in this study do not serve to identify the proteinase target or targets for SERP-1 that are responsible for its *in vivo* effects, they confirm that it is likely to be a trypsin-like proteinase with specificity for cleaving after a basic residue. In addition, our results shed some light on the mechanistic parameters that govern the inhibitory specificity of SERP-1, while highlighting the fact that the specificity of a serpin for inhibiting one or another proteinase may be significantly influenced by reaction conditions at the site of action, and particularly by the relative concentrations of proteinase and serpin. The ability of SERP-1 to dampen the inflammatory response to the virus infection offers clues to the roles that serine proteinases may be playing in these processes. A better understanding of the underlying mechanisms may help in the development of anti-inflammatory drugs based on serpins and small molecule proteinase inhibitors.

*Appendix A* extends the discussion of the inhibitory mechanism of serpins using the kinetic data reported herein for SERP-1. A linear free energy relationship is observed between  $\log K_I$  and  $\log k_{inh}$  and the implications of this to the serpin inhibitory mechanism are discussed in greater detail.

**Scheme II-1**



**Table II-1: Summary of the Interactions of Wild-Type and R319A/N320A Mutant SERP-1 with Various Proteinases as Determined by SDS-PAGE and Western Blot Analysis.** Purified SERP-1 (180 nM) was incubated at 37 °C for 2 hrs with a slight molar excess of each proteinase (100 mM Tris-HCl, 100 mM NaCl, 2 mM CaCl<sub>2</sub>, 0.005% Triton X-100, pH 7.5). The products were separated by SDS-PAGE and detected by Western blot using polyclonal anti-SERP-1 antiserum (see Figure II-2). Abbreviations for the enzymes are as follows: tPA = tissue-type plasminogen activator; uPA = urokinase-type plasminogen activator; hNE = human neutrophil elastase; pTrypsin and hTrypsin = porcine and human trypsins; pPE = porcine pancreatic elastase; bChymotrypsin = bovine chymotrypsin. The symbol *I* indicates the observation of a high molecular weight band, relative to that for unreacted SERP-1, indicating the formation of an SDS-stable inhibited complex between SERP-1 and the enzyme. The observation of such a complex was invariably accompanied by the formation of some amount of a lower molecular weight species corresponding to SERP-1 that has been proteolytically cleaved in or near the RCL (see Figure II-2). The symbol *S* indicates that only lower molecular weight species and unreacted SERP-1 were observed in the product mixture, indicating that SERP-1 was proteolytically cleaved without the concomitant formation of a detectable high molecular weight complex. The symbol *N* indicates that incubation with the proteinase resulted in no apparent change in the SERP-1 band, even when retested at higher concentrations of enzyme or for longer incubation times. Cases in which the interaction was not tested are denoted *n.d.*

TABLE II-1

**SUMMARY OF THE INTERACTIONS OF WILD-TYPE AND R319A/N320A MUTANT SERP-1 WITH VARIOUS PROTEINASES AS DETERMINED BY SDS-PAGE AND WESTERN BLOT ANALYSIS.**

| Enzyme                | Wild Type SERP-1       |                     | R319A/N320A   |
|-----------------------|------------------------|---------------------|---------------|
|                       | Present Study          | Lomas <i>et al.</i> | Present Study |
| Plasmin               | <i>I</i>               | <i>I</i>            | <i>N</i>      |
| tPA                   | <i>I</i>               | <i>I</i>            | <i>N</i>      |
| uPA                   | <i>I</i>               | <i>I</i>            | <i>N</i>      |
| Thrombin              | <i>I</i>               | <i>S</i>            | <i>N</i>      |
| Factor Xa             | <i>I</i>               | <i>S</i>            | <i>N</i>      |
| C1s                   | <i>(N)<sup>a</sup></i> | <i>I</i>            | <i>N</i>      |
| hNE                   | <i>S</i>               | <i>S</i>            | <i>S</i>      |
| Kallikrein            | <i>N</i>               | <i>N</i>            | <i>n.d.</i>   |
| Cathepsin G           | <i>S</i>               | <i>N</i>            | <i>S</i>      |
| <i>p</i> Trypsin      | <i>n.d.</i>            | <i>S</i>            | <i>n.d.</i>   |
| <i>h</i> Trypsin      | <i>S</i>               | <i>n.d.</i>         | <i>S</i>      |
| <i>p</i> PE           | <i>n.d.</i>            | <i>S</i>            | <i>n.d.</i>   |
| <i>b</i> Chymotrypsin | <i>S</i>               | <i>S</i>            | <i>S</i>      |
| Subtilisin            | <i>S</i>               | <i>S</i>            | <i>n.d.</i>   |
| Thermolysin           | <i>S</i>               | <i>S</i>            | <i>n.d.</i>   |
| Factor XIIa           | <i>N</i>               | <i>n.d.</i>         | <i>N</i>      |
| Factor D              | <i>N</i>               | <i>n.d.</i>         | <i>N</i>      |
| C3 convertase         | <i>N</i>               | <i>n.d.</i>         | <i>N</i>      |
| Cathepsin B           | <i>N</i>               | <i>n.d.</i>         | <i>n.d.</i>   |
| Cathepsin D           | <i>N</i>               | <i>n.d.</i>         | <i>n.d.</i>   |

<sup>a</sup> No higher molecular weight complex was observed in this study, but evidence of weak inhibition was seen in kinetic experiments (see text).

**Table II-2: Observed Kinetic Parameters for the Reaction of SERP-1 with Six Inhibited Proteinases.** Values for the apparent inhibition constant,  $K_{I(app)}$ , the apparent second-order rate constant for inhibition,  $k_{app}$ , and the measured rate constant for dissociation of the inhibited complex,  $k_{diss(exp.)}$ , were determined in slow binding inhibition experiments as shown in Figures 3 and 4 (100 mM Tris-HCl, 100 mM NaCl, 2 mM CaCl<sub>2</sub>, 0.005% Triton X-100, pH 7.5, 37 °C). Both  $K_{I(app)}$  and  $k_{app}$  are corrected for the competitive binding of chromogenic substrate in the slow binding inhibition assays as described in the text. Calculated values for the rate constant for dissociation of the inhibited complex,  $k_{diss(calc.)}$ , were determined using the equation  $k_{diss(calc.)} = K_{I(app)}k_{app}$ , as described in the text. Stoichiometry of inhibition (SI) values were measured as shown in Figure 5 (100 mM Tris-HCl, 100 mM NaCl, 2 mM CaCl<sub>2</sub>, 0.005% Triton X-100, pH 7.5, 37 °C). The notation *n.d.* indicates that the given experimental data were not obtained.

TABLE II

**OBSERVED KINETIC PARAMETERS FOR THE REACTION OF SERP-1  
WITH SIX INHIBITED PROTEINASES.**

| Enzyme    | $K_{I(\text{app})}$ (nM)         | $k_{\text{app}}$ ( $\text{M}^{-1}\text{s}^{-1}$ ) | $k_{\text{diss}(\text{calc.})}$ ( $\text{s}^{-1}$ ) | $k_{\text{diss}(\text{exp.})}$ ( $\text{s}^{-1}$ ) | SI                     |
|-----------|----------------------------------|---------------------------------------------------|-----------------------------------------------------|----------------------------------------------------|------------------------|
| Plasmin   | $0.44 \pm 0.05$                  | $4.8 \pm 0.5 \times 10^4$                         | $2 \pm 0.4 \times 10^{-5}$                          | $3 \pm 0.4 \times 10^{-5}$                         | $2.0 \pm 0.5$          |
| tPA       | $0.14 \pm 0.02$                  | $4.3 \pm 0.4 \times 10^4$                         | $7 \pm 0.8 \times 10^{-6}$                          | $6 \pm 0.5 \times 10^{-6}$                         | $\approx 2^{\text{a}}$ |
| uPA       | $0.16 \pm 0.01$                  | $5.0 \pm 0.6 \times 10^4$                         | $8 \pm 0.7 \times 10^{-6}$                          | <i>n.d.</i>                                        | $1.4 \pm 0.1$          |
| Thrombin  | $0.13 \pm 0.02$                  | $2.6 \pm 0.4 \times 10^4$                         | $3 \pm 0.4 \times 10^{-6}$                          | $2 \pm 0.2 \times 10^{-6}$                         | $13 \pm 3$             |
| Factor Xa | $4.3 \pm 0.4$                    | $1.7 \pm 0.5 \times 10^3$                         | $7 \pm 0.7 \times 10^{-6}$                          | $8 \pm 0.4 \times 10^{-6}$                         | $3.2 \pm 0.5$          |
| CIs       | $\approx 200 \pm 100^{\text{b}}$ | $\approx 3 \pm 2 \times 10^2^{\text{b}}$          | $\approx 6 \pm 5 \times 10^{-5}$                    | <i>n.d.</i>                                        | <i>n.d.</i>            |

<sup>a</sup> SI for reaction with tPA was estimated from the relative intensities of the bands for complexed versus cleaved SERP-1, visualized by Western blotting, to have a value of  $\approx 2$  with lower and upper limits of 1.5 and 3 (see text).

<sup>b</sup> The  $K_{I(\text{app})}$  and  $k_{\text{app}}$  values for CIs were estimated from measurements made at a single SERP-1 concentration of 100 nM (see text).

**Table II-3: Final Kinetic Parameters for the Reaction of SERP-1 with Six Inhibited Proteinases After Correction for the Effects of Partitioning in the Branched Kinetic Mechanism.** Values for the true second-order rate constant for inhibition,  $k_{inh}$ , were calculated from the  $k_{app}$  values in Table II by correcting for the effect of partitioning in the branched kinetic mechanism using the formula  $k_{inh} = k_{app}SI$ , as described in the text. Similarly, true  $K_i$  values were calculated from the  $K_{i(app)}$  values in Table II using the formula  $K_i = K_{i(app)}/SI$ . Values for  $k_{diss}$  are not affected by partitioning, and so were taken directly from Table II; the directly-determined experimental values are used except for reaction with uPA and with CIs, for which the calculated values ( $k_{diss(calc.)}$ ) are given. The partitioning ratio  $k_3/k_4$  was calculated using the formula  $SI = 1 + k_3/k_4$ , as described in the text. The notation *n.d.* indicates that the experimental data were not obtained (see text).

TABLE III

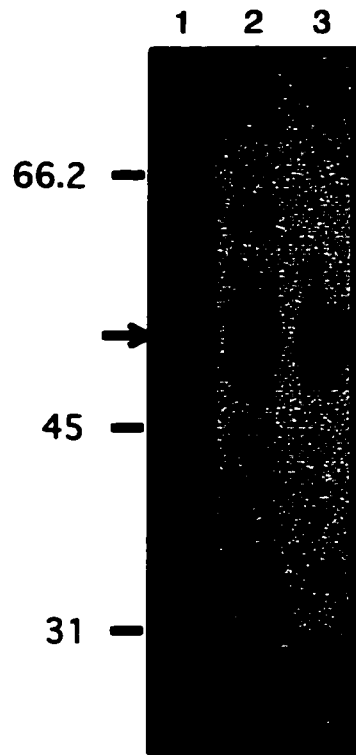
**FINAL KINETIC PARAMETERS FOR THE REACTION OF SERP-1 WITH SIX INHIBITED PROTEINASES AFTER CORRECTION FOR THE EFFECTS OF PARTITIONING IN THE BRANCHED KINETIC MECHANISM.**

| Enzyme    | $K_I$ (nM)             | $k_{inh.}$ ( $M^{-1}s^{-1}$ )   | $k_{diss}$ ( $s^{-1}$ )          | $k_3/k_4$       |
|-----------|------------------------|---------------------------------|----------------------------------|-----------------|
| Plasmin   | $0.22 \pm 0.04$        | $9.6 \pm 2.5 \times 10^4$       | $3 \pm 0.4 \times 10^{-5}$       | $1.0 \pm 0.4$   |
| tPA       | $0.07 \pm 0.01$        | $8.6 \pm 4.3 \times 10^4$       | $6 \pm 0.5 \times 10^{-6}$       | $\approx 1.0^a$ |
| uPA       | $0.11 \pm 0.02$        | $7.0 \pm 0.5 \times 10^4$       | $8 \pm 0.7 \times 10^{-6}$       | $0.4 \pm 0.1$   |
| Thrombin  | $0.01 \pm 0.002$       | $3.4 \pm 0.7 \times 10^5$       | $2 \pm 0.2 \times 10^{-6}$       | $12 \pm 3$      |
| Factor Xa | $1.3 \pm 0.3$          | $5.4 \pm 1.0 \times 10^3$       | $8 \pm 0.4 \times 10^{-6}$       | $2.2 \pm 0.5$   |
| C1s       | $\approx 100 \pm 80^b$ | $\approx 6 \pm 5 \times 10^2^b$ | $\approx 6 \pm 5 \times 10^{-5}$ | n.d.            |

<sup>a</sup>  $k_3/k_4$  for the reaction with tPA was calculated from the estimated SI shown in Table II.

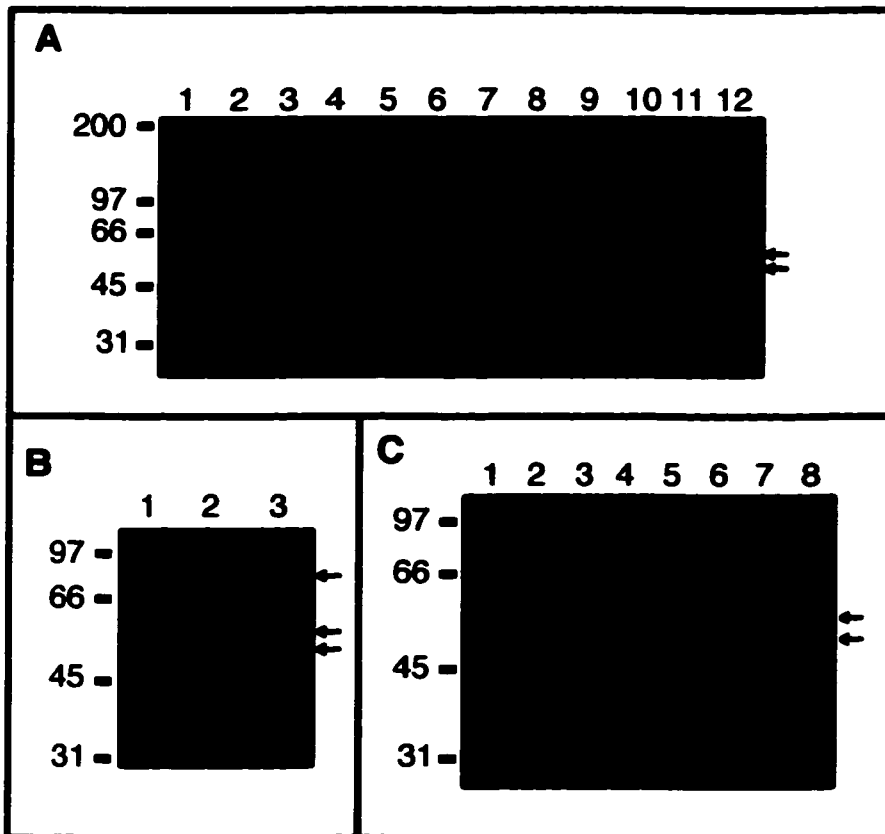
<sup>b</sup>  $K_I$  and  $k_{inh.}$  values for C1s were estimated using the values for  $K_{I(app)}$  and  $k_{app}$  given in Table II, and an assumed SI of  $\approx 2$ . The large uncertainties in the values reflect the fact that the true SI could be as low as 1 or as high as 10, but is unlikely to significantly exceed 10 based on the ability of Lomas *et al.* (1993) to detect a shifted complex by Western blot.

**Figure II-1: SERP-1 Purification.** Silver stained SDS-polyacrylamide gel showing stages of purification of the SERP-1 protein. The arrow indicates the position at which SERP-1 migrates, corresponding to an apparent m.w. of  $\approx 55$  kDa. Lane 1: crude supernatant from cells infected with VV-S1. Lane 2: pooled fractions recovered from MonoQ FPLC anion exchange chromatography. Lane 3: SERP-1 fraction recovered from gel filtration chromatography on a Superdex 75 column.

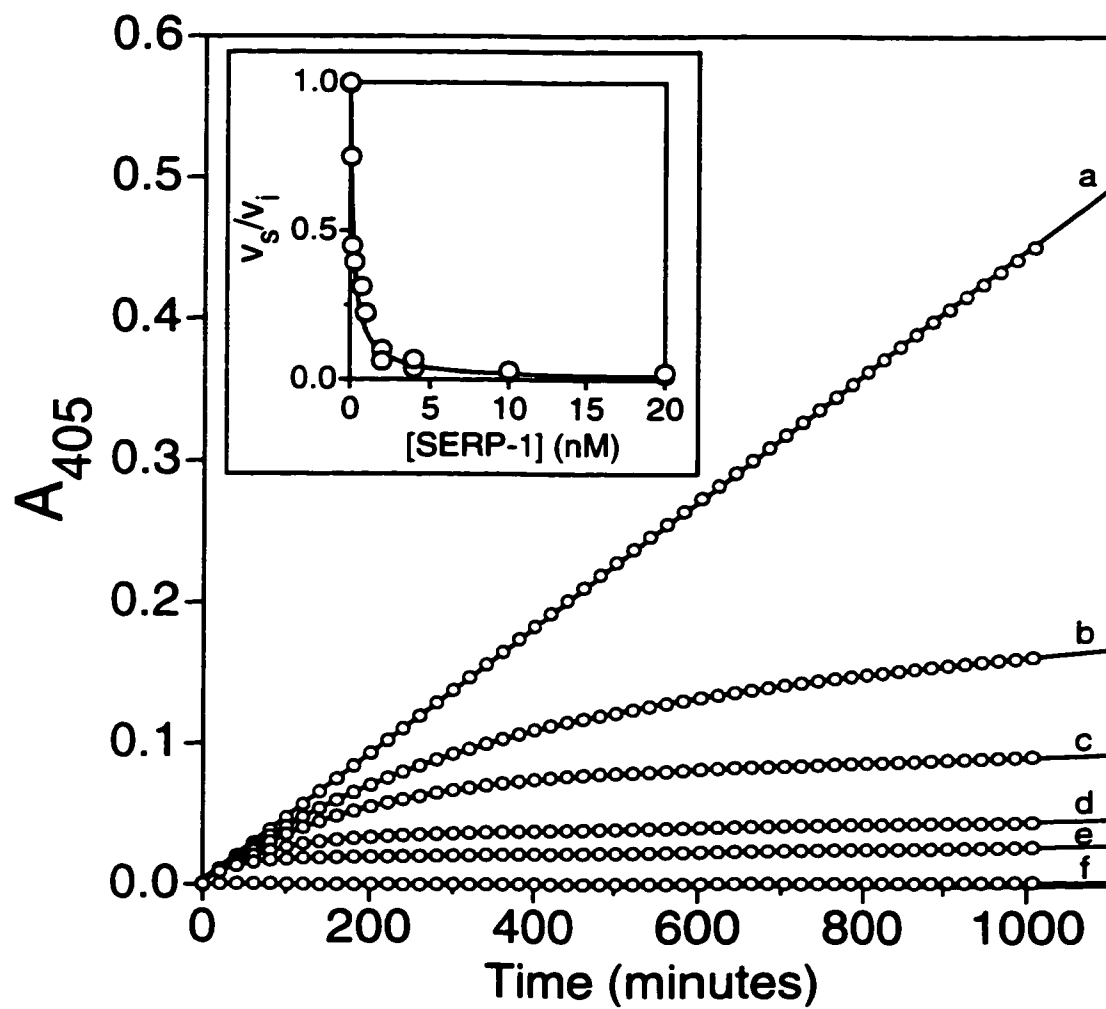




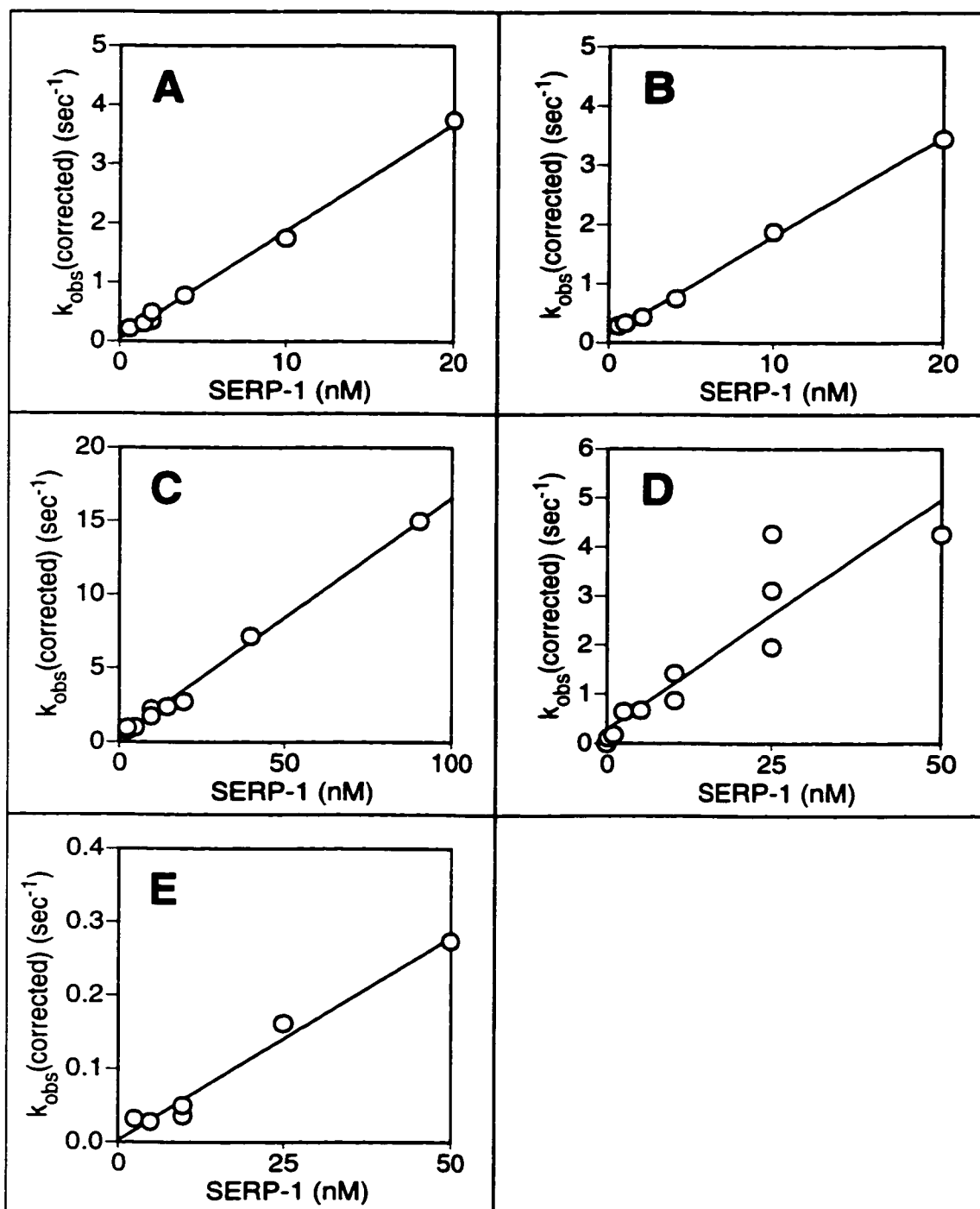
**Figure II-2. SERP-1 forms SDS-stable complexes with uPA, tPA, plasmin, thrombin and factor Xa.** **A.** SERP-1 forms SDS-stable complexes with plasmin, tPA, uPA, and factor Xa. Purified SERP-1 (lane 1) was incubated for 2 hrs with a slight molar excess of the following proteinases: plasmin (lane 2), uPA (lane 3), tPA (lane 4), thrombin (lane 5), factor Xa (lane 6), human trypsin (lane 7), human neutrophil elastase (lane 8), cathepsin G (lane 9), C1s (lane 10), factor D (lane 11), and factor  $\beta$ XIIa (lane 12). The products were separated by non-reducing SDS-PAGE and detected by Western blot using polyclonal anti-SERP-1 antiserum. Uncleaved SERP-1 is visible as a  $\approx$ 55 kDa band in lanes 1, 2, 4, 6, 10, 11, and 12. SERP-1 cleaved in or near the RCL appears as a  $\approx$ 50 kDa band in lanes 3, 4, 5, 6, 7, 8, and 9. Higher molecular weight bands indicating the formation of SDS-stable complexes between SERP-1 and the proteinase appear in lanes 2, 3, 4, and 6. **B.** SERP-1 forms a short-lived SDS-stable complex with thrombin. Purified SERP-1 (180 nM) was incubated with approximately equimolar human thrombin for 2 minutes (lane 1), 12 minutes (lane 2), or 60 minutes (lane 3). A small amount of a high molecular weight complex of 90 kDa, corresponding to the size expected for a complex between thrombin and SERP-1, is seen at 2 and 12 minutes, but both uncleaved SERP-1 and the complex have disappeared by 60 minutes leaving only the band at  $\approx$ 50 kDa that corresponds to SERP-1 cleaved at or near the RCL. **C.** SERP-1 mutant R319A/N320A does not react with the proteinases that form complexes with wild-type SERP-1. Purified R319A/N320A (lane 1) was incubated with an excess of plasmin (lane 2), uPA (lane 3), tPA (lane 4), factor Xa (lane 5), thrombin (lane 6), human neutrophil elastase (lane 7) and cathepsin G (lane 8), and the resulting products were separated by SDS-PAGE and visualized by Western blotting. Intact R319A/N320A appears as  $\approx$ 55 kDa band seen in lanes 1 through 6, while cleavage at or near the RCL yields a product of  $\approx$ 50 kDa seen in lanes 7 and 8.



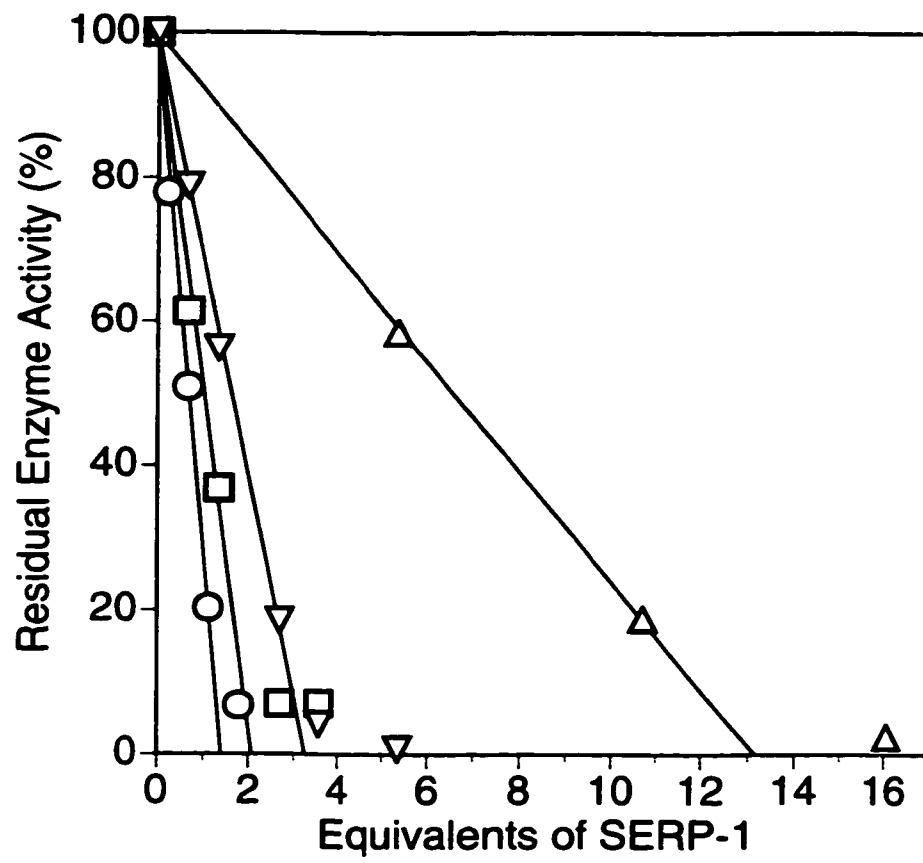
**Figure II-3: Progress curves for inhibition of tissue-type plasminogen activator (tPA) by various concentrations of SERP-1.** The enzyme (25 pM) was incubated with SERP-1 (0-20 nM) in the presence of the chromogenic substrate Chromozym-TPA (0.25 mM; 100 mM Tris-HCl, 100 mM NaCl, 2 mM CaCl<sub>2</sub>, 0.005% Triton X-100, pH 7.5, 37 °C). Reaction was followed at 405 nm. Curve (a) shows the rate of substrate turnover in the absence of SERP-1. Curves (b)-(e) show progress curves for the time-dependent inhibition of tPA activity by reaction with 2, 4, 10, and 20 nM SERP-1. Curve (f) shows the very slow background hydrolysis of Chromozym-TPA in the absence of tPA. Experimental progress curves are shown by the symbols, which for clarity show only every 10th time-point; the solid lines represent the best fit of the data to equation 1, from which were determined values for the initial and final velocities of substrate turnover,  $v_i$  and  $v_s$ , and the observed rate constant for the approach to steady state,  $k_{obs}$ , at each concentration of SERP-1. The inset plot shows the dependence of the final steady-state velocity of substrate turnover relative to uninhibited enzyme,  $v_s/v_i$ , determined from fitting individual progress curves from several experiments to Equation 1, on the concentration of SERP-1. The solid line represents the best fit to equation 2, from which was determined a value of  $K_{I(app)} = 0.14$  nM for the inhibition of tPA by SERP-1 (see text).



**Figure II-4: Second order rate constant determination.** Second-order replots for the reactions of SERP-1 with **A**, tPA; **B**, uPA; **C**, plasmin; **D**, thrombin; and **E**, factor Xa. For each enzyme, the observed rate constant for the approach to steady state,  $k_{obs}$ , was determined at various concentrations of SERP-1 in slow-binding inhibition experiments such as that shown in Figure II-3. The resulting values were corrected as described in the text to compensate for the competitive binding of chromogenic substrate in the slow-binding inhibition assay. Plots of  $k_{obs}(\text{corrected})$  against the concentration of SERP-1 are linear, and the slopes give values for  $k_{app}$ , the apparent second-order rate constant for the reaction of each enzyme with SERP-1, which are given in Table II-2.



**Figure II-5: Stoichiometry of Inhibition.** Stoichiometry of inhibition for the reactions of uPA (○), plasmin (□), factor Xa (▽) and thrombin (Δ) with SERP-1. Fixed concentrations of each enzyme were incubated with SERP-1 at various molar ratios for 1 hour at 37 °C (100 mM Tris-HCl, 100 mM NaCl, 2 mM CaCl<sub>2</sub>, 0.005% Triton X-100, pH 7.5), except Xa for which a 10 hour incubation was required to reach the final level of inhibition. At the end of the incubation, residual proteinase activity in each reaction was determined in a chromogenic substrate assay. In all cases, control experiments at longer reaction times were performed to show that reaction was complete, and that incubation under these conditions did not cause any significant loss of activity in the enzyme only control. The stoichiometry of inhibition (SI) for each proteinase corresponds to the minimum number of equivalents of SERP-1 required to achieve complete inhibition of enzyme activity, and was determined by extrapolating residual proteinase activity to its intercept on the x axis as shown by the solid lines in the figure. SI values for each enzyme were measured a minimum of 3 times in at least 2 independent experiments, and are given in Table II-2.





**REFERENCES:**

- Blanton, R. E., Licate, L. S., and Aman, R. A. (1994). Characterization of a native and recombinant *Schistosoma haematobium* serine protease inhibitor gene product. *Mol. Biochem. Parasitol.* **63**: 1-11.
- Block, W., Upton, C., and McFadden, G. (1985). Tumorigenic poxviruses: genomic organization of malignant rabbit virus, a recombinant between Shope fibroma virus and myxoma virus. *Virology* **140**: 113-24.
- Carrell, R. W., and Boswell, D. R. (1986). Serpins: A family of serine protease inhibitors. In *Protease Inhibitors*, A. Barrett and G. Salvesen, eds. (Amsterdam: Elsevier), pp. 403.
- Carrell, R. W., Pemberton, P. A., and Boswell, D. R. (1987). The Serpins: Evolution and Adaptation in a Family of Protease Inhibitors. In *Cold Spring Harbor Symposia on Quantitative Biology: Cold Spring Harbor Laboratory*.
- Christensen, S., Valnickova, Z., Thogersen, I. B., Pizzo, S. V., Nielsen, H. R., Roepstorff, P., and Enghild, J. J. (1995). Sodium dodecyl sulfate-stable complexes between serpins and active or inactive proteinases contain the region COOH-terminal to the reactive site loop. *J. Biol. Chem.* **270**: 14859-62.
- Davidson, A. J., and Moss, B. (1990). New vaccinia virus recombination plasmids incorporating a synthetic late promoter for high level expression of foreign proteins. *Nucleic Acids Res.* **18**: 4285-4286.
- Engh, R. A., Huber, R., Bode, W., and Schulze, A. J. (1995). Divining the serpin inhibition mechanism: suicide substrate 'spring'? *Tr. Biol. Technol.* **13**.
- Fenner, F., and Meyers, K. (1978). Myxoma virus and myxomatosis in retrospect: The first quarter century of a new disease. In *Viruses and the Environment*,

- E. Kurstok and K. Maramorosch, eds. (New York: Academic Press), pp. 539-570.
- Fenner, F., and Ratcliffe, F. N. (1965). *Myxomatosis* (London: Cambridge University Press).
- Gettins, P., Patston, P. A., and Schapira, M. (1993). The role of conformational change in serpin structure and function. *Bioessays* **15**: 461-7.
- Gettins, P. G., Patston, P., and Olson, S. (1996). *Serpins: structure, function and biology* (Austin: R. G. Landes Co.).
- Ghendler, Y., Arnon, R., and Fishelson, Z. (1994). *Schistosoma mansoni*: Isolation and characterization of Smpi56, a novel serine protease inhibitor. *Exp. Parasitol.* **78**: 121-131.
- Gill, S. C., and von Hippel, P. H. (1989). *Anal. Biochem.* **182**: 319-326.
- Gils, A., and Declerck, P. J. (1997). Proteinase specificity and functional diversity in point mutants of plasminogen activator inhibitor 1. *J. Biol. Chem.* **272**: 12662-12666.
- Hewick, R. M., Hunkapillar, M. W., Hood, L. E., and Dreyer, W. J. (1981). A gas-liquid solid phase peptide and protein sequenator. *J. Biol. Chem.* **256**: 7990-7997.
- Hood, D. B., Huntington, J. A., and Gettins, P. G. (1994). Alpha 1-proteinase inhibitor variant T345R. Influence of P14 residue on substrate and inhibitory pathways. *Biochemistry* **33**: 8538-47.
- Huntington, J. A., Fan, B., Karlsson, K. E., Deinum, J., Lawrence, D. A., and Gettins, P. G. (1997). Serpin conformational change in ovalbumin, enhanced reactive center loop insertion through hinge region mutations. *Biochemistry* **36**: 5432-5440.

- Jameson, G. W., Roberts, D. V., Adams, R., W., Kyle, W. S. A., and Elmore, D. T. (1973). Determination of the operational molarity of solutions of bovine alpha-chymotrypsin, trypsin, thrombin and factor Xa by spectrofluorimetric titration. *Biochem. J.* **131**: 107-117.
- Laemelli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**: 680.
- Lawrence, D. A., Ginsburg, D., Day, D. E., Berkenpas, M. B., Verhamme, I. M., Kvassman, J.-O., and Shore, J. D. (1995). Serpin-protease complexes are trapped as stable acyl-enzyme intermediates. *J. Biol. Chem.* **270**: 25309-25312.
- Lomas, D. A., Evans, D. L., Upton, C., McFadden, G., and Carrell, R. W. (1993). Inhibition of plasmin, urokinase, tissue plasminogen activator, and C1S by a myxoma virus serine proteinase inhibitor. *J. Biol. Chem.* **268**: 516-21.
- Lucas, A., Liu, L., Macen, J. L., Nash, P. D., Dai, E., Stewart, M., Yan, W., Graham, K., Etches, W., Boshkov, L., Nation, P. N., Humen, D., Hobman, M., and McFadden, G. (1996). Virus-encoded serine proteinase inhibitor SERP-1 inhibits atherosclerotic plaque development following balloon angioplasty. *Circulation* **94**: 2890-2900.
- Macen, J. L., Upton, C., Nation, N., and McFadden, G. (1993). SERP1, a serine proteinase inhibitor encoded by myxoma virus, is a secreted glycoprotein that interferes with inflammation. *Virology* **195**: 348-63.
- Maksymowych, W. P., Nation, N., Nash, P. D., Macen, J., Lucas, A., McFadden, G., and Russell, A. S. (1996). Amelioration of antigen-induced arthritis in rabbits treated with a secreted viral serine proteinase inhibitor. *J. Rheumatol.* **23**: 878-882.

- Matheson, N. R., van, H. H., and Travis, J. (1991). Evidence for a tetrahedral intermediate complex during serpin-proteinase interactions. *J. Biol. Chem.* **266**: 13489-91.
- McFadden, G. (1995). *Viroceptors, virokines and related immune modulators encoded by DNA Viruses* (Austin, Texas: R. G. Landes Co.).
- McFadden, G., Graham, K., Ellison, K., Barry, M., Macen, J., Schreiber, M., Mossman, K., Nash, P., Lalani, A., and Everett, H. (1995). Interruption of cytokine networks by poxviruses: lessons from myxoma virus. *J. Leukoc. Biol.* **57**: 731-8.
- Morgenstern, K. A., Sprecher, C., Holth, L., Foster, D., Grant, F. J., Ching, A., and Kisiel, W. (1994). Complementary DNA cloning and kinetic characterization of a novel intracellular serine proteinase inhibitor: mechanism of action with trypsin and factor Xa as model proteinases. *Biochemistry* **33**: 3432-41.
- Morrison, J. F., and Walsh, C. T. (1988). *Adv. Enzymol.* **61**: 201-301.
- Muldbjerg, M., Markussen, S., Magnusson, S., and Halkier, T. (1993). *Blood Coagul. Fibrinolysis* **4**: 47-54.
- Nash, P., Lucas, A., and McFadden, G. (1997). SERP-1, a poxvirus-encoded serpin, is expressed as a secreted glycoprotein that inhibits the inflammatory response to myxoma virus infection. In *Chemistry and Biology of Serpins*, F. C. Church, D. D. Cunningham, D. Ginsburg, M. Hoffman, S. R. Stone and D. M. Tollefsen, eds. (New York: Oxford University Press), pp. 195-205.
- O' Malley, K., Nair, S., Rubin, H., and Cooperman, B. (1997). The kinetic mechanism of serpin-proteinase complex formation. *J. Biol. Chem.* **272**: 5354-5359.

- Olson, S. T., Bjork, I., and Shore, J. D. (1993). Kinetic characterization of heparin-catalyzed and uncatalyzed inhibition of blood coagulation proteinases by antithrombin. *Methods Enzymol.* **222**: 525-559.
- Patston, P. A., Gettins, P., Beechem, J., and Schapira, M. (1991). Mechanism of serpin action: evidence that C1 inhibitor functions as a suicide substrate. *Biochemistry* **30**: 8876-82.
- Patston, P. A., and Gettins, P. G. W. (1994). A database of recombinant wild-type and mutant serpins. *Thromb. Haemost.* **72**: 166-179.
- Patston, P. A., and Gettins, P. G. W. (1996). Significance of secondary structure predictions on the reactive center loop region of serpins: a model for the folding of serpins into a metastable state. *FEBS Lett.* **383**: 87-92.
- Patston, P. A., Gettins, P. G. W., and Schapira, M. (1994). Serpins are suicide substrates: implications for the regulation of proteolytic pathways. *Semin. Thromb. Hemost.* **20**: 410-416.
- Potempa, J., Korzus, E., and Travis, J. (1994). The serpin superfamily of proteinase inhibitors: structure, function, and regulation. [Review]. *J. Biol. Chem.* **269**: 15957-60.
- Rubin, H. (1996). Serine protease inhibitors (SERPINS): where mechanism meets medicine. *Nature Medicine* **2**: 632-633.
- Rubin, H., Plotnick, M., Wang, Z. M., Liu, X., Zhong, Q., Schechter, N. M., and Cooperman, B. S. (1994). Conversion of alpha 1-antichymotrypsin into a human neutrophil elastase inhibitor: demonstration of variants with different association rate constants, stoichiometries of inhibition, and complex stabilities. *Biochemistry* **33**: 7627-33.
- Schechter, I., and Berger, A. (1967). *Biochem. Biophys. Res. Commun.* **27**: 100-107.

- Schechter, N. M., Plotnick, M., Selwood, T., Walter, M., and Rubin, H. (1997). Diverse effects of pH on the inhibition of human chymase by serpins. *J. Biol. Chem.* **272**: 24499-24507.
- Stein, P. E., and Carrell, R. W. (1995). What do dysfunctional serpins tell us about molecular mobility and disease. *Nat. Struct. Biol.* **2**: 96-113.
- Stone, S. R., and Hermans, J. M. (1995). Inhibitory mechanism of serpins. Interaction of thrombin with antithrombin and protease nexin 1. *Biochemistry* **34**: 5164-72.
- Travis, J., and Salvesen, G. S. (1983). Human plasma proteinase inhibitors. *Annual Reviews in Biochemistry* **52**: 655-709.
- Turner, P. C., Musy, P. W., and Moyer, R. W. (1995). Poxvirus serpins. In *In Viroceptors, Virokines and Related Immune Modulators Encoded By DNA Viruses*, G. McFadden, ed. (Austin, TX: R.G. Landes Company), pp. 67-88.
- Upton, C., Macen, J. L., Wishart, D. S., and McFadden, G. (1990). Myxoma virus and malignant rabbit fibroma virus encode a serpin-like protein important for virus virulence. *Virology* **179**: 618-631.
- Wilczynska, M., Fa, M., Karolin, J., Ohlsson, P.-I., Johansson, L., and Ny, T. (1997). Structural insights into serpin-protease complexes reveal the inhibitory mechanism of serpins. *Nat. Struct. Biol.* **4**: 354-356.
- Wilczynska, M., Fa, M., Ohlsson, P.-I., and Ny, T. (1995). The inhibition mechanism of serpins. *J. Biol. Chem.* **270**: 29652-29655.
- Wright, H. T., and Scarsdale, J. N. (1994). Structural basis for serpin inhibitor activity. *Proteins: Structure, Function and Genetics* **22**: 210-225.

Yenbutr, P., and Scott, A. L. (1995). Molecular cloning of a serine proteinase inhibitor from *Brugia malayi*. *Infection & Immunity* **63**: 1745-53.

Zhou, Q., Snipas, S., Orth, K., Muzio, M., Dixit, V. M., and Salvesen, G. S. (1997). Target protease specificity of the viral serpin crmA. *J. Biol. Chem.* **272**: 7797-7800.

**CHAPTER III**

**REACTIVE CENTRE MUTANTS OF SERP-1 EXHIBIT LOSS OF *IN VITRO*  
INHIBITORY ACTIVITY**

"The great tragedy of science - the slaying of a beautiful hypothesis by an ugly fact."  
- *Aldous Huxley* -

*Volo, Non Valeo*



## INTRODUCTION

The serpin (*serine proteinase inhibitor*) family of structurally homologous, single chain proteins are of considerable interest to biochemists for both their remarkable functional characteristics, as well as their unusual folding and conformational properties. Members of the serpin family regulate the activity of a wide variety of serine proteinases and thereby play a critical role in a host of biological processes (Rubin, 1996; Travis and Salvesen, 1983). Present understanding of the serpin mechanism of inhibition suggests that the serpin and proteinase form a covalent complex during the attempted substrate-like hydrolysis of an exposed peptide loop at a specific site. This peptide loop, known as the reactive-centre loop (RCL), ranges in size from approximately 20 to 25 residues in length, and presents a specific peptide bond (P1-P1') to the target proteinase for attempted hydrolysis. As such, serpins are considered to be mechanism based, suicide substrate inhibitors that form a covalent complex with the target proteinase through a branched pathway mechanism that can lead to either a stable inhibited complex, or cleavage of the serpin (Engh et al., 1995; O' Malley et al., 1997; Wilczynska et al., 1997; Wilczynska et al., 1995). This is in sharp contrast to other classes of proteinase inhibitors such as the Kunitz, Kazal and Bowman-Birk inhibitors which form tight, but reversible, non-covalent complexes with their target proteinases (Bode and Huber, 1992; Laskowski and Kato, 1980; Stone et al., 1997). While almost all other proteins fold into the most energetically favorable, lowest free-energy state (Baker,

1998; Murray et al., 1998; Vajda et al., 1997), serpins fold into a kinetically trapped metastable conformation (Dinner and Karplus, 1998; Patston and Gettins, 1996). Several lines of evidence suggest that inhibition requires the interaction of the P1 residue with the active site of a target proteinase which must trigger a conformational change in the serpin (Carrell et al., 1994; Gettins et al., 1993; Huntington et al., 1997; Plotnick et al., 1996). This conformational change involves the insertion of the RCL into  $\beta$ -sheet A (see Figure I-8) of the serpin body and is likely accompanied by the distortion of the active site of the proteinase which prevents the rapid completion of catalytic hydrolysis (Plotnick et al., 1996). The resulting serpin-proteinase complex is stable to dissociation by SDS, heat or urea and is therefore thought to contain a covalent bond between O $\gamma$  of the proteinase active site serine and the carbonyl carbon of the serpin P1 residue, existing as an acyl-enzyme (Lawrence et al., 1995; Wilczynska et al., 1995). The stable complex is long-lived, with an *in vitro* half-life ranging from hours to days, but does eventually hydrolyze to release active enzyme and inactivated serpin. In an *in vivo* environment, such a long half-life would almost always result in removal and destruction of the inhibited complex.

The role that RCL residues play in determining the kinetics of complex formation and breakdown is not entirely clear. However, since these residues are either in contact with, or in close proximity to the active site of the proteinase, they are assumed to play a role in serpin specificity. A number of studies have implicated RCL residues in determining the rate of complex formation. Replacement of the P3-

P3' residues of  $\alpha$ 1-antichymotrypsin (ACT) with the corresponding residues from human neutrophil elastase (hNE) inhibiting serpin,  $\alpha$ <sub>1</sub>-proteinase inhibitor ( $\alpha$ <sub>1</sub>-PI), converts ACT from a substrate to an inhibitor of hNE (Rubin et al., 1994). The ACT/PI chimera containing the P3-P3' residues had a  $k_{inh}$  for hNE inhibition greater than that observed with an ACT variant containing only the P1 residue of  $\alpha$ <sub>1</sub>-PI. This indicates that residues other than the P1-P1' are important in complex formation and stabilization, presumably by virtue of their interaction with the extended substrate binding site of the proteinase (Rubin et al., 1994). Other studies have implicated RCL residues extending from P6 to P3' in binding of serpins to their target proteinases (Patston and Gettins, 1994; Plotnick et al., 1997).

P1 variants have been examined with a large number of serpins, with the conclusion that this residue plays a critical role in determining the specificity of the serpin for its target proteinase. P1 modifications also act to determine the stoichiometry of inhibition by altering the relative rates of the substrate and inhibitory reactions. A P1 Met to Arg variant of  $\alpha$ 1-proteinase inhibitor has specificity for thrombin and activated protein C (APC) rather than elastase (Heeb et al., 1990), while replacing the complete P7-P3' RCL of  $\alpha$ 1-PI with the corresponding residues from antithrombin (AT) results in a chimeric serpin that inhibits thrombin but not APC (Hopkins et al., 1995). Also, a Leu to Arg mutation in the P1 of heparin co-factor II results in an improved rate of inhibition of thrombin (Derechin et al., 1990).

The P7-P3' residues of the RCL of SERP-1 was inserted in a backbone of  $\alpha$ 1-antitrypsin and  $\alpha$ 1-antichymotrypsin to create AT/SERP1 and ACT/SERP1 chimeras, respectively (Bottomley and Stone, 1998). AT/SERP1 inhibited plasmin, tissue-type plasminogen activator (tPA), urokinase (uPA), thrombin and Factor Xa with second order association rate constants of approximately  $10^4 \text{ M}^{-1}\text{s}^{-1}$  and a stoichiometry of inhibition of approximately 1 in all cases. ACT/SERP1, however, formed SDS-stable complexes with only plasmin and thrombin with association rates that were 100-fold slower than AT/SERP1 and with higher SI values (Bottomley and Stone, 1998). The RCL of ACT and ACT/SERP1 contains four more residues than the RCL of AT or AT/SERP1. Replacement of these four residues (VETR) into the AT/SERP1 to form AT/SERP1(VETR) resulted in a chimeric serpin that inhibited tPA, plasmin and thrombin with rate constants 10-fold lower than AT/SERP1 while the SI remained around 1 (Bottomley and Stone, 1998). This suggests that the additional residues effect the rate of initial complex formation by placing the RCL in a non-ideal conformation, and implies that both RCL length and serpin scaffold are important in defining the inhibitory characteristics of a serpin.

In this study a panel of chimeric serpins utilizing the SERP-1 scaffold and RCL residues from a variety of known serpins were constructed. These create chimeras that are universally poorer inhibitors than the parental SERP-1, but may distinguish between the inhibition of tPA/thrombin and uPA/plasmin.

**MATERIALS AND METHODS:**

**Materials:** The human enzyme tissue-type plasminogen activator (tPA, two chain form), was obtained from Sigma. Proteinase substrate Chromozym-TPA was purchased from Boehringer-Mannheim. Anti-SERP-1 monoclonal antibodies AQ.H9 and AG.F11 were kindly provided by Leona Ling (Biogen, Inc.).

**Construction of SERP-1 RCL Mutants:** RCL mutants and chimeras in the SERP-1 backbone were constructed using a multi-stage mutagenesis and cloning approach (Figure III-1). Mutagenesis of SERP-1 was achieved with the aid of the Altered Sites II mutagenesis system (Promega). Site-directed mutagenesis of SERP-1 was employed to insert a silent AlwNI site at the P8-P7 codon boundary (nucleotide position 934 of the SERP-1 ORF) without altering the SERP-1 protein sequence so that AlwNI and BglI could be employed to excise the P7-P2' region of the RCL. At the same time, the upstream endogenous AlwNI site at nucleotide position 795 of the open reading frame (ORF) was disrupted with a silent mutation that did not alter the protein translation. A BamHI/HindIII fragment containing the complete SERP-1 ORF was cloned into the pAlter-1 vector (Promega). Purified pAlter-SERP-1 was denatured and annealed to three mutagenic oligonucleotides simultaneously. These were the ampicillin resistance repair oligonucleotide (Promega), and two oligonucleotides used for SERP-1 RCL cassette mutagenesis: PNAIwRC3 (GGGGAACGACGGCGTCGTCAGACACTGCCATCACCTCATCC) that maintains a Ser with codon TCA and Thr with codon ACT and PNAIwKO2:

(GACGCCCTCCAGAGATTAGGGGTGCGAGACGC)

that maintains Leu with codon TTA. Mutagenic mismatch positions are indicated in bold.

The resulting construct, termed pAS-Alw was subcloned into pUC-19 to generate pUC-Salw. This allows construction of RCL cassette mutants of SERP-1 by cloning in synthetic oligonucleotide pairs in place of the endogenous P7-P2' region. Synthetic oligonucleotides were designed as complementary pairs to create the recombinant RCLs (Table III-1). These oligonucleotide pairs were designed such that upon ligation into pUC-SAlw, they would reconstitute the AlwNI site, but not the BglI site. Oligonucleotide pairs were added together, denatured, and allowed to slowly anneal by cooling at a rate of 1°C/min in a Turner PCR machine. The pUC-SAlw was digested with either AlwNI and BsaI or BglI and BsaI and the large fragment isolated and purified from each digest. Three way ligations were performed to incorporate the synthetic oligonucleotide pairs into the pUC-SAlw backbone reconstituted using the fragments isolated from the AlwNI/BsaI and BglI/BsaI digests described above. RCL chimeras were isolated on the basis of the deletion of the RCL BglI site and reconstitution of the RCL AlwNI site, and confirmed by DNA sequencing. RCL chimeric mutants were then sub-cloned into the pMJ-601 vector using BamHI and HindIII endonucleases to allow expression in vaccinia virus.

**Expression of SERP-1 mutants from recombinant vaccinia virus:** The complete SERP-1 RCL mutant gene, including the signal sequence, was inserted into the thymidine kinase locus of vaccinia virus strain WR under the control of a strong,

synthetic late promoter by homologous recombination as described (Macen et al., 1993), in order to produce recombinant viruses termed VV-S1(PAI), VV-S1(Apl), VV-S1(MN), VV-S1(LN), VV-S1(RM), and VV-S1(Ala)<sub>6</sub>. At late times (>8 hours) after infection of BGMK cells with VV-S1mut viruses, SERP-1 accumulates as a stable glycoprotein in the culture supernatant. Expression was confirmed by Western blot analysis using polyclonal rabbit anti-SERP-1 serum. At 20 hours post-infection, the supernatant was collected and SERP-1 was purified as described below.

**Purification of wild type and mutant SERP-1:** BGMK cells ( $2 \times 10^8$  cells) were adsorbed with vaccinia virus expressing wild-type or chimeric SERP-1 at a multiplicity of infection of 1 pfu/cell for 2 hours at 37°C in 10 ml of DMEM containing 10% newborn calf serum. The inoculum was removed, and the cells were washed three times with 50 ml of sterile PBS to remove medium and serum proteins. Serum-free DMEM (15 ml) was added to each bottle and the infection was allowed to proceed for 20 hours at 37°C. The culture supernatant was collected, spun at 5000 x g to pellet cells and cell debris, and stored at -20°C. The medium containing secreted viral proteins was concentrated approximately 50-fold using an Amicon pressure cell equipped with a 10 kDa cut-off membrane, and was then dialyzed against 25 mM Tris, pH 8.0 at 4°C using Spectrapore dialysis tubing with a 30 kDa molecular weight cut-off. The dialyzed samples were spun at 14000 x g to remove precipitates, loaded onto a MonoQ anion exchange column (Pharmacia), and eluted with a linear salt gradient (0 - 300 mM NaCl in 25 mM Tris, pH 8.0). Fractions were

analyzed by SDS-PAGE, and those containing SERP-1 were identified by immunoblotting using anti-SERP-1 antiserum (Macen et al., 1993), pooled, and concentrated to 1 ml using Centriprep 10 concentrators (Amicon). This material was further purified on a Superdex 75 gel filtration column (Pharmacia) (150 mM NaCl, 25 mM Tris, pH 8.0). Fractions were collected and analyzed for SERP-1 by SDS-PAGE, visualizing by silver staining and immunoblotting using anti-SERP-1 mAb AQ.H9.

**Determination of SERP-1 concentration by ELISA:** Flat bottom 96 well ELISA plates (Nunc) were coated with 100  $\mu$ l of anti-SERP-1 mAb AG.F11 at 1  $\mu$ g/ml in 50 mM carbonate buffer, pH 9.6 overnight at 4°C. Prior to use, plates were washed 3 times with wash buffer (PBS, 0.1% (v/v) Tween-20) and incubated with 200  $\mu$ L blocking buffer (PBS, 2% (w/v) BSA, 0.05% Tween-20) for 2 hours. SERP-1 standards and samples were diluted in assay buffer (PBS, 1% BSA (w/v), 0.01% Tween-20) and 50  $\mu$ L added to triplicate wells. SERP-1 standards consisted of highly purified SERP-1 with the concentration determined using  $A_{280}$  and a molar extinction coefficient of 32,700  $M^{-1}cm^{-1}$  (Nash et al., 1998). Samples and standards were incubated on the plate for 1 hour, washed 3 times with wash buffer and 50  $\mu$ L of 1mg/ml biotinylated AQ.H9 (Biogen, Inc.) in assay buffer was added and incubated for 45 min. The plate was washed again and 50  $\mu$ L of 1  $\mu$ g/ml streptavidin-HRP in assay buffer was added and incubated for 45 min. The plate was then washed 3 times and 100  $\mu$ L of chromogenic substrate (0.5 mg/ml OPD (Sigma), 0.003 % hydrogen peroxide, 0.05 M citric acid, 0.05 M  $NaPO_4$  buffer, pH 5.0) was added. When color development was complete, the reaction was stopped with the



addition of 100  $\mu$ L of 2M H<sub>2</sub>SO<sub>4</sub> and the absorbance at 490 nm was determined using a BioRad ELISA plate reader.

**Western blot analysis of the products of reaction of SERP-1 with proteinases:**

SERP-1 (180 nM) was incubated with a slight molar excess of each proteinase for the times indicated in a total reaction volume of 10  $\mu$ l (100 mM NaCl, 2 mM CaCl<sub>2</sub>, 0.005% Triton X-100, 100 mM Tris-HCl, pH 7.5). Control experiments established that both SERP-1 and the proteinases are stable under these conditions. Reactions were quenched by boiling for 5 minutes in SDS-loading buffer containing 100 mM dithiothreitol and 2% SDS. Samples were separated on 4 - 20% linear gradient or 10% Tris-glycine SDS polyacrylamide gels (Novex) using the Laemmli buffer system (Laemmli, 1970). Proteins were transferred to Hybond C-extra (Amersham) nitrocellulose by electroblotting, blots were blocked in TBS (150 mM NaCl, 2.5 mM KCl, 25 mM Tris-HCl, pH 7.4) containing 5% (w/v) skimmed milk powder and 0.1% (v/v) Tween-20 for >2 hours, and SERP-1 was detected by incubating for 1 hour with 0.01% (v/v) mAb AQ.H9 in blocking buffer. After washing with TBS containing 0.1% (v/v) Tween-20, a secondary goat anti-rabbit antibody conjugated to horseradish peroxidase (BioRad) was applied for 1 hour. Immunoreactive SERP-1 species were visualized using ECL detection (Amersham) on Kodak X-ray detection type film. Reactions that showed complete cleavage of SERP-1 by the proteinase, with no evidence of the formation of a stable SERP-1-enzyme complex, were repeated with shorter incubation times in order to see whether reactions proceeded via a transient inhibited complex. Reactions in which the serpin appeared unchanged

at the end of the incubation period were repeated at higher enzyme concentrations and for longer incubation periods.

**Slow binding inhibition kinetics:** Inhibition progress curves were obtained by incubating a limiting concentration of tPA (125 pM) with various concentrations of SERP-1 (0.125 to 100 nM) in the presence of Chromozym-tPA chromogenic substrate. In all cases, serpin was present at  $\geq 10$ -fold excess over proteinase in order to achieve pseudo-first-order conditions with respect to SERP-1. Concentrations of enzyme and substrate were chosen so that  $\leq 10\%$  of the substrate was hydrolyzed over the entire duration of the assay. Reactions were performed at 37°C in a total volume of 800  $\mu$ l (100 mM Tris-HCl, 100 mM NaCl, 2 mM CaCl<sub>2</sub>, 0.005% Triton X-100, pH 7.5) in plastic cuvettes sealed with Parafilm. The time-dependent inhibition of each enzyme by SERP-1 was monitored by following the rate of production of *p*-nitroaniline (pNA) at 405 nm at 1 or 5 minute intervals for a period of 1000 minutes, using a CARY 1 spectrophotometer equipped with a six-cell cuvette holder.

## RESULTS

SERP-1 chimeric mutants were constructed in which the RCL was altered within the SERP-1 scaffold. This was accomplished with the aid of site-directed mutagenesis and subsequent cloning of synthetic oligonucleotide pairs (Table III-1)

to replace the entire RCL from P7 to P2' as summarized in Figure III-1. The pUC-SAlw vector allows the complete replacement of the P7 to P2' region of the SERP-1 RCL, and can therefore be used for the creation of RCL mutants other than those discussed here.

Seven SERP-1 chimeras were constructed to partially or completely mimic the RCL sequence found in a variety of known cellular serpins (Figure III-2). The SERP-1(PAI) chimera contains the P7 to P2' region of plasminogen activator inhibitor 2 (PAI-2) within the SERP-1 scaffold, while the SERP-1(Apl) chimera contains the complete P7 to P2' region of the potent plasmin inhibitor, antiplasmin. A series of smaller RCL changes were also made, focusing on the critical P1 and P1' residues of SERP-1. The SERP-1(MN) chimera contains a single change of arginine to methionine at the P1 residue. A methionine at the P1 position is characteristic of  $\alpha$ 1-proteinase inhibitor, or antitrypsin, a particularly potent serpin. Similarly, the SERP-1(LN) chimera converts the P1 from arginine to leucine to resemble the P1 of antichymotrypsin. The SERP-1(RM) mutant alters the P1' residue from asparagine to methionine. A P1' asparagine is thus far unique among serpins, and converting this position to a methionine conforms to the P1-P1' of antiplasmin. Finally, the SERP-1(DC) chimera converts the P1 and P1' from Arg-Asn to Asp-Cys, making it equivalent to the P1-P1' found in the orthopoxvirus intracellular serpin crmA. CrmA is a potent inhibitor of caspases such as interleukin 1- $\beta$  converting enzyme (ICE), and of the serine proteinase granzyme B. As a counterpart to the P1-P1' Ala-Ala mutant (SAA), SERP-1(Ala)<sub>6</sub> mutates P7 to P2 to alanine, replacing all of the

residues except for the P1-P1' with alanines. The RCL changes made are summarized in Figure III-2.

In order to produce sufficient amounts of SERP-1 chimera protein, all seven mutants were expressed from a recombinant vaccinia virus expression system. Although this system does not produce large quantities of protein, it has been shown to express functionally active SERP-1, while yeast and bacterial systems cannot. The production of recombinant vaccinia virus expressing SERP-1 chimeras is detailed in Figure III-3. Following plaque purification, SERP-1 chimeras were confirmed by sequencing of the SERP-1 gene amplified by PCR. The resulting viruses were termed VV-S1(Apl), VV-S1(PAI), VV-S1(MN), VV-S1(LN), VV-S1(RM), VV-S1(DC), and VV-S1(Ala)<sub>6</sub>. SERP-1 chimeric proteins were purified to between 80% and 95% purity as described. A detailed description of the purification scheme and SDS-PAGE silver stain gels showing the degree of purity can be found in Chapter II.

#### ***Interactions observed on SDS-PAGE***

The SERP-1(Apl) chimera resulted in a non-inhibitory serpin that was subject to cleavage by uPA, tPA, plasmin, thrombin, factor Xa, trypsin, chymotrypsin, human neutrophil elastase and cathepsin G (Figure III-4). In no cases were SDS-stable complexes observed between SERP-1(Apl) and any of the proteinases tested. A band with an apparent molecular weight of 50 kDa, corresponding to serpin cleaved within the RCL, was seen in all cases, indicating that this chimera is still capable of interacting with all of the serine proteinases but fails to form a stable inhibited complex.

By contrast, the SERP-1(PAI) chimera failed to interact with uPA, tPA, or plasmin and did not produce either a stable complex, or a cleaved serpin product (Figure III-5). This was not expected as the P1 arginine of this chimera is identical to that found in both wild-type SERP-1 and the SERP-1(Apl) mutant which are inhibitors of these proteinases. A small amount of SDS-stable complex was observed upon incubation with thrombin. Despite this, SERP-1(PAI) is subject to cleavage by human neutrophil elastase and cathepsin G, exhibiting a product of approximately 50 kDa, consistent with cleavage at or near the RCL. SERP-1(PAI) is also cleaved by trypsin and chymotrypsin without formation of an inhibited complex.

The two P1 mutants of SERP-1 exhibit somewhat different inhibitory phenotypes. SERP-1(LN) fails to form SDS-stable inhibited complexes with any of the eight enzymes tested (Figure III-6). Cleaved product was observed in the interaction between SERP-1(LN) and each of the proteinases, indicating that it does interact in as a substrate in all cases. SERP-1(MN) formed SDS-stable complexes with tPA or thrombin. However, no inhibited complex was observed with either plasmin or uPA, both of which cleaved SERP-1(MN) without forming a visible inhibited complex. SERP-1(MN) was also cleaved by trypsin, chymotrypsin, hNE and cathepsin G (Table III-2).

Mutation of the P1' residue of SERP-1 to methionine to create SERP-1(RM) resulted in an inhibitory profile that resembled SERP-1(MN). While SDS-stable high molecular weight complexes were observed with both tPA and thrombin, the extremely small amount of such complex suggests that SERP-1(RM) is a relatively

poor inhibitor of these proteinases. SERP-1(RM) failed to form inhibited complexes with, and was cleaved by uPA, plasmin, trypsin, hNE, cathepsin G and chymotrypsin (Figure III-7). Mutation of the P1-P1' to aspartate-cysteine to create SERP-1(DC) resulted in a serpin with properties similar to those observed for the P1-P1' alanine-alanine mutant described in Chapter II. SERP-1(DC) was subject to cleavage by trypsin, hNE, cathepsin G and chymotrypsin, but failed to interact with uPA, tPA, plasmin or thrombin (Figure III-8).

SERP-1(Ala)<sub>6</sub> failed to form SDS-stable complexes with any of the proteinases tested, but a 50 kDa cleaved form was observed upon incubation with uPA, tPA, plasmin, thrombin, trypsin and chymotrypsin. Incubation of SERP-1(Ala)<sub>6</sub> with hNE and cathepsin G failed to exhibit either substrate or inhibition activity, and no interaction was observed.

#### ***Slow binding inhibition of tPA***

Although none of the SERP-1 RCL mutants appeared to be candidates for proteinase inhibition with reasonably fast association rates, we attempted to quantify the degree of tPA inhibition using the technique of slow-binding inhibition. SERP-1(RM), SERP-1(MN), SERP-1(Apl), and SERP-1(DC) were all examined for their ability to inhibit tPA under slow binding conditions. In none of these cases was inhibition observed at the concentrations of serpin that we were able to achieve in an 800  $\mu$ L volume. Using the equations derived in Chapter II, this allows us to place limits on the second order rate constant for the inhibition of tPA by these mutants. Thus, for SERP-1(RM), SERP-1(MN), SERP-1(Apl), and SERP-1(DC), the apparent

second order association rate constant,  $k_{app}$ , must be less than  $10^3 \text{ M}^{-1}\text{s}^{-1}$ . Although SERP-1(RM) and SERP-1(MN) form SDS-stable inhibited complexes with tPA that were visualized by western blot, the rate of inhibition of tPA by these chimeras is considerably slower than was observed for wild-type SERP-1.

## DISCUSSION

Although much progress has been made in elucidating the serpin mechanism of inhibition in general, there is still only a rudimentary understanding of the structural elements that determine the activity of a serpin against specific target proteinases. Previous investigations have demonstrated that the reactive centre loop residues around the P1-P1' can influence complex formation and inhibition properties (Bottomley and Stone, 1998; Cooperman et al., 1993; Gettins et al., 1996; Hopkins et al., 1995; Plotnick et al., 1997; Sherman et al., 1992). Residues within the RCL are predicted to interact with the extended substrate binding site of the target proteinase, and therefore are thought to play an important role in determining the inhibitory activity of the serpin. In the current study, we examine the effects of mutations within the RCL on the ability of SERP-1 to form SDS-PAGE stable complexes with a panel of eight serine proteinases. By examining the inhibitory profile of these chimeras we can gain an understanding of the degree that the

SERP-1 scaffold participates in complex formation and to what degree specific amino acid residues within the RCL contribute to the inhibitory activities of SERP-1.

The SERP-1(PAI) chimera failed to inhibit uPA, tPA and plasmin, and did not appear to interact with these enzymes, despite the presence of an arginine at the P1 position. This is somewhat unexpected as these enzymes cleave after basic amino acids and would be expected to recognize a P1 arginine and result in either a substrate or inhibitor interaction. Although this could be a result of aberrant folding of the RCL of SERP-1(PAI), the appearance of a 50 kDa cleaved form of this chimera upon incubation with hNE and cathepsin G, and an apparent SDS-stable product observed upon incubation with thrombin suggests that these enzymes are still able to efficiently interact with the RCL. Since the chimeras are secreted from infected cells as mature glycoproteins, it is unlikely that generalized incorrect folding has occurred, as such proteins would be unlikely to escape the stringent controls present in the endoplasmic reticulum. While PAI-2 has the additional feature of spontaneously entering a latent conformation that is unable to inhibit proteinases, this is generally believed to be largely a function of the PAI-2 scaffold, and so we consider it unlikely that this is the case with SERP-1(PAI).

By contrast, SERP-1(Apl) is seen as a substrate by all of the enzymes tested. This chimera also contains a P1 arginine, suggesting that in this case, this position is susceptible to cleavage. Despite the fact that antiplasmin is an efficient inhibitor of plasmin, uPA and tPA, the SERP-1(Apl) chimera failed to inhibit any of the enzymes



tested, suggesting that the SERP-1 scaffold is sufficiently different from that of antiplasmin.

Mutations within the P1-P1' region of SERP-1 had variable effects on the inhibition profile of the chimeras. SERP-1(LN) failed to form inhibited complexes with, but was cleaved by all eight of the proteinases listed in Table III-2. SERP-1(DC) failed to interact with uPA, tPA, plasmin or thrombin, but was still cleaved by trypsin, hNE, cathepsin G and chymotrypsin. SERP-1(RM) and SERP-1(MN) formed SDS-stable complexes with tPA and thrombin, though not with any of the other enzymes tested. The absence of inhibited complexes with uPA and plasmin for the SERP-1(RM) and SERP-1(MN) chimeric serpins presents an interesting discrepancy in the inhibition profile compared to wild-type SERP-1. Since the inhibition of tPA and thrombin by SERP-1(RM) and SERP-1(MN) appears to be much weaker than that observed with wt SERP-1, with  $k_{app}$  below  $10^3 M^{-1} s^{-1}$  in both cases, it is possible that plasmin and uPA inhibition have dropped below the threshold of detection. Alternatively, it is possible that these mutations affect the stoichiometry of inhibition (SI), and that the SI for uPA and plasmin is so high that no inhibited complex can be detected. In the absence of larger quantities of purified material, this has thus far not been tested. One final possibility is that the RCL mutants have accelerated both the association and dissociation rate such that inhibition cannot be observed due to a short half-life of the inhibited complex. A mutation in the RCL of crmA aimed at producing a more potent inhibitor of caspases 3 and 8 resulted in rapid degradation of crmA and a subsequently poor inhibitor

(Ekert et al., 1999). This suggests that an optimal substrate site in place of the RCL peptide creates a poorer, rather than a more efficient inhibitor.

Taken as a whole, this set of RCL chimeras within the SERP-1 scaffold demonstrate that the activity of the SERP-1 protein is dependent on both the RCL and serpin scaffold. The RCL region of other serpins, and even small changes in the P1 and P1' residues are not compatible with SERP-1 function.

**TABLE III-1**

**OLIGONUCLEOTIDES USED TO CONSTRUCT RCL MUTANT CHIMERAS IN THE SERP-1 BACKBONE**

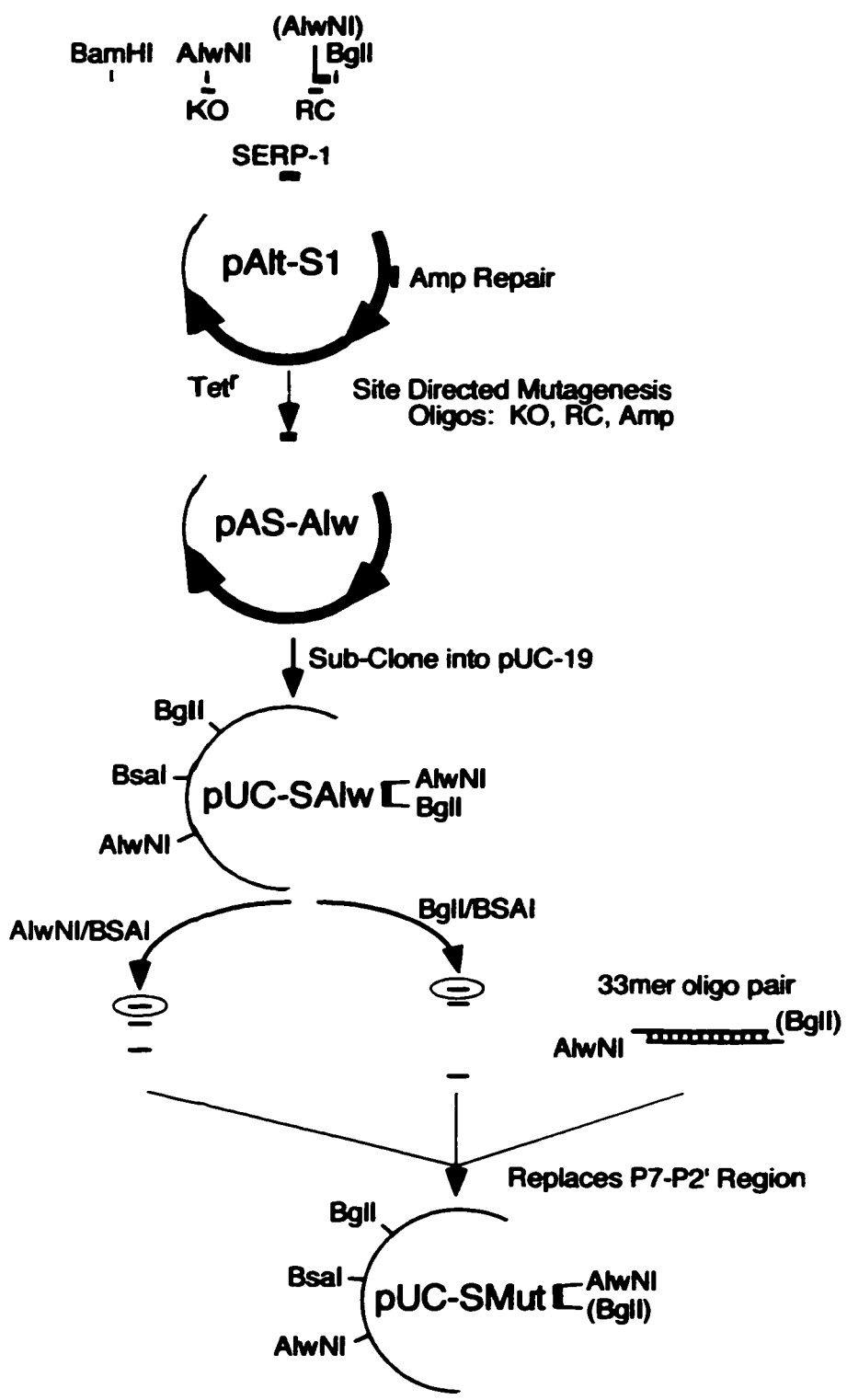
| <b>Oligo Name</b> | <b>Sequence</b>                      | <b>RCL Created</b>           | <b>RCL Sequence (P7-P2')</b> |
|-------------------|--------------------------------------|------------------------------|------------------------------|
| PN3PAI2+          | CTGGTGGAGTAATGACAGGGGCGGAACGGGCCTCA  | P7-P2' of PAI-2              | GGVMTGRTG                    |
| PN4PAI2-          | GGCCCGTTCCGCCCTGTCACTACTCCACCAGTGT   |                              |                              |
| PN1SApl+          | CTACTTCTATCGCCCATGTCCGGTATGTCTCTCA   | P7-P2' of Antiplasmin        | TSIAMSRMS                    |
| PN2SApl-          | GAGACATACGGGACATGGCGATAGAAGTAGTGT    |                              |                              |
| PN5S(Ala)6+       | CTGCTGCGCGCAGCTGCGGCCAGGAAACGGCACTCA | P1 Leucine                   | AITLIPLNA                    |
| PN6S(Ala)6-       | GTGCGTTCTGCGCCGAGCTGCGGCAGCAGTGT     |                              |                              |
| PN5SMN+           | CTGCCATCACCCCTCATCCCCATGAACGGCACTCA  | P1 Methionine                | AITLI PMNA                   |
| PN5SMN-           | GTGCGTTCATGGGGATGAGGGTGATGGCAGTGT    |                              |                              |
| PN11SRM+          | CTGCCATCACCCCTCATCCCCAGGATGGCACTCA   | P1' Methionine               | AITLI PRMA                   |
| PN12SRM12-        | GTGCCATCCTGGGGATGAGGGTGATGGCAGTGT    |                              |                              |
| PN7SDC+           | CTGCCATCACCCCTCATCCCCCGCATGTGCACTCA  |                              |                              |
| PN8SDC-           | GTGCACAGTCGGGGATGAGGGTGATGGCAGTGT    |                              |                              |
| PN9SLeu+          | CTGCCATCACCCCTCATCCCCCTGAACGGCACTCA  |                              |                              |
| PN10SLeu-         | GTGCGTTCAGGGGATGAGGGTGATGGCAGTGT     | P7-P2 (Alanine) <sub>6</sub> | AAAAAARNA                    |

**TABLE III-2**  
**SUMMARY OF SERP-1 RCL MUTANTS EMSAS WITH PROTEINASES**

|                     | (PAI2) | (Apl) | PILeu (LN) | PI Met (MN) | P1' Met (RM) | P1-P1' DC | (Ala) <sub>6</sub> |
|---------------------|--------|-------|------------|-------------|--------------|-----------|--------------------|
| <b>uPA</b>          | N      | S     | S          | S           | S            | N         | S                  |
| <b>tPA</b>          | N      | S     | S          | I           | I            | N         | S                  |
| <b>Plasmin</b>      | N      | S     | S          | S           | S            | N         | S                  |
| <b>Thrombin</b>     | I      | S     | S          | I           | I            | N         | S                  |
| <b>Trypsin</b>      | S      | S     | S          | S           | S            | S         | S                  |
| <b>hNE</b>          | S      | S     | S          | S           | S            | S         | N                  |
| <b>Cathepsin G</b>  | S      | S     | S          | S           | S            | S         | N                  |
| <b>Chymotrypsin</b> | S      | S     | S          | S           | S            | S         | S                  |

Abbreviations for the enzymes are as follows: tPA = tissue-type plasminogen activator; uPA = urokinase-type plasminogen activator; hNE = human neutrophil elastase; pTrypsin and hTrypsin = porcine and human trypsins; pPE = porcine pancreatic elastase; bChymotrypsin = bovine chymotrypsin. The symbol *I* indicates the observation of a high molecular weight band, relative to that for unreacted SERP-1, indicating the formation of an SDS-stable inhibited complex between SERP-1 and the enzyme. The observation of such a complex was invariably accompanied by the formation of some amount of a lower molecular weight species corresponding to SERP-1 that has been proteolytically cleaved in or near the RCL. The symbol *S* indicates that only lower molecular weight species and unreacted SERP-1 were observed in the product mixture, indicating that SERP-1 was proteolytically cleaved without the concomitant formation of a detectable high molecular weight complex. The symbol *N* indicates that incubation with the proteinase resulted in no apparent change in the SERP-1 band, even when retested at higher concentrations of enzyme or for longer incubation times. Cases in which the interaction was not tested are denoted *n.d.*

**Figure III-1: Construction of SERP-1 RCL chimeras.** SERP-1 was cloned into the pAlter-1 vector containing a defective ampicillin resistance gene. Site directed mutagenesis was employed to create a silent AlwNI site at 934bp of the SERP-1 ORF, while destroying the AlwNI site at 795bp and repairing the ampicillin resistance gene. The resulting mutant pAS-Alw was subcloned into pUC-19 to create pUC-SAlw. Fragments isolated from pUC-SAlw treated with AlwNI/BsaI or BglI/BsaI endonucleases were combined with annealed oligonucleotide pairs and AlwNI and BglI complementary overhangs and reconstituting the P7-P2' region of the RCL in a three way ligation. The resulting SERP-1 scaffold/RCL chimeras were identified by the loss of the BglI site and confirmed by nucleotide sequencing.



**Figure III-2: Amino acid sequence of the P8-P4' Reactive centre loop of SERP-1 chimeras.** Existing serpins: SAA is the P1-P1' ala-ala mutant of SERP-1; Spi-2/crmA, interleukin 1- $\beta$  converting enzyme inhibitor from cowpox virus; PAI-1, plasminogen activator inhibitor 1; PAI-2, plasminogen activator inhibitor 2; Act, antichymotrypsin;  $\alpha$ 1-PI,  $\alpha$ 1 proteinase inhibitor. SERP-1 variants: MN, P1 methionine is that of  $\alpha$ 1-proteinase inhibitor; Apl, P7 to P3' of antiplasmin; PAI2, P7-P3' of PAI-2; (Ala)<sub>6</sub>, replacement of P7 to P2 of SERP-1 with alanine; DC, P1-P1' of Spi-2/crmA; LN, P1 leucine to resemble anti-chymotrypsin; RM, P1-P1' of antiplasmin and PAI-1.

P8-P7-P6-P5-P4-P3-P2-P1-P1'-P2'-P3'-P4'

**Existing Serpins:**

|               |   |   |   |   |   |   |   |          |          |   |   |   |
|---------------|---|---|---|---|---|---|---|----------|----------|---|---|---|
| SERP-1        | T | A | I | T | L | I | P | <b>R</b> | <b>N</b> | A | L | T |
| SAA           | T | A | I | T | L | I | P | <b>A</b> | <b>A</b> | A | L | T |
| Spi-2/crma    | A | T | C | A | L | V | A | <b>D</b> | <b>C</b> | A | S | T |
| PAI-1         | T | A | V | I | V | S | A | <b>R</b> | <b>M</b> | A | P | E |
| PAI-2         | T | G | V | I | M | T | G | <b>R</b> | <b>T</b> | G | H | G |
| Antiplasmin   | A | T | S | I | A | M | S | <b>R</b> | <b>M</b> | S | L | S |
| C1 inhibitor  | A | S | A | I | S | V | A | <b>R</b> | <b>T</b> | L | L | V |
| Act           | T | A | V | I | K | I | L | <b>S</b> | <b>A</b> | A | L | V |
| $\alpha$ 1-PI | M | F | L | E | A | I | P | <b>M</b> | <b>S</b> | I | P | P |

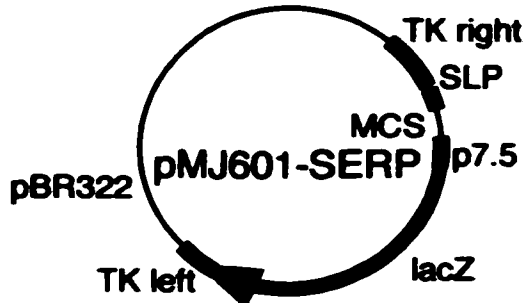
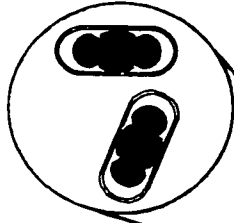
**SERP-1 Variants:**

|                    |   |   |   |   |   |   |   |          |          |   |   |   |
|--------------------|---|---|---|---|---|---|---|----------|----------|---|---|---|
| MN                 | T | A | I | T | L | I | P | <b>M</b> | <b>N</b> | A | L | T |
| AP1                | T | T | S | I | A | M | S | <b>R</b> | <b>M</b> | S | L | T |
| PAI2               | T | G | V | A | M | T | G | <b>R</b> | <b>T</b> | G | L | T |
| (Ala) <sub>6</sub> | T | A | A | A | A | A | A | <b>R</b> | <b>N</b> | A | L | T |
| DC                 | T | A | I | T | L | I | P | <b>D</b> | <b>C</b> | A | L | T |
| LN                 | T | A | I | T | L | I | P | <b>L</b> | <b>N</b> | A | L | T |
| RM                 | T | A | I | T | L | I | P | <b>R</b> | <b>M</b> | A | L | T |



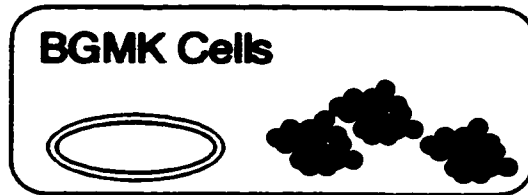
**Figure III-3: Construction of recombinant vaccinia virus.** SERP-1/RCL chimeras were subcloned into pMJ-601 under the control of a synthetic late promoter.  $\beta$ -galactosidase under the control of an early/late p7.5 promoter and the SERP-1 gene are flanked by the left and right arms of the vaccinia virus thymidine kinase gene to allow targeted insertion into the thymidine kinase locus. Following infection with vaccinia virus (strain WR), BGMK cells were transfected with pMJ-SERP-1/RCL mutants. After 24 hours the virus was harvested and used to infect H143 cells in the presence of BUdR. Following three blind rounds of selection of H143 cells in the presence of BUdR, blue plaques were isolated through a further three rounds of plaque purification to obtain pure recombinant virus. The presence of the SERP-1/RCL mutant insert was confirmed by PCR and subsequent sequencing.

Vaccinia Virus WR



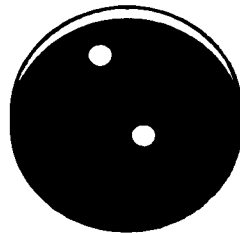
SERP-1

Infect & Transfect



Harvest

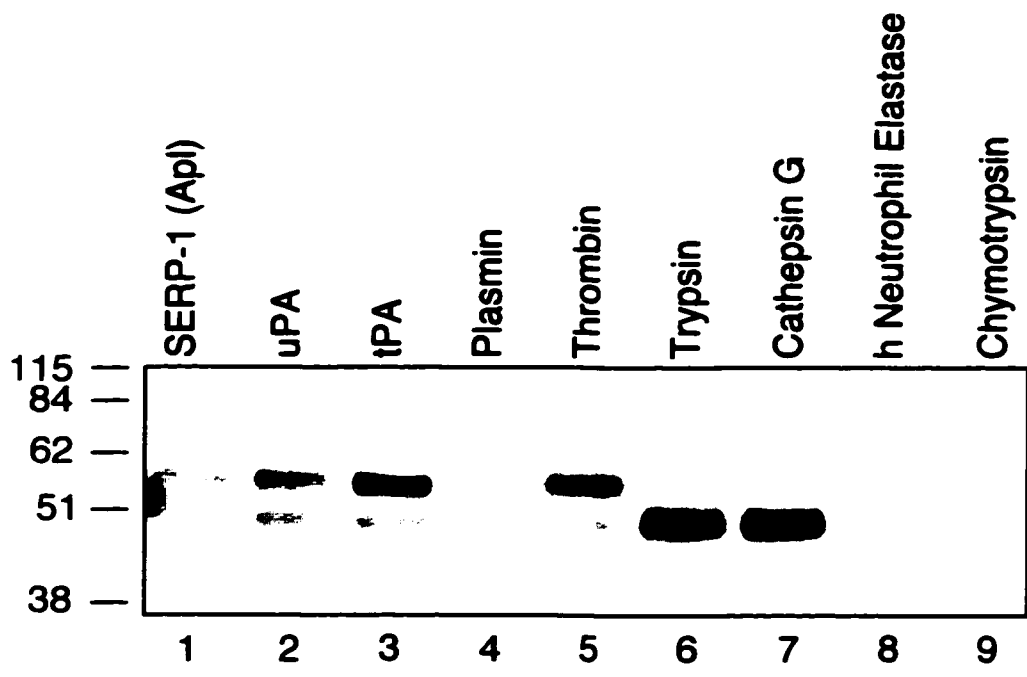
Select on H143 using BUdR (3 rds)



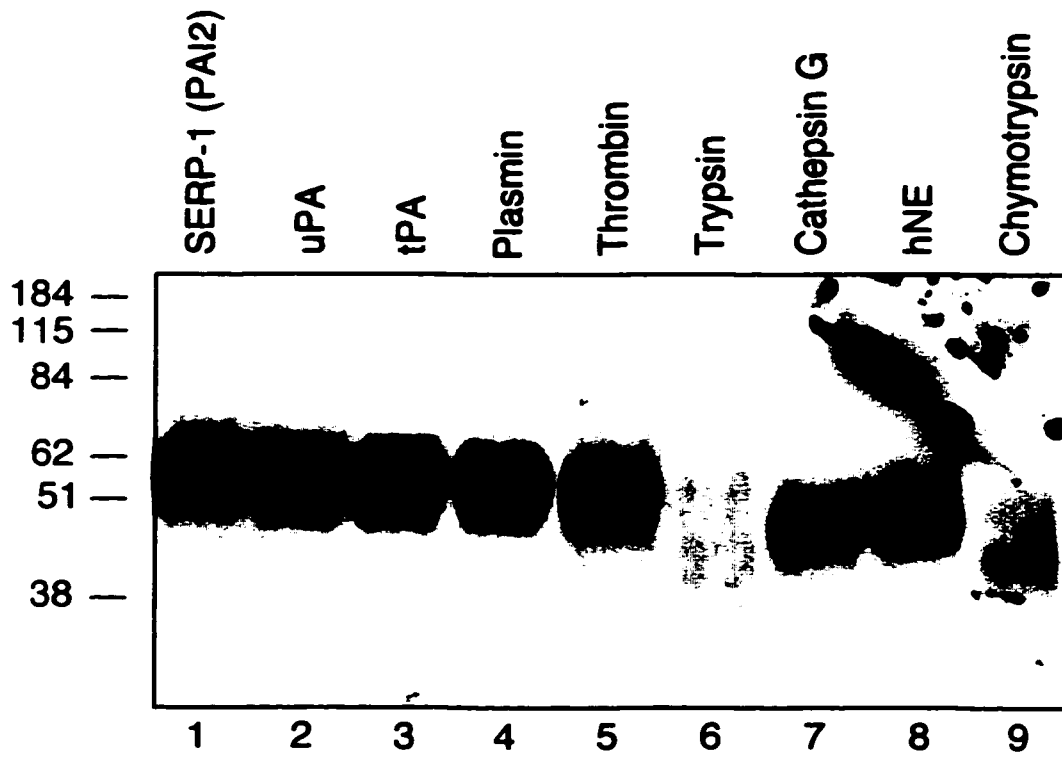
Pick Blue Plaques (3 rds)

Test by PCR  
All Blues

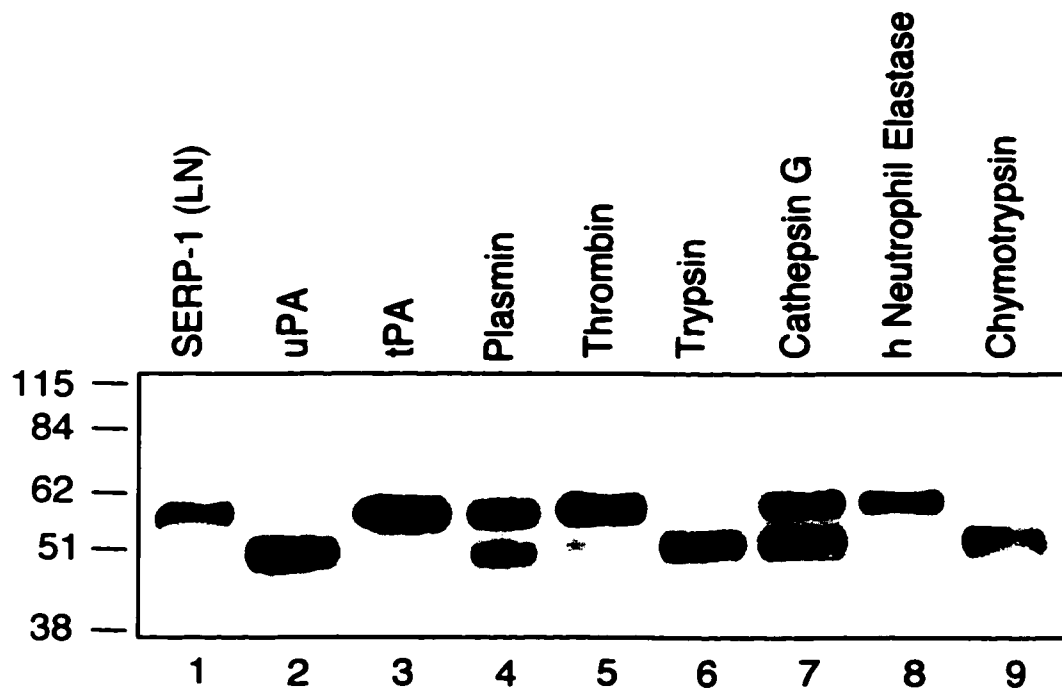
**Figure III-4: Inhibitory profile of SERP-1(Apl).** SERP-1(Apl) was incubated with uPA, tPA, plasmin, thrombin, trypsin, hNE, cathepsin G and chymotrypsin for 1 hour at 37°C. The reaction mixture was diluted into SDS-gel loading buffer, boiled and separated on 12% polyacrylamide gels by electrophoresis. Following SDS-PAGE, the gel was electroblotted onto Hybond C nitrocellulose, blocked overnight in 5% skim milk/TBS-Tween and SERP-1 bands detected by western blotting using mAb AQ.H9. SERP-1(Apl) appears at 55 kDa while SERP-1(Apl) cleaved at or near the RCL appears as a band of approximately 50 kDa.



**Figure III-5: Inhibitory profile of SERP-1(PAI).** SERP-1(PAI) was incubated with uPA, tPA, plasmin, thrombin, trypsin, hNE, cathepsin G and chymotrypsin for 1 hour at 37°C. The reaction mixture was diluted into SDS-gel loading buffer, boiled and separated on 12% polyacrylamide gels by electrophoresis. Following SDS-PAGE, the gel was electroblotted onto Hybond C nitrocellulose, blocked overnight in 5% skim milk/TBS-Tween and SERP-1 bands detected by western blotting using mAb AQ.H9. SERP-1(PAI) appears at 55 kDa while SERP-1(PAI) cleaved at or near the RCL appears as a band of approximately 50 kDa.

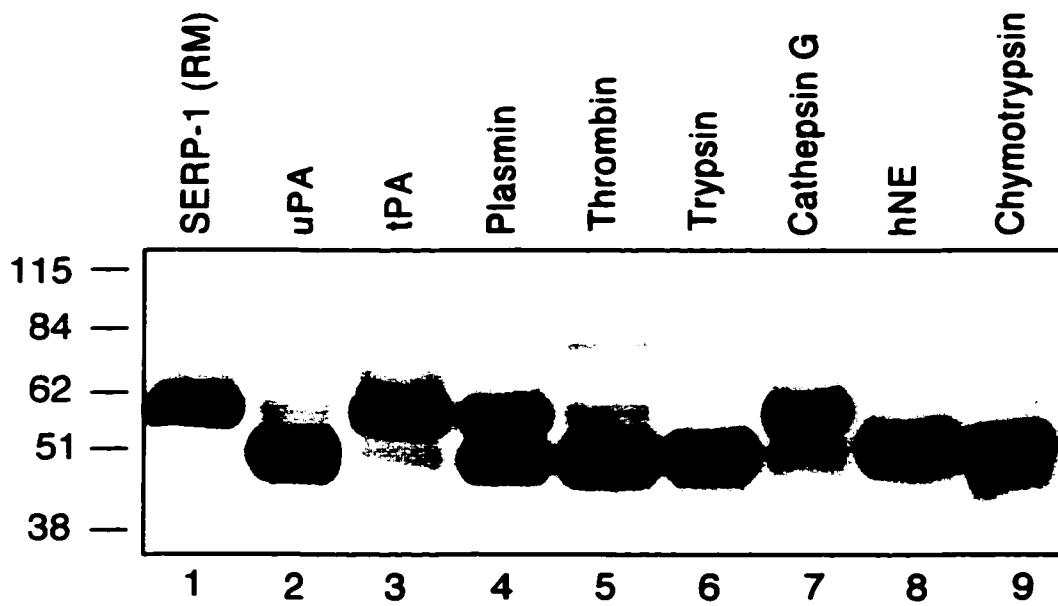


**Figure III-6: Inhibitory profile of SERP-1(LN).** SERP-1(LN) was incubated with uPA, tPA, plasmin, thrombin, trypsin, hNE, cathepsin G and chymotrypsin for 1 hour at 37°C. The reaction mixture was diluted into SDS-gel loading buffer, boiled and separated on 12% polyacrylamide gels by electrophoresis. Following SDS-PAGE, the gel was electroblotted onto Hybond C nitrocellulose, blocked overnight in 5% skim milk/TBS-Tween and SERP-1 bands detected by western blotting using mAb AQ.H9. SERP-1(LN) appears at 55 kDa while SERP-1(LN) cleaved at or near the RCL appears as a band of approximately 50 kDa.

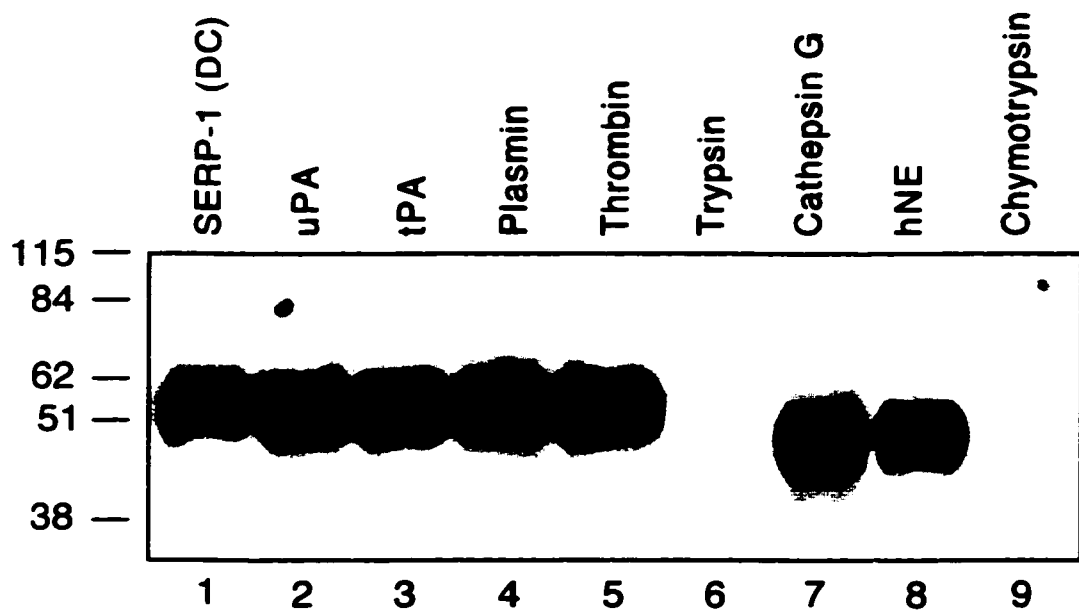




**Figure III-7: Inhibitory profile of SERP-1(RM).** SERP-1(RM) was incubated with uPA, tPA, plasmin, thrombin, trypsin, hNE, cathepsin G and chymotrypsin for 1 hour at 37°C. The reaction mixture was diluted into SDS-gel loading buffer, boiled and separated on 12% polyacrylamide gels by electrophoresis. Following SDS-PAGE, the gel was electroblotted onto Hybond C nitrocellulose, blocked overnight in 5% skim milk/TBS-Tween and SERP-1 bands detected by western blotting using mAb AQ.H9. SERP-1(RM) appears at 55 kDa while SERP-1(RM) cleaved at or near the RCL appears as a band of approximately 50 kDa.



**Figure III-8: Inhibitory profile of SERP-1(DC).** SERP-1(DC) was incubated with uPA, tPA, plasmin, thrombin, trypsin, hNE, cathepsin G and chymotrypsin for 1 hour at 37°C. The reaction mixture was diluted into SDS-gel loading buffer, boiled and separated on 12% polyacrylamide gels by electrophoresis. Following SDS-PAGE, the gel was electroblotted onto Hybond C nitrocellulose, blocked overnight in 5% skim milk/TBS-Tween and SERP-1 bands detected by western blotting using mAb AQ.H9. SERP-1(DC) appears at 55 kDa while SERP-1(DC) cleaved at or near the RCL appears as a band of approximately 50 kDa.



**REFERENCES**

- Baker, D. (1998). Metastable states and folding free energy barriers. *Nat. Struct. Biol.* **5**: 1021-1024.
- Bode, W., and Huber, R. (1992). Natural protein proteinase-inhibitors and their interaction with proteinases. *Eur. J. Biochem.* **204**: 433-451.
- Bottomley, S. P., and Stone, S. R. (1998). Protein engineering of chimeric serpins: an investigation into effects of the serpin scaffold and reactive centre loop length. *Prot. Eng.* **11**: 1243-1247.
- Carrell, R. W., Whisstock, J., and Lomas, D. A. (1994). Conformational changes in serpins and the mechanism of alpha 1-antitrypsin deficiency. *American Journal of Respiratory & Critical Care Medicine* **150**: (published erratum appears in Am J Respir Crit Care Med 1995 Mar;151(3 Pt 1):926).
- Cooperman, B. S., Stavridi, E., Nickbarg, E., Rescorla, E., Schechter, N. M., and Rubin, H. (1993). Antichymotrypsin interaction with chymotrypsin. Partitioning of the complex. *J. Biol. Chem.* **268**: 23616-25.
- Derechin, V. M., Blinder, M. A., and Tollefsen, D. M. (1990). Substitution of arginine for Leu444 in the reactive site of heparin cofactor II enhances the rate of thrombin inhibition. *J. Biol. Chem.* **265**: 5623-5628.

- Dinner, A. R., and Karplus, M. (1998). A metastable state in folding simulations of a protein model. *Nat. Struct. Biol.* **5**: 236-241.
- Ekert, P. G., Silke, J., and Vaux, D. L. (1999). *EMBO J.* **18**: 330-338.
- Engh, R. A., Huber, R., Bode, W., and Schulze, A. J. (1995). Divining the serpin inhibition mechanism: suicide substrate 'spring'? *Tr. Biol. Technol.* **13**.
- Gettins, P., Patston, P. A., and Schapira, M. (1993). The role of conformational change in serpin structure and function. *Bioessays* **15**: 461-7.
- Gettins, P. G., Patston, P., and Olson, S. (1996). Serpins: structure, function and biology (Austin: R. G. Landes Co.).
- Heeb, M. J., Bischoff, R., Courtney, M., and Griffin, J. H. (1990). Inhibition of activated protein C by recombinant  $\alpha$ 1-antitrypsin variants with substitution of arginine or leucine for methionine. *J. Biol. Chem.* **265**: 2365-2369.
- Hopkins, P. C. R., Crowther, D. C., Carrell, R. W., and Stone, S. R. (1995). Development of a novel recombinant serpin with potential antithrombotic properties. *J. Biol. Chem.* **270**: 11866-11871.
- Huntington, J. A., Fan, B., Karlsson, K. E., Deinum, J., Lawrence, D. A., and Gettins, P. G. (1997). Serpin conformational change in ovalbumin, enhanced reactive center loop insertion through hinge region mutations. *Biochemistry* **36**: 5432-5440.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**: 680.

Laskowski, M., and Kato, I. (1980). Protein inhibitors of proteinases. *Annu. Rev. Biochem.* **49**: 593-626.

Lawrence, D. A., Ginsburg, D., Day, D. E., Berkenpas, M. B., Verhamme, I. M., Kvassman, J.-O., and Shore, J. D. (1995). Serpin-protease complexes are trapped as stable acyl-enzyme intermediates. *J. Biol. Chem.* **270**: 25309-25312.

Macen, J. L., Upton, C., Nation, N., and McFadden, G. (1993). SERP1, a serine proteinase inhibitor encoded by myxoma virus, is a secreted glycoprotein that interferes with inflammation. *Virology* **195**: 348-63.

Murray, A. J., Head, J. G., Barker, J. J., and Brady, R. L. (1998). Engineering an intertwined form of CD2 for stability and assembly. *Nat. Struct. Biol.* **5**: 778-782.

Nash, P., Whitty, A., Handwerker, J., Macen, J., and McFadden, G. (1998). Inhibitory specificity of the anti-inflammatory myxoma virus serpin, Serp-1. *J. Biol. Chem.* **273**: 20982-20991.

O' Malley, K., Nair, S., Rubin, H., and Cooperman, B. (1997). The kinetic mechanism of serpin-proteinase complex formation. *J. Biol. Chem.* **272**: 5354-5359.

Patston, P. A., and Gettins, P. G. W. (1994). A database of recombinant wild-type and mutant serpins. *Thromb. Haemost.* **72**: 166-179.

Patston, P. A., and Gettins, P. G. W. (1996). Significance of secondary structure predictions on the reactive center loop region of serpins: a model for the folding of serpins into a metastable state. *FEBS Lett.* **383**: 87-92.

Plotnick, M. I., Mayne, L., Schechter, N. M., and Rubin, H. (1996). Distortion of the active site of chymotrypsin complexed with a serpin. *Biochemistry* **35**: 7586-90.

Plotnick, M. I., Schechter, N. M., Wang, Z. M., Liu, X., and Rubin, H. (1997). Role of P6-P3' region of the serpin reactive loop in the formation and breakdown of the inhibitory complex. *Biochemistry* **36**: 14601-14608.

Rubin, H. (1996). Serine protease inhibitors (SERPINS): where mechanism meets medicine. *Nature Medicine* **2**: 632-633.

Rubin, H., Plotnick, M., Wang, Z. M., Liu, X., Zhong, Q., Schechter, N. M., and Cooperman, B. S. (1994). Conversion of alpha 1-antichymotrypsin into a human neutrophil elastase inhibitor: demonstration of variants with different association rate constants, stoichiometries of inhibition, and complex stabilities. *Biochemistry* **33**: 7627-33.



Sherman, P. M., Lawrence, D. A., Yang, A. Y., Vandenberg, E. T., Paielli, D., Olson, S. T., Shore, J. D., and Ginsburg, D. (1992). Saturation mutagenesis of the plasminogen activator inhibitor-1 reactive center. *J. Biol. Chem.* **267**: 7588-95.

Stone, S. R., Whisstock, J. C., Bottomley, S. P., and Hopkins, P. C. (1997). SERPINS: a mechanistic class of their own. *Adv Exp Med Biol* **425**: 5-15.

Travis, J., and Salvesen, G. S. (1983). Human plasma proteinase inhibitors. *Annual Reviews in Biochemistry* **52**: 655-709.

Vajda, S., Sippl, M., and Novotny, J. (1997). Empirical potentials and functions for protein folding and binding. *Curr. Opin. Struct. Biol.* **7**: 222-228.

Wilczynska, M., Fa, M., Karolin, J., Ohlsson, P.-I., Johansson, L., and Ny, T. (1997). Structural insights into serpin-protease complexes reveal the inhibitory mechanism of serpins. *Nat. Struct. Biol.* **4**: 354-356.

Wilczynska, M., Fa, M., Ohlsson, P.-I., and Ny, T. (1995). The inhibition mechanism of serpins. *J. Biol. Chem.* **270**: 29652-29655.

**CHAPTER IV****POST-TRANSLATIONAL MODIFICATION OF THE MYXOMA VIRUS  
ANTI-INFLAMMATORY SERPIN, SERP-1**

If the fool would persist in his folly he would become wise.

*- William Blake -*

## INTRODUCTION

Glycosylation is a key determinant in immunological recognition and signaling events involved in the immune response to an invading parasite (Kukuruzinska and Lennon, 1998). Sialylated structures in particular are critical as the ligands of the immunological signaling molecules of the CD62 class of E-, P-, and L-selectins, and CD22 (Hennet et al., 1998; Nakamura et al., 1998; Traving and Schauer, 1998). It is not surprising, therefore, that a variety of pathogenic organisms employ molecular mimicry of glycosylated moieties to assist in the evasion of the immune system of the infected host. For example, the sialic acid-containing capsules of *N. meningitidis*, *E. coli* K1, and group B streptococi are important virulence factors (Bitter-Suermann, 1993; Timmis et al., 1985). While the marked effects of bacteria and parasite encoded sialyltransferase genes in conferring increased virulence has been well documented (Bozue et al., 1999; Mandrell et al., 1988; Schauer et al., 1995), it has only been recently that a bona fide sialyltransferase has been identified from a virus (Jackson et al., 1999).

Myxoma virus, a leporipoxvirus member of the poxvirus family of double stranded DNA viruses, has recently been reported to encode and express a functional sialyltransferase with homology to cellular  $\alpha$ 2,3-sialyltransferases (Jackson et al., 1999). Myxoma virus evolved in New World rabbits in which it causes a relatively benign and non-lethal disease characterized by persistent dermal lesions (Fenner, 1990). However, when myxoma virus infects Old World, or

European, rabbits (*Oryctolagus cuniculus*) a highly lethal disease state known as myxomatosis is produced (Fenner and Meyers, 1978; Fenner and Ratcliffe, 1965). The disease of myxomatosis is characterized by fulminating primary and secondary lesions, and extensive immunosuppression resulting in death of infected rabbits within 2 weeks caused by supervening Gram negative infections (McFadden et al., 1995). One of the most intriguing aspects of myxoma virus is its striking ability to systematically disable the immune and inflammatory responses of the infected host. The study of this virus has identified a wide range of immunomodulatory proteins capable of subverting specific aspects of the hosts immune system (Nash et al., 1999), one of which is SERP-1.

SERP-1 is expressed as a secreted glycoprotein possessing three predicted sites for N-linked glycosylation (Figure IV-1) that belongs to the serpin family of serine proteinase inhibitors (Nash et al., 1997; Upton et al., 1990). Serpins are involved in maintaining homeostasis in a variety of systems including the modulation of inflammatory responses, complement activation, fibrinolysis, thrombosis, and development (Rubin, 1996). Given the critical role of proteinases in diverse activities, it is not surprising that some viruses have utilized serpins in order to disrupt proteinase-regulated processes that would otherwise be antagonistic to the virus (Turner et al., 1995). Serpins have been described from a number of members of the poxvirus family, but SERP-1 remains the only example of a virus-encoded serpin that is secreted as a processed soluble glycoprotein from infected

cells (Turner et al., 1995). SERP-1 serves to dampen the *in vivo* inflammatory response to virus infection, and previous studies have shown that a targeted disruption of the SERP-1 gene results in greater inflammation of the primary lesion, more rapid resolution of the infection, and attenuation of virus virulence upon infection of immunocompetent hosts (Macen et al., 1993; Upton et al., 1990). SERP-1 has also been shown to inhibit a number of serine proteinases *in vitro*, including urokinase, tissue-type plasminogen activator, plasmin and thrombin (Lomas et al., 1993; Nash et al., 1998). Purified SERP-1 protein has also proven effective as an anti-inflammatory agent in models of coronary restenosis following balloon angioplasty (Lucas et al., 1996), and in antigen-induced arthritis (Maksymowych et al., 1996).

The role of post-translational modification of virus encoded immunomodulatory proteins in the complex processes of infection, immune evasion and antigenicity remains poorly understood. Previous studies have shown that SERP-1 is N-glycosylated, and that secretion is blocked by tunicamycin, a drug that selectively blocks the addition of N-linked oligosaccharides (Macen et al., 1993). This suggests that SERP-1 secretion is dependent upon correct and complete glycosylation. Furthermore, the discovery of a functional  $\alpha$ 2,3-sialyltransferase encoded by myxoma virus that is capable of modifying N-linked oligosaccharide chains by the addition of negatively charged sialic acid moieties (Jackson et al., 1999), suggests that secreted viral proteins such as SERP-1 may be

targets for terminal sialylation of N-linked glycans. Knock-out analysis of the myxoma virus sialyltransferase gene revealed that it is required for enhanced viral virulence, though the actual role of sialylation by the MST3N gene product during infection has not yet been characterized (Jackson et al., 1999).

We have investigated the nature of the post-translational glycosylation of the myxoma virus virulence factor, SERP-1. We show that SERP-1 processing and trafficking is dependent upon glycosylation, specifically at Asn<sub>172</sub>, and to a lesser degree at Asn<sub>99</sub>. Furthermore, we demonstrate for the first time that SERP-1 is the first example of a secreted virulence factor that is post-translationally modified by a virus encoded glycosyltransferase, though this does not affect the ability of SERP-1 to inhibit proteinases *in vitro*.

## **EXPERIMENTAL PROCEDURES**

**Materials:** The human enzyme tissue-type plasminogen activator (tPA, two chain form), was obtained from Sigma. Proteinase substrate Chromozym-TPA was purchased from Boehringer-Mannheim. Anti-SERP-1 monoclonal antibodies AQ.H9 and AG.F11 were kindly provided by Leona Ling (Biogen, Inc.). Neuraminidase (sialidase) was obtained from Boehringer Mannheim and used as per the manufacturers instructions.

**Viruses and Cell Lines:** The complete SERP-1 gene, including the signal sequence, was inserted into the thymidine kinase locus of vaccinia virus strain Western Reserve (WR) under the control of a synthetic late promoter utilizing the pMJ-601 plasmid by homologous recombination as described (Macen et al., 1993), in order to produce the recombinant virus termed VV-S1. BGMK cells were obtained from Dr. S. Dales, and infected with VV-S1 or Myxoma virus strain. Supernatants and cells were harvested at 20h post-infection. SERP-1 accumulates as a stable glycoprotein in the culture supernatant at late times (>8h) post-infection. SERP-1 was purified from VV-S1 supernatants as described (Nash et al., 1998). SERP-1 from CHO cells expressing SERP-1 was a gift from Leonna Ling (Biogen, Inc.). Myxoma viruses Lu169, L14Lz, MSTN<sup>-</sup>, and MSTNRev were described previously (Jackson et al., 1999).

**Purification of SERP-1 from virus infections:** BGMK cells ( $2 \times 10^8$  cells) were adsorbed with VV-S1, myxoma virus Lu169 (Lausanne) or MSTN<sup>-</sup> at a multiplicity of infection (moi) of 1.0 for 2 hours at 37°C in 10 ml of DMEM containing 10% newborn calf serum. The inoculum was removed, and the cells were washed three times with 50 ml of sterile phosphate buffered saline (PBS) to remove medium and serum proteins. Serum-free DMEM (15 ml) was added to each bottle and the infection was allowed to proceed for 20 hours at 37°C. The culture supernatant was collected, spun at 5000 x g to pellet cells and cell debris, and stored at -20°C. The medium containing secreted viral proteins was concentrated approximately 50-fold

using an Amicon pressure cell equipped with a 10 kDa cut-off membrane, and was then dialyzed against 25 mM Tris, pH 8.0 at 4°C using Spectrapore dialysis tubing with a 30 kDa molecular weight (MW) cut-off. The dialyzed samples were spun at 14000 x g to remove precipitates, loaded onto a MonoQ anion exchange column (Pharmacia), and eluted with a linear salt gradient (0 - 300 mM NaCl in 25 mM Tris, pH 8.0). Fractions were separated by SDS-PAGE, and those containing SERP-1 were identified by immunoblotting using anti-SERP-1 mAb AQ.H9 (Biogen, Inc), pooled, and concentrated to 1 ml using Centriprep 10 concentrators (Amicon), and further purified on a Superdex 75 gel filtration column (Pharmacia) (150 mM NaCl, 25 mM Tris, pH 8.0). Fractions were collected and monitored for SERP-1 by SDS-PAGE, and visualized by either silver staining or immunoblotting using anti-SERP-1 mAb AQ.H9 (Biogen, Inc.).

**<sup>35</sup>S-Met/Cys Pulse-Chase:** BGMK cells were grown in 6 well tissue culture plates to 80% confluency (approximately 10<sup>6</sup> cells/well). Cells were infected by myxoma virus (Lausanne) or VV-S1 at an moi of 10 in an adsorption volume of 0.5 ml for 1 hour at 37°C. Following virus adsorption, the cells were washed with PBS and allowed to recover in 1.0 ml of DMEM supplemented with 10% NBCS. At 8 hours post-infection the cells were washed 3 times with sterile PBS and starved in 1.0 ml of serum-free Met-/Cys- media for 30 minutes at 37°C. The cells were then pulsed with 500 µCi of <sup>35</sup>S-Met/Cys and incubated for 15 minutes to allow protein labelling. The cells were then washed 3 times with sterile PBS, and 1.0 ml serum-



free media was added. Cells were harvested at appropriate times post-pulse by incubating with SSC (150 mM NaCl, 15 mM NaCitrate, pH 7.0) and pipetting repeatedly, and transferred to microfuge tubes. Cells were pelleted by centrifuging at top speed in a microcentrifuge for 3 minutes and then resuspended in 0.5ml of lysis buffer (1% NP-40, 150 mM NaCl, and 50 mM Tris-HCl, pH 8.0). Cell lysis was allowed to proceed for 60 minutes at 4°C with constant agitation. Cell nuclei were removed by centrifugation at top speed in a microcentrifuge for 15 minutes. The supernatant containing the soluble portion of the cell lysates was transferred to a clean tube and the pellet containing the nuclei and cell debris was discarded.

**Immunoprecipitation of SERP-1:** Protein A-Sepharose beads (Sigma) were pre-swollen in lysis buffer and washed 3 times in lysis buffer prior to preparing a slurry of 50% (v/v) protein A beads in lysis buffer. Anti-SERP-1 monoclonal antibody AQH9 was added to the soluble portion of the cell lysate or supernatant (see above) to a final concentration of 3µg/ml and incubated at 4°C for 60 minutes with constant agitation. Protein A slurry (30 µL) was then added to each sample and incubated for a 30 minutes at 4°C with constant agitation. The beads were then recovered by briefly centrifuging in a microcentrifuge. The supernatant was discarded and beads washed 3 times with 100 µL of Lysis buffer.

**Endoglycosidase-H treatment:** Following immunoprecipitation of SERP-1, the protein A beads with SERP-1 bound were subjected to Endoglycosidase H treatment. Half of the beads were re-suspended in 50 µL of denaturing buffer

(NEB) and boiled for 10 minutes. 5  $\mu$ L of 10X G5 buffer (NEB) was added, followed by 5  $\mu$ L of Endo H<sub>r</sub> (NEB), and the samples incubated at 37°C for 60 minutes. Samples were then boiled in denaturing/reducing SDS-PAGE gel loading buffer and separated by SDS-PAGE. SERP-1 was visualized by drying the gels onto filter paper and exposing X-ray film (Kodak) at -80°C.

**Preparation of N-Glycosylation Site Mutants:** Mutagenesis of SERP-1 was achieved with the aid of the Altered Sites II mutagenesis system (Promega). Briefly, a BamHI/HindIII fragment containing the complete SERP-1 open reading frame (ORF) was cloned into the pAlter-1 vector (Promega). Purified pAlter-SERP-1 was denatured and annealed to mutagenic oligonucleotides. The three oligonucleotides used for SERP-1 glycosylation-site mutagenesis were:

Nmut1 (CCGATACGTCTACGCCGAAAGCGACAACG) allowing an AAC to GCC change that results in asparagine to alanine mutation at position 28; Nmut2 (GAGTTCTCCTCGCGTTTCGCT-ACCTCCGTGCAAC) allowing an AAT to GCT change that destroyed an NruI site and caused an asparagine to alanine change at residue 99; Nmut3 (CCTTTTTATTCCGGAGCGGCTACATAACAAGG) allowing an AAC to GCG change that introduced a BsrB1 site and mutated asparagine to alanine at amino acid residue 172. These oligonucleotides were used individually or in combination to produce SERP-1 mutants designated N28A, N99A, N172A and tmNA (triple mutant). SERP-1 glycosylation mutants were isolated by restriction endonuclease digestion and confirmed by nucleotide

sequencing. Mutants were then sub-cloned into the pMJ-601 vector to allow expression in vaccinia virus (see above).

**Western Blot Analysis of SERP-1:** Samples were separated on Tris-glycine SDS 10% polyacrylamide gels using the Laemmli buffer system (Laemmli, 1970), and transferred to Hybond C-extra (Amersham) nitrocellulose by semi-dry electroblotting. Blots were blocked in TBS (150 mM NaCl, 2.5 mM KCl, 25 mM Tris-HCl, pH 7.4) containing 5% (w/v) skimmed milk powder and 0.1% (v/v) Tween-20 for >2 hours, and SERP-1 was detected by incubating for 1 hour with 0.005% (v/v) monoclonal anti-SERP-1 anti-serum AQH9 (Biogen, Inc) in blocking buffer. After washing with TBS containing 0.1% (v/v) Tween-20, a secondary goat anti-mouse antibody conjugated to horseradish peroxidase (BioRad) was applied for 1 hour. Immunoreactive SERP-1 species were visualized using ECL detection (Amersham) on Kodak X-ray detection type film.

**Isoelectric focusing gels:** Isoelectric focusing was accomplished on IEF Ready-Gels (BioRad). Sample loading buffer consisted of 50% glycerol and gels were run with a 7mM phosphoric acid anode buffer, and a cathode buffer consisting of 20mM lysine free base (Sigma), 20 mM arginine free base (Sigma). IEF gels were run for 3 hours at 200 volts in mini-protean II gel units (BioRad). The relative pI positions were determined with IEF marker proteins (BioRad). Following IEF separation, the gels were soaked in 1% SDS for 30 minutes,

followed by 10 minutes in transfer buffer, and transferred by semi-dry electrophoretic transfer as described above.

**Determination of SERP-1 concentration by ELISA:** Flat bottom 96 well ELISA plates (Nunc) were coated with 100  $\mu$ L of anti-SERP-1 mAb AG.F11 at 1  $\mu$ g/ml in 50 mM carbonate buffer, pH 9.6 overnight at 4°C. Prior to use, plates were washed 3 times with wash buffer (PBS, 0.1% (v/v) Tween-20) and incubated with 200  $\mu$ L blocking buffer (PBS, 2% (w/v) BSA, 0.05% Tween-20) for 2 hours. SERP-1 standards and samples were diluted in assay buffer (PBS, 1% BSA (w/v), 0.01% Tween-20) and 50  $\mu$ L added to triplicate wells. SERP-1 standards consisted of highly purified SERP-1 with the concentration was determined using  $A_{280}$  and a molar extinction coefficient of 32,700  $M^{-1}cm^{-1}$  (Nash et al., 1998). Samples and standards were incubated in the plate for 1 hour, washed 3 times with wash buffer and 50  $\mu$ L of 1mg/ml biotinylated AQ.H9 (Biogen, Inc.) in assay buffer was added and incubated for 45 min. The plate was washed again and 50  $\mu$ L of 1  $\mu$ g/ml streptavidin-HRP in assay buffer was added and incubated for 45 min. The plate was then washed 3 times and 100  $\mu$ L of chromogenic substrate (0.5 mg/ml OPD (Sigma) , 0.003 % hydrogen peroxide, 0.05 M citric acid, 0.05 M  $NaPO_4$  buffer, pH 5.0) was added. When color development was complete, the reaction was stopped with the addition of 100  $\mu$ L of 2M  $H_2SO_4$ , and the absorbance at 490 nm determined using a BioRad ELISA plate reader.

**Slow binding inhibition kinetics:** Inhibition progress curves were obtained by incubating a limiting concentration of tPA (125 pM) with various concentrations of SERP-1 (0.125 to 100 nM) in the presence of Chromozym-tPA chromogenic substrate. In all cases, serpin was present at  $\geq 10$ -fold excess over proteinase in order to achieve pseudo-first-order conditions with respect to SERP-1. Concentrations of enzyme and substrate were chosen so that  $\leq 10\%$  of the substrate was hydrolyzed over the entire duration of the assay. Reactions were performed at 37°C in a total volume of 800  $\mu$ l (100 mM Tris-HCl, 100 mM NaCl, 2 mM CaCl<sub>2</sub>, 0.005% Triton X-100, pH 7.5) in plastic cuvettes sealed with Parafilm. The time-dependent inhibition of each enzyme by SERP-1 was monitored by following the rate of production of *p*-nitroaniline (pNA) at 405 nm at 1 or 5 minute intervals for a period of 1000 minutes, using a CARY 1 spectrophotometer equipped with a six-cell cuvette holder. Each set of six reactions comprised five reactions containing various concentrations of SERP-1 and one control reaction containing enzyme and substrate with no SERP-1. In the [SERP-1] = 0 controls, progress curves were observed to be linear, indicating that the enzyme activity was stable over the course of the reaction. For each enzyme, two to five sets of measurements spanning at least 6 different concentrations of SERP-1 were used to calculate the kinetic constants. The reactions were allowed to proceed until the steady state velocity of pNA formation was attained, and the progress curves for each reaction were then fitted to the integrated rate equation for slow binding inhibition

(Equation 1) (Morrison and Walsh, 1988; Stone and Hermans, 1995), in which  $A_t$  is the absorbance at 405 nm at time  $t$ , due to the evolution of  $p$ -nitroaniline;  $k_{obs}$  is the apparent first-order rate constant for the inhibition of enzyme by SERP-1;  $v_i$  and  $v_s$  are the initial and steady-state velocities for reaction of the chromogenic substrate; and  $A_0$  is the initial absorbance at 405 nm.

$$A_t = v_s t + \left( \frac{v_i - v_s}{k_{obs}} \right) (1 - e^{-k_{obs} t}) + A_0 \quad \text{Equation 1}$$

Values for  $v_i$ ,  $v_s$ , and  $k_{obs}$  were obtained for the progress curves measured at each SERP-1 concentration using nonlinear regression analysis (DeltaGraph 4.0).

**$K_M$  determination for chromogenic proteinase substrate:** Estimates of  $K_M$  for the reaction of tPA with chromogenic substrate Chromozym-tPA were determined directly under the conditions used for the slow-binding inhibition experiments. A  $K_M$  value for the reactions of tPA with Chromozym-tPA was found to be 160  $\mu$ M.

## RESULTS

The intracellular trafficking of secreted proteins begins with the co-translational translocation of the nascent polypeptide chain into the lumen of the ER. This step requires a signal sequence, which is cleaved by signal peptidase in the lumen of the ER, and is therefore not present on the mature protein. We

confirmed the predicted cleavage of the signal sequence from the mature SERP-1 polypeptide by sequencing the N-terminus of the purified secreted protein. A sequence of RDIGLWTR was determined, which corresponds to encoded residues Arg<sub>14</sub>-Arg<sub>21</sub> of the SERP-1 sequence and is consistent with the predicted signal peptidase cleavage site at Cys<sub>13</sub>-Arg<sub>14</sub>.

**SERP-1 secretion occurs only after modification of glycosylation:** To examine the rate of SERP-1 processing, virus infected cells were pulse labelled with <sup>35</sup>S-methionine/cystine and the levels of intracellular and secreted <sup>35</sup>S-labeled SERP-1 monitored by immunoprecipitation and SDS-PAGE autoradiography. In order to monitor the movement of SERP-1 from the ER to the Golgi, immunoprecipitated SERP-1 samples were subjected to endoglycosidase-H treatment. This deglycosylates only the immature ER or cis-Golgi form of SERP-1, while leaving mature SERP-1 that has migrated as far as the trans-Golgi, intact. Figure IV-2 shows immunoprecipitated SERP-1 at various times following a 30 minute <sup>35</sup>S-Met/Cys pulse. Intracellular SERP-1 from cells infected with either myxoma virus or a vaccinia vector that over-expresses SERP-1 (VV-S1) appears as an immature glycosylated band that is fully sensitive to endoglycosidase H treatment (Figure IV-2 panels A and B), and likely represents the initial ER-resident glycosylated form of SERP-1. This Endo-H sensitive immature SERP-1 remains the predominant intracellular form of SERP-1 for the 4 hour (Myxoma virus Figure IV-2 panel B) and 6 hour (VV-S1 Figure IV-2 panel A) chase periods, at which point we detect

the first appearance of an Endo-H resistant species that migrates with an apparent molecular weight of 55 kDa on SDS-PAGE. This Endo-H resistant intracellular form is the mature SERP-1 just prior to secretion, and is consistent with the appearance of mature Endo-H resistant SERP-1 in the supernatant at around the same time for both VV-S1 (Figure IV-2 panel B) and myxoma virus (Figure IV-2, panel D). By eight hours post-pulse, virtually all SERP-1 labeled during the pulse has been secreted as a mature Endo-H resistant glycoprotein and no immature species remains in either the supernatant or cell lysates.

Treatment with Brefeldin A, a fungal metabolite that selectively blocks anterograde transport from the ER to Golgi apparatus blocked SERP-1 secretion (data not shown). This is consistent with SERP-1 following the default pathway for secretion rather than an alternate Golgi-independent pathway as has been reported for selected viral proteins (Schreiber et al., 1996) and at least one serpin PAI-2 (Ritchie and Booth, 1998). Brefeldin A treatment resulted in quantitative intracellular accumulation of SERP-1 as an immature glycosylated species, presumably located within the collapsed endoplasmic reticulum (data not shown)

**SERP-1 secretion requires glycosylation at Asn<sub>178</sub>:** In order to determine the sites at which SERP-1 is N-glycosylated, as well as to examine whether these sites are actually required for protein secretion, mutant SERP-1 constructs were engineered with each of the three putative N-glycosylation sites (Figure 1) disrupted by mutation to alanine (N28A, N99A and N172A). A fourth SERP-1 construct was



engineered lacking all three putative N-glycosylation sites, referred to as triple mutant asparagine to alanine (tmNA). The resulting SERP-1 mutants were produced in a recombinant vaccinia virus expression system as described in *Experimental Methods*. Figure IV-3A shows a Western blot of the secreted SERP-1 from each of the glycosylation-mutant constructs. The N28A mutation caused an increased mobility of SERP-1 on SDS-PAGE indicating a partial reduction in the degree of glycosylation, although the protein still migrates more slowly than the N-glycosidase F treated SERP-1 sample. In addition, the N28A mutation does not appear to impair secretion, as the secreted SERP-1 levels are equivalent to that of the wild-type. In contrast, the N99A mutant caused demonstrably reduced levels of SERP-1 secretion, as well as exhibiting decreased glycosylation. The N172A mutant failed to be secreted at all, as did the tmNA construct, indicating that glycosylation at Asn<sub>172</sub> is an absolute requirement for SERP-1 secretion from virus-infected cells. In order to confirm that the intracellular forms of these proteins were being produced, lysates were also examined by Western blotting (Fig. 3B). In addition,  $\beta$ -galactosidase expression was tested to confirm that all viruses were producing comparable amounts of protein (data not shown). N28A and N99A proteins appeared as intracellular glycosylated species, presumably retained primarily within the ER or cis-Golgi. N172A and tmNA were present in much smaller steady-state levels, and only in a non-glycosylated form. It appears that the

absence of Asn<sub>172</sub> results in a form of SERP-1 protein that is either unstable or rapidly targeted for degradation.

**SERP-1 is modified by the myxoma virus sialyltransferase:** Myxoma virus has been demonstrated to encode a functional sialyltransferase encoded by the MST3N gene, although the biological function of this glycosyltransferase enzyme is not yet known (Jackson et al., 1999). To investigate the effect of the MST3N gene product on the SERP-1, we compared SERP-1 secreted from cells infected with the parental Lausanne strain (Lul69), a control lacZ<sup>-</sup> variant of the parental virus (L14Lz), MST3N knock-out virus (MSTN<sup>-</sup>) and a MSTN<sup>-</sup> revertant virus (MSTN<sup>-</sup> Rev). On reducing SDS-PAGE, secreted SERP-1 harvested from the MST3N knock-out virus exhibits increased mobility (Figure IV-4A, lane 3), suggesting that SERP-1 is directly modified by the MST3N gene product in wild-type myxoma virus. To confirm that this alteration in mobility is due to glycosylation differences and not other changes in the protein, SERP-1 samples from the lacZ<sup>+</sup> parental virus (L14Lz) and the MST3N<sup>-</sup> virus were digested with N-glycosidase F to remove all N-linked glycosylation (Figure IV-4B). The deglycosylated SERP-1 from both viruses exhibits an identical apparent molecular mass of 42 kDa confirming that the mobility difference observed for MSTN<sup>-</sup>-derived SERP-1 was due to oligosaccharide variations.

**Sialylation alters the negative charge of secreted SERP-1:** To further investigate the effect of SERP-1 modification by the MST3N gene product, we compared the

isoelectric point (pI) distribution of SERP-1 species from parental, MSTN<sup>-</sup>, revertant viruses, and recombinant SERP-1 produced from vaccinia virus and CHO systems. The diffuse band on SDS-PAGE is evidence that SERP-1 glycosylation exhibits some heterogeneity, and the mature secreted SERP-1 appears as several distinct charged species when separated by isoelectric focusing (Figure IV-5A, lanes 1, 2, 4). Wild-type myxoma virus SERP-1 resolves into two major distinct bands at pI ~5.4 and ~4.5. By contrast, SERP-1 from the MSTN<sup>-</sup> virus appears as a predominant species at pI ~4.5 with a ladder from pI ~4.5 to 5.4 (Figure IV-5A, lane 3). This ladder effect has been observed for other sialylated glycoproteins, and is due to the progressive addition of neuraminic acid residues with each band varying by 0.05-0.5 pH units (*Beeley, 1985*). Recombinant SERP-1 purified from either vaccinia virus or CHO cells exhibits a pI of ~5.0 (Figure IV-5A, lanes 5, 6). This data indicates that the MST3N gene product modification of SERP-1 shifts the pI to ~5.4. The lower species (pI ~4.5) observed from all of the myxoma virus derived products, as well as the pI ~5.0 observed for vaccinia virus or CHO cell produced SERP-1, are likely the result of cellular sialyltransferases, as the pI values are significantly lower than predicted for the mature SERP-1 protein based on its amino acid sequence (pI = 6.29).

**Cellular sialyltransferases also act upon SERP-1:** From the data above, it was suspected that cellular sialyltransferases act on SERP-1 in addition to the MST3N modification. SERP-1 from myxoma parental, MSTN<sup>-</sup>, revertant viruses and

recombinant protein from vaccinia virus and CHO systems, was subjected to neuraminidase in order to cleave all sialic acid from the protein. The mobility shift shown by blot analysis (Figure IV-5B) indicates that SERP-1 from each source was susceptible to neuraminidase digestion. Thus, SERP-1 is modified by both the MST3N gene product and still-unidentified host sialyltransferases in myxoma virus infected cells and exclusively by the host enzyme(s) in VV-S1 infected cells.

**Sialylation/Glycosylation variations in SERP-1 do not affect *in vitro* proteinase inhibition:** SERP-1 has been shown to function *in vitro* as an inhibitor of a number of plasma serine proteinases (Lomas et al., 1993; Nash et al., 1998). To test the effect of sialylation and glycosylation variations upon SERP-1 proteinase inhibition function, we attempted to quantify the effectiveness of various isolates of SERP-1 at inhibiting tPA *in vitro* using the techniques of slow binding inhibition kinetics (Morgenstern et al., 1994; Nash et al., 1998; Stone and Hermans, 1995). The kinetics of tPA inhibition were measured using purified SERP-1 derived independently from vaccinia virus (VV-S1), CHO cells, myxoma virus (Lausanne), and the MSTN<sup>-</sup> strain of myxoma. The proteinase was incubated with various concentrations of SERP-1 in the presence of a chromogenic substrate (Chromozym-tPA). The time-dependent inactivation of the enzyme through reaction with SERP-1 was monitored continuously by following the accompanying decrease in the rate of substrate turnover. Experiments were carried out at a low concentration of enzyme (125 pM) so that the reaction could be followed for many

hours without significantly depleting the chromogenic substrate. SERP-1 was always present at concentrations that were in >10-fold excess with respect to the proteinase, thus giving rise to pseudo-first-order conditions with respect to SERP-1. The progress curves were fitted to Equation 1 (Stone and Hermans, 1995) to obtain values for the rate constant for the approach to the steady state,  $k_{obs}$ , and the velocity of substrate turnover at the steady state,  $v_s$ , as described in *Experimental Procedures*. Values for the apparent steady state rate constant,  $K_p$ , and the apparent second order rate constant,  $k_{ass}$ , were determined from this data (Nash et al., 1998). Figure IV-6 shows the dependence of  $k_{obs}$  on the concentration of SERP-1 for the reaction of tPA with purified SERP-1 from VV-S1, CHO cells, vMyxWT, and vMyxMSTN<sup>-</sup>. The slopes of the “best fits” to the data in Figure IV-5 give values for the apparent second order rate constants for the reaction of tPA with purified SERP-1 from VV-S1, CHO cells, vMyxWT, and vMyxMSTN<sup>-</sup> of  $k_{ass} = 4.3 \pm 0.4 \times 10^4$ ,  $4.5 \pm 0.6 \times 10^4$ ,  $4.2 \pm 0.5 \times 10^4$ ,  $4.5 \pm 0.6 \times 10^4 \text{ M}^{-1}\text{s}^{-1}$  respectively, after correction for competitive binding of the chromogenic substrate during the reaction (Stone and Hermans, 1995). The apparent second order rate constants, steady state inhibition constants and dissociation constants for the reaction of tPA with purified SERP-1 from VV-S1, CHO cells, vMyxWT, and vMyxMSTN<sup>-</sup> are presented in Table I. No significant variation is apparent in the kinetics of tPA inhibition for any of the SERP-1 variants tested, indicating that alterations in the glycosylation and sialylation state of SERP-1 do not alter *in vitro* proteinase inhibition.

## **DISCUSSION**

SERP-1 is the only known virally encoded secreted serine proteinase inhibitor, and thus acts in the extracellular milieu of infected tissue. In previous studies, SERP-1 has been shown to be an important virulence factor in the pathogenesis of myxomatosis (Macen et al., 1993; Upton et al., 1990). Furthermore, highly purified SERP-1 protein has been shown to act as an anti-inflammatory agent in distinct models of inflammation (Lucas et al., 1996; Maksymowych et al., 1996). The effects of SERP-1 are manifest even at extremely low doses, suggesting that it inhibits a rare serine proteinase essential for establishing an early inflammatory response in these model systems (Nash et al., 1998). SERP-1 is a moderately potent inhibitor of the plasma serine proteinases uPA, tPA, plasmin, thrombin, factor Xa and C1s, and the inhibition kinetics for these reactions have been described in great detail (Lomas et al., 1993; Nash et al., 1998). There seems to be a further level of complexity in the action of SERP-1 in virus infected tissues as the protein is the target of the myxoma virus encoded sialyltransferase, and glycosylation at Asn<sub>172</sub> is essential for secretion. The sialylation of SERP-1 by the myxoma MST3N sialyltransferase, which has recently been shown to be a bona fide virus encoded glycosyltransferase (Jackson et al., 1999), is the first reported example of a virally-encoded glycosyltransferase that acts on a secreted virus encoded virulence factor.

We have shown here that correct glycosylation of SERP-1 is essential for the mature protein to avoid being targeted for rapid degradation and efficient trafficking out of the ER. Mutation of the glycosylation site at Asn<sub>178</sub>, caused both failure of SERP-1 to appear as a secreted protein, and the rapid degradation of intracellular SERP-1 in the ER and secretory pathway. This is in keeping with the role of the endoplasmic reticulum as a site for conformation-based quality control (Bergeron et al., 1998; Kopito, 1997; Sakaguchi, 1997; Zhang et al., 1997). It is now well established that damaged or misfolded proteins are removed from the early secretory pathway and degraded by the proteasome (Kouchi et al., 1998; Kowalski et al., 1998; Parodi, 1999). Recent studies have implicated the chaperone/lectin calnexin in recognizing specific oligosaccharide motifs on misfolded proteins (Bergeron et al., 1998; Liu et al., 1999; Zhang et al., 1997). This suggests that the removal and degradation of proteins from the ER may be triggered by changes in glycosylation-state alone, at least in certain circumstances.

Glycosylation, and in particular the addition of sialic acid residues to the oligosaccharide termini, have significant roles in a variety of cellular and immune responses. These include immunological recognition or masking of antigens, initiation of inflammatory responses (Kasky, 1995), and cell-specific adhesion events (Tedder et al., 1995). A wide variety of viral stealth strategies has been described in the past few years (Barry and McFadden, 1997; Spriggs, 1994; Zinkernagel, 1996), and poxviruses such as myxoma virus have served as

paradigms in this emerging field (Nash et al., 1999). Myxoma virus has already been documented to encode at least five distinct anti-apoptotic proteins (Barry and McFadden, 1998; Barry and McFadden, In Press; McFadden and Barry, 1998), to downregulate CD4 and class I MHC (Barry et al., 1995; Boshkov et al., 1992), and produce a wide range of soluble cytokine receptor homologs (McFadden et al., 1998).

The demonstrated attenuated virulence of the MST3N<sup>-</sup> knock-out virus (Jackson et al., 1999) could, in theory, be due to an increased antigenic profile for secreted and cell-associated virus encoded anti-immune molecules or, alternatively, it may be the result of decreased half-life of virulence factors within the infected tissues. Sialylation of SERP-1 by the MST3N gene product may also be required to localize the mature secreted protein to the surface of specific target cells. The low effective doses of SERP-1, both in animal models of inflammation and in virus-infected lesions, have lead to speculation that it acts at low concentrations to block a proteinase-mediated pro-inflammatory cascade set up by rare sentinel cells of the immune system (Nash et al., 1998). Since SERP-1 functions biochemically as a serine proteinase inhibitor, it acts in a concentration dependent manner. Localization to the surface of target cells would raise the effective local concentration of SERP-1, resulting in a more effective inhibition of pro-inflammatory proteinase cascades. It would also sequester the serpin away from a plasma environment of competing proteinases, thereby avoiding interaction with



inappropriate target enzymes that would either deplete, or irrevocably cleave SERP-1.

There are a variety of potential ligand-receptor interactions that would facilitate localization of a sialylated viral protein to the surface of target immune cells. The sialoadhesin proteins are members of the immunoglobulin superfamily that act as sialylation dependent adhesion molecules. Among the sialoadhesion family members are the macrophage/myeloid marker CD33, and sialoadhesin (Sn) which are expressed on activated macrophages at sites of infection (Crocker et al., 1995; Kelm et al., 1996; Powell and Varki, 1995). Both of these receptors recognize and bind  $\text{Sia}\alpha 2,3\text{Gal}\beta 1,3\text{GlcNAc}$  and  $\text{Sia}\alpha 2,3\text{Gal}\beta 1,4\text{GlcNAc}$  which are among the products of the MST3N enzymatic activity (M. Palcic, personal communication). In addition, the MST3N gene product could act in association with a cellular  $\text{GlcNAc}\alpha 1,3/4\text{-fucosyltransferase}$  to form sialyl-Lewis a and sialyl-Lewis x which act as ligands for the adhesion proteins E-, P- and L-selectins involved in inflammation and immune responses (Varki, 1997). Interestingly, the MST3N gene product does appear to be able to transfer sialic acid to the fucosylated acceptors  $\text{Le}^x$  and  $\text{Le}^a$  (M. Palcic, personal communication), a unique activity that distinguishes the MST3N gene product from all known mammalian sialyltransferases. Thus, sialylation of SERP-1 by the myxoma virus encoded  $\alpha 2,3\text{-sialyltransferase}$  has the potential to dramatically alter the actions of SERP-1

in complex biological tissues while not effecting the proteinase inhibition by SERP-1 *in vitro*.

Inactivation of the MST3N gene in myxoma virus results in a reduced virulence in immunocompetent hosts, but does not cause any defects in virus replication in tissue culture (Jackson et al., 1999). This is consistent with the MST3N gene product acting on secreted myxoma virulence factors and regulating antigenicity, biological half-life, or localization within complex tissue systems. Many bacteria and parasites express sialyltransferase activities in order to mask antigenic sites (Varki, 1993). The observation that the MST3N gene product is not the only sialyltransferase responsible for modifying SERP-1 is intriguing. The MST3N sialyltransferase creates a distinct sialylated species of SERP-1 with a consistent charge resulting in a protein with a  $pI = 5.4$ . This differs from the more acidic ( $pI = 4.5$ ) species observed in the absence of the MST3N gene product in either myxoma virus, vaccinia virus or CHO cells, that is produced through the compensating action of cellular sialyltransferases. This suggests that it is not simply the degree of charge imparted by sialylation that is important to myxoma virus, but rather that the particular linkage resulting from the MST3N sialyltransferase may be the critical factor. Further investigation of this area will be required before the effects of glycosylation and sialylation on SERP-1 *in vivo* function and myxoma virus virulence can be fully ascertained.

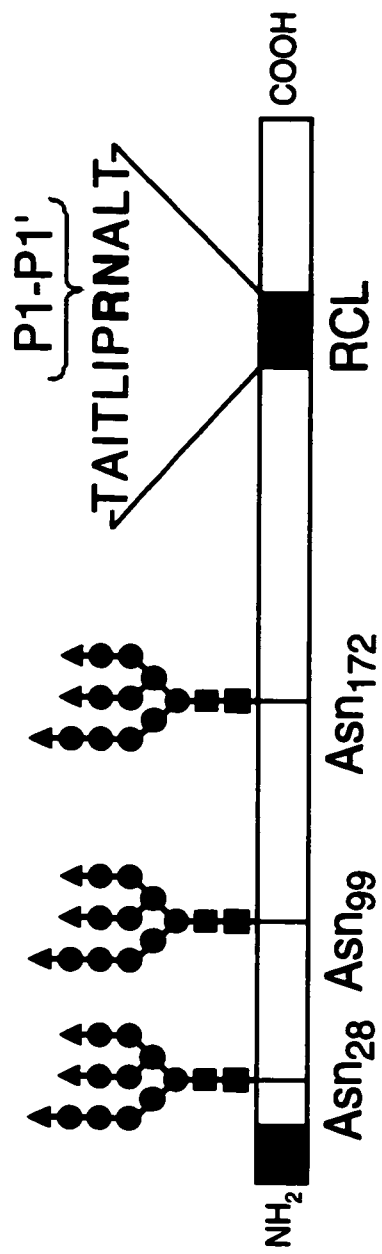
TABLE IV-1

**OBSERVED KINETIC PARAMETERS FOR THE REACTION OF SERP-1  
GLYCOSYLATION VARIANTS WITH TPA.**

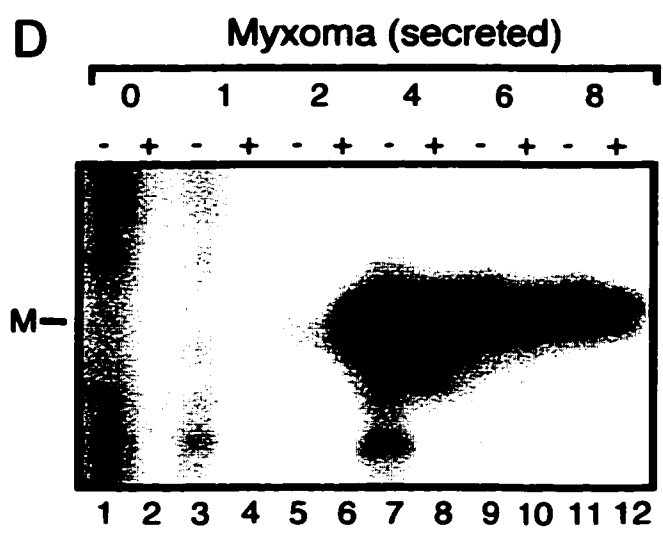
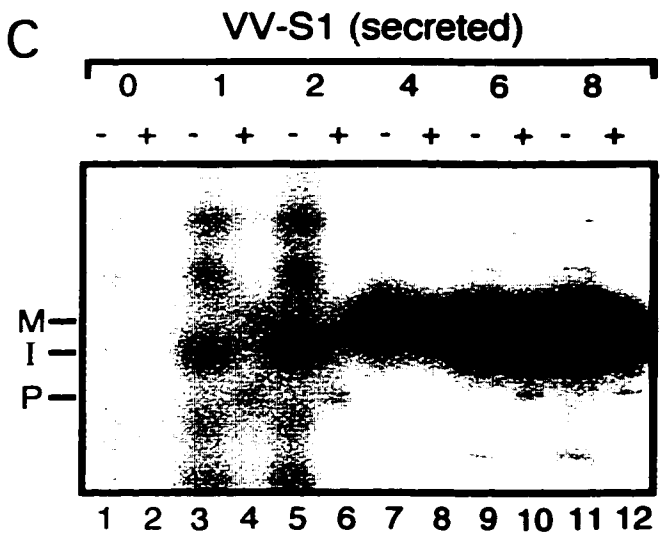
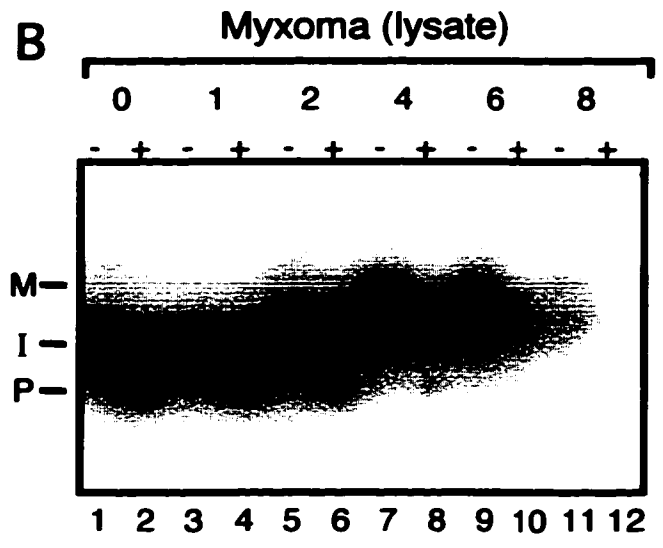
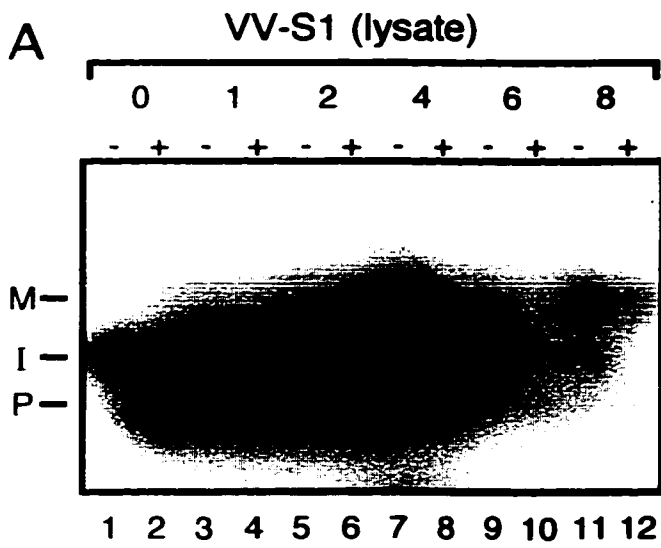
| <b>Source</b>                | <b><math>K_{I(app)}</math> (nM)</b> | <b><math>k_{app}</math> (<math>M^{-1}s^{-1}</math>)</b> |
|------------------------------|-------------------------------------|---------------------------------------------------------|
| VV-S1 (vaccina virus)        | $0.14 \pm 0.02$                     | $4.3 \pm 0.4 \times 10^4$                               |
| CHO cells                    | $0.13 \pm 0.03$                     | $4.5 \pm 0.6 \times 10^4$                               |
| Myxoma (wt)                  | $0.16 \pm 0.04$                     | $4.2 \pm 0.5 \times 10^4$                               |
| Myxoma (MST3N <sup>-</sup> ) | $0.15 \pm 0.04$                     | $4.5 \pm 0.6 \times 10^4$                               |

Values for the apparent inhibition constant,  $K_{I(app)}$  and the apparent second-order rate constant for inhibition,  $k_{app}$  were determined in slow binding inhibition experiments (100 mM Tris-HCl, 100 mM NaCl, 2 mM CaCl<sub>2</sub>, 0.005% Triton X-100, pH 7.5, 37 °C). Both  $K_{I(app)}$  and  $k_{app}$  are corrected for the competitive binding of chromogenic substrate in the slow binding inhibition assays as described in Nash et al. (1998).

**Figure IV-1: Glycosylation sites on SERP-1.** Schematic representation of SERP-1 indicating the three putative N-glycosylation sites at asparagine residues 28, 99, and 172. Hypothetical glycosylation structures are shown at these positions. The reactive center loop (RCL) of SERP-1 is indicated as a gray box near the C-terminus and the P1 (Arg, R) and P1' (Asn, N) sites are indicated. The N-terminal signal sequence is shaded in black with the cleavage site indicated.



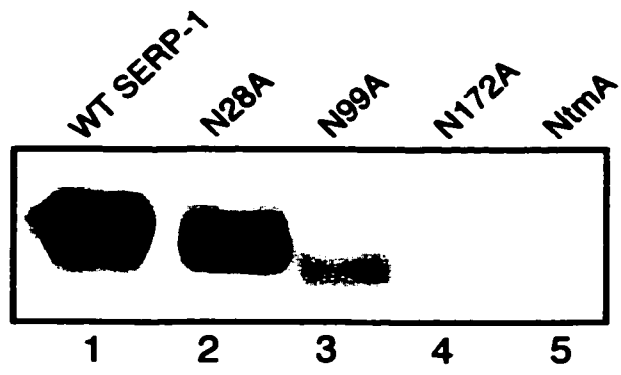
**Figure IV-2: Endoglycosidase H resistance of intracellular and secreted SERP-1.** Pulse-chase time-course for the processing and secretion of SERP-1 from BGMK cells infected with VV-S1 (panels A and C) and myxoma virus (panels B and D). At 10 hours post-infection, cells were pulse labeled with 500  $\mu$ Ci of  $^{35}$ S-Met/Cys and incubated for 15 minutes. The cells were washed and then both cells (panels A and B) and secreted proteins (panels C and D) were harvested at times of 0 (lanes 1 & 2), 1 hour (lanes 3 & 4), 2 hours (lanes 5 & 6), 4 hours (lanes 7 & 8), 6 hours (lanes 9 & 10), and 8 hours (lanes 11 & 12) hours post-labeling (times are indicated above each panel). Samples were either treated with endoglycosidase H (+) or left untreated (-). Mature, glycosylated SERP-1 appears as a 55 kDa species (M), immature glycosylated SERP-1 appears as a 48 kDa species (I), while SERP-1 deglycosylated following Endo H treatment appears as a 42 kDa species (P).



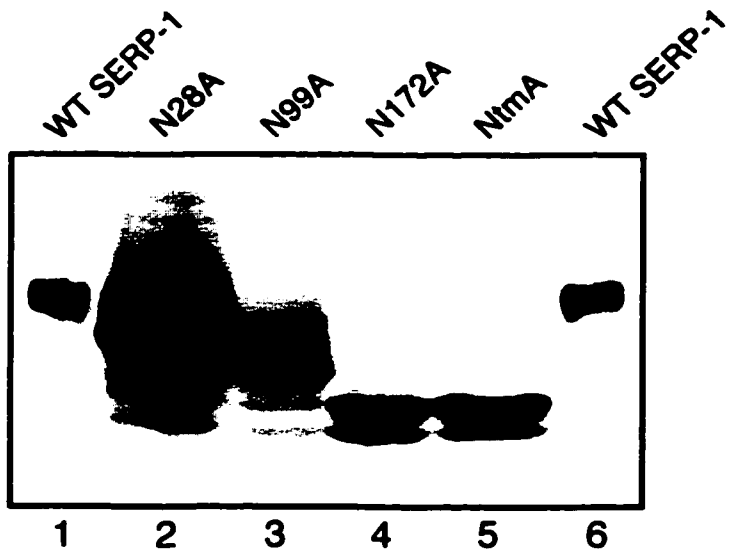
**Figure IV-3: Secretion and degradation of SERP-1 glycosylation mutants.**  
SERP-1 mutants N28A (lane 2), N99A (lane 3), N128A (lane 4) and NtmA (lane 5) were expressed from vaccinia virus and culture supernatants (panel A), and cell lysates (panel B) collected, separated on SDS-PAGE and detected by western blotting with anti-SERP-1 mAb AQ.H9.



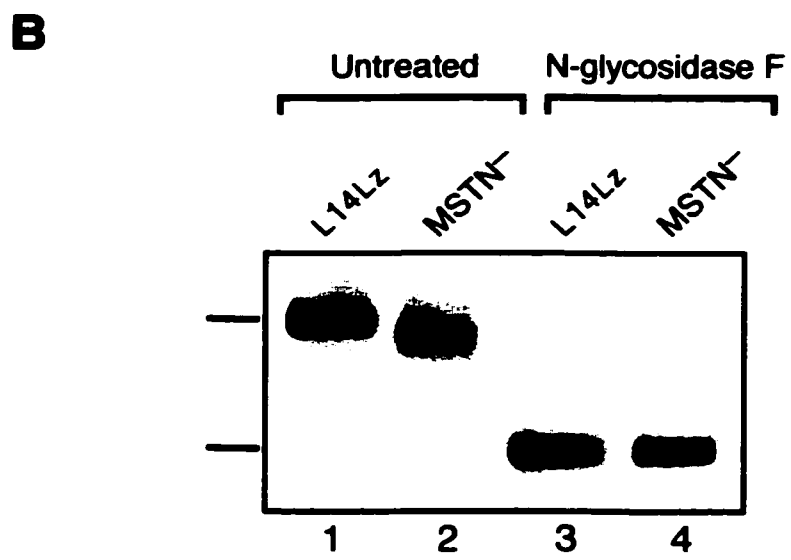
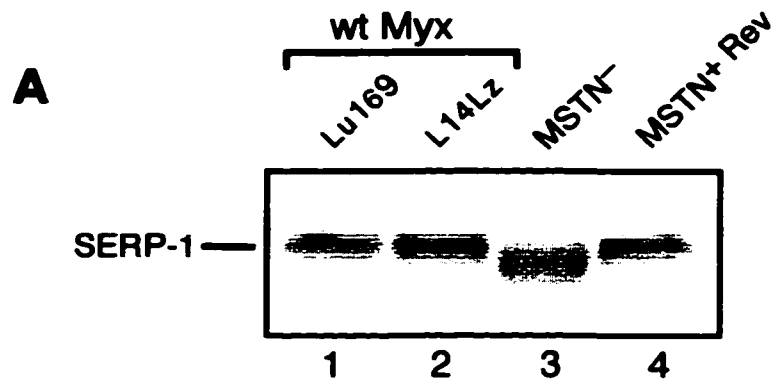
**A**



**B**

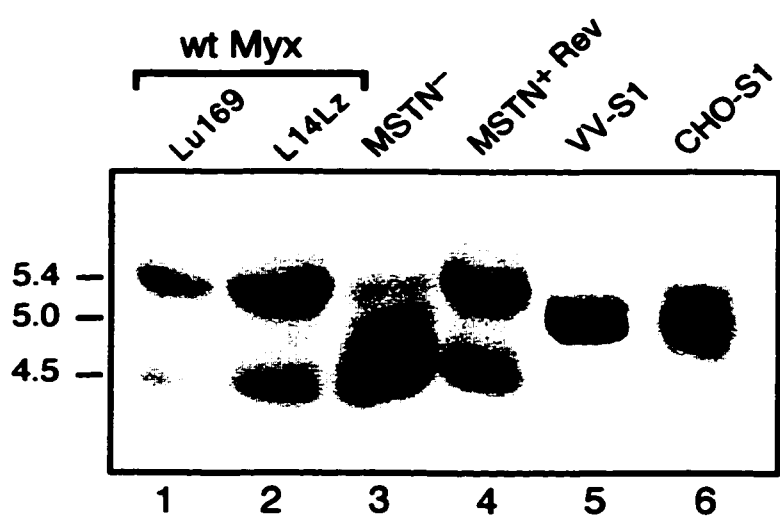


**Figure IV-4: SERP-1 is sialylated by the MST3N gene product.** *A.* Culture supernatant from BGMK cells infected with wild-type myxoma virus Lu169 (lane 1), a control myxoma virus construct with a LacZ cassette, L14Lz (lane 2), myxoma virus in which the sialyltransferase gene is disrupted by the lacZ cassette, MSTN<sup>-</sup> (lane 3), or the MST3N<sup>-</sup> revertant virus (lane 4) were collected and concentrated 10 fold. Following separation by SDS-PAGE, samples were visualized by Western blotting using anti-SERP-1 mAb AQ.H9. *B.* Culture supernatants from L14Lz (lanes 1 & 3), or MSTN<sup>-</sup> (lanes 2 & 4) were concentrated 10 fold and treated with N-glycosidase F (lane 3 & 4). Samples were separated by SDS-PAGE and visualized by Western blotting with anti-SERP-1 mAb AQ.H9.

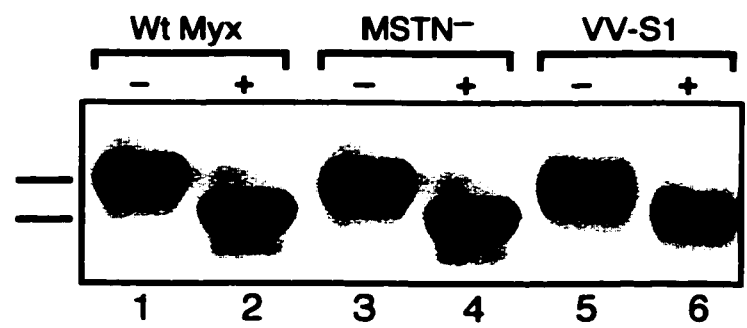


**Figure IV-5: Sialylation of SERP-1 by the MST3N gene product alters overall negative charge of the mature secreted protein.** *A.* SERP-1 produced from BGMK cells infected with myxoma virus strains Lu169 (lane 1), L14Lz (lane 2), MSTN<sup>-</sup> (lane 3), the MST3N<sup>-</sup> revertant virus (lane 4), or from VV-S1 (lane 5) and uninfected CHO cells engineered to overexpress SERP-1 (lane 6) were separated by isoelectric focussing, and detected by Western blotting with anti-SERP-1 mAb AQ.H9. The *pI* standards 4.5, 5.0 and 5.4 are indicated with dashes. *B.* Both cellular and viral sialyltransferases modify SERP-1. SERP-1 secreted from BGMK cells infected with myxoma virus (lanes 1 & 2), MSTN<sup>-</sup> (lanes 3 & 4), and VV-S1 (lanes 5 & 6) were subjected to treatment with neuraminidase (lanes 2, 4, 6). Samples were separated by SDS-PAGE and detected by Western blotting using mAb AQ.H9.

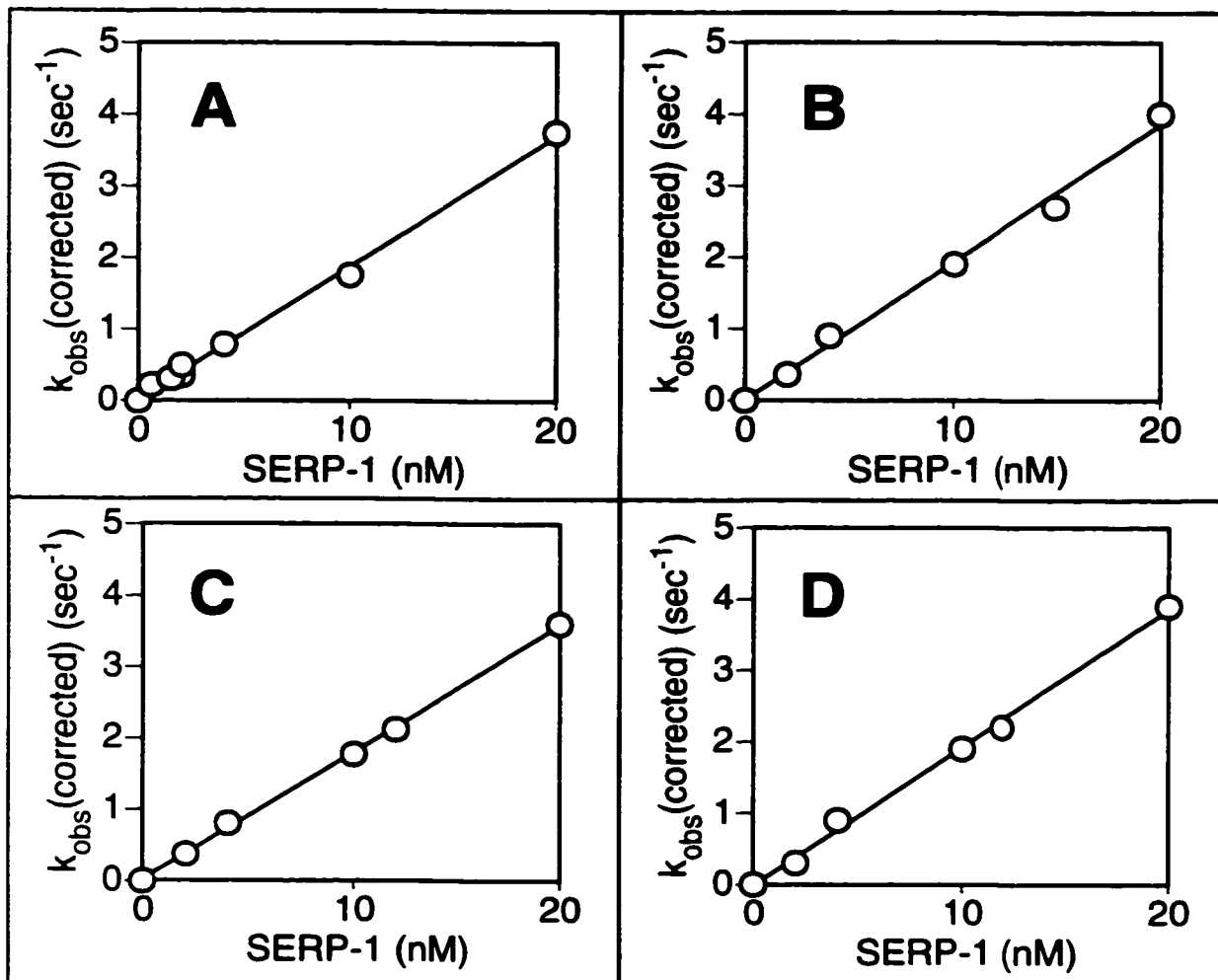
**A**



**B**



**Figure IV-6: Second order rate constant determination. Second order rate constant determination of SERP-1 forms with variable sialylation.** Second-order replots for the reactions of tPA with secreted SERP-1 expressed and purified from **A**, VV-S1; **B**, CHO cells expressing SERP-1; **C**, wild-type myxoma virus; **D**, MSTN<sup>-</sup> myxoma virus. For each sample of SERP-1, the observed rate constant for the approach to steady state,  $k_{obs}$ , was determined at various concentrations of SERP-1 in slow-binding inhibition experiments with tPA. The resulting values were corrected as described in the text to compensate for the competitive binding of chromogenic substrate in the slow-binding inhibition assay. Plots of  $k_{obs}$ (corrected) against the concentration of SERP-1 are linear, and the slopes give values for  $k_{app}$ , the apparent second-order rate constant for the reaction of each enzyme with SERP-1, which are given in Table IV-1.



**REFERENCES**

- Barry, M., Lee, S. F., Boshkov, L., and McFadden, G. (1995). Myxoma virus induces extensive CD4 downregulation and dissociation of p56<sup>lck</sup> in infected rabbit CD4+ T lymphocytes. *J. Virol.* **69**: 5243-5251.
- Barry, M., and McFadden, G. (1998). Apoptosis regulators from DNA viruses. *Current Opinion in Immunology* **10**: 422-430.
- Barry, M., and McFadden, G. (In Press). The regulation of apoptosis by poxviruses. In *Effects of Microbes on the Immune System*, E. W. Cunningham and R. Fujinami, eds.: Lippincott-Raven Publishers).
- Barry, M., and McFadden, G. (1997). Virokines and viroceptors. In *Cytokines in Health and Disease*, D. G. Remick and J. S. Friedland, eds. (New York: Marcel Dekker, Inc.), pp. 251-261.
- Beeley, J. G. (1985). Glycoprotein and proteoglycan techniques, Volume 16, R. H. Burdon and P. H. van Knippenberg, eds. (Amsterdam: Elsevier).
- Bergeron, J. J., Zapun, A., Ou, W. J., Hemming, R., Parlati, F., Cameron, P. H., and Thomas, D. Y. (1998). The role of the lectin calnexin in conformation independent binding to N-linked glycoproteins and quality control. *Adv. Exp. Med. Biol.* **435**: 105-116.



Bitter-Suermann, D. (1993). . In Polysialic acid: from microbes to man, J. Roth, U. Rutishauer and F. A. Troy, eds. (Basel, Switzerland: Birkhauser Verlag), pp. 11-24.

Boshkov, L. K., Macen, J. L., and McFadden, G. (1992). Virus-induced loss of class I MHC antigens from the surface of cells infected with myxoma virus and malignant rabbit fibroma virus. *J. Immunol.* **148**: 881-7.

Bozue, J. A., Tullius, M. V., Wang, J., Gibson, B. W., and Munson, R. S. (1999). Haemophilus ducreyi produces a novel sialyltransferase. *J. Biol. Chem.* **274**: 4106-4114.

Crocker, P. R., Freeman, S., Gordon, S., and Kelm, S. (1995). Sialoadhesin binds preferentially to cells of the granulocytic lineage. *J. Clin. Invest.* **95**: 635-643.

Fenner, F. (1990). Poxviruses. In Virology, B. N. Fields, ed. (New York: Raven Press, Ltd.), pp. 2113-2133.

Fenner, F., and Meyers, K. (1978). Myxoma virus and myxomatosis in retrospect: The first quarter century of a new disease. In Viruses and the Environment, E. Kurstok and K. Maramorosch, eds. (New York: Academic Press), pp. 539-570.

Fenner, F., and Ratcliffe, F. N. (1965). Myxomatosis (London: Cambridge University Press).

- Hennet, T., Chui, D., Paulson, J. C., and Marth, J. D. (1998). Immune regulation by the ST6Gal sialyltransferase. *Proc. Natl. Acad. Sci. USA* **95**: 4504-4509.
- Jackson, R. J., Hall, D. F., and Kerr, P. J. (1999). Myxoma virus encodes an  $\alpha$ 2,3-sialyltransferase that enhances virulence. *J. Virol.* **73**: 2376-2384.
- Kasky, L. A. (1995). Selectin-carbohydrate interactions in the initiation of the inflammatory response. *Annu. Rev. Biochem.* **64**: 113-139.
- Kelm, S., Schauer, R., and Crocker, P. R. (1996). The sialoadhesins: a family of sialic acid-dependent cellular recognition molecules within the immunoglobulin superfamily. *Glycoconjugate J.* **13**: 913-926.
- Kopito, R. R. (1997). ER quality control: the cytoplasmic connection. *Cell* **88**: 427-30.
- Kouchi, Z., Kinouchi, T., Sorimachi, H., Ishiura, S., and Suzuki, K. (1998). The deletion of the C-terminal tail and addition of an endoplasmic reticulum targeting signal to Alzheimer's amyloid precursor protein change its localization, secretion, and intracellular proteolysis. *Eur. J. Biochem.* **258**: 291-300.
- Kowalski, J. M., Parekh, R. N., Mao, J., and Wittrup, K. D. (1998). Protein folding stability can determine the efficiency of escape from endoplasmic reticulum quality control. *J. Biol. Chem.* **273**: 19453-19458.

Kukuruzinska, M. A., and Lennon, K. (1998). Protein N-glycosylation: molecular genetics and functional significance. *Critical Reviews in Oral Biology and Medicine* **9**: 415-448.

Laemelli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**: 680.

Liu, Y., Choudhury, P., Cabral, C. M., and Sifers, R. N. (1999). Oligosaccharide modification in the early secretory pathway directs the selection of a misfolded glycoprotein for degradation by the proteasome. *J. Biol. Chem* **274**: 5861-5867.

Lomas, D. A., Evans, D. L., Upton, C., McFadden, G., and Carrell, R. W. (1993). Inhibition of plasmin, urokinase, tissue plasminogen activator, and C1S by a myxoma virus serine proteinase inhibitor. *J. Biol. Chem.* **268**: 516-21.

Lucas, A., Liu, L., Macen, J. L., Nash, P. D., Dai, E., Stewart, M., Yan, W., Graham, K., Etches, W., Boshkov, L., Nation, P. N., Humen, D., Hobman, M., and McFadden, G. (1996). Virus-encoded serine proteinase inhibitor SERP-1 inhibits atherosclerotic plaque development following balloon angioplasty. *Circulation* **94**: 2890-2900.

Macen, J. L., Upton, C., Nation, N., and McFadden, G. (1993). SERP1, a serine proteinase inhibitor encoded by myxoma virus, is a secreted glycoprotein that interferes with inflammation. *Virology* **195**: 348-63.

Maksymowych, W. P., Nation, N., Nash, P. D., Macen, J., Lucas, A., McFadden, G., and Russell, A. S. (1996). Amelioration of antigen-induced arthritis in rabbits treated with a secreted viral serine proteinase inhibitor. *J. Rheumatol.* **23**: 878-882.

Mandrell, R. E., Griffiss, J. M., and Macher, B. A. (1988). *J. Exp. Med.* **168**: 107-126.

McFadden, G., and Barry, M. (1998). How poxviruses oppose apoptosis. *Semin. Virology* **8**: 429-442.

McFadden, G., Graham, K., Ellison, K., Barry, M., Macen, J., Schreiber, M., Mossman, K., Nash, P., Lalani, A., and Everett, H. (1995). Interruption of cytokine networks by poxviruses: lessons from myxoma virus. *J. Leukoc. Biol.* **57**: 731-8.

McFadden, G., Lalani, A., Everett, H., Nash, P., and Xu, X. (1998). Virus encoded-receptors for cytokines and chemokines. *Seminars in Cell and Developmental Biology* **9**: 359-368.

Morgenstern, K. A., Sprecher, C., Holth, L., Foster, D., Grant, F. J., Ching, A., and Kisiel, W. (1994). Complementary DNA cloning and kinetic characterization of a novel intracellular serine proteinase inhibitor: mechanism of action with trypsin and factor Xa as model proteinases. *Biochemistry* **33**: 3432-41.

Morrison, J. F., and Walsh, C. T. (1988). *Adv. Enzymol.* **61**: 201-301.

Nakamura, M., Kudo, T., Narimatsu, H., Furukawa, Y., Kikuchi, J., Asakura, S., Yang, W., Iwase, S., Hatake, K., and Miura, Y. (1998). Single glycosyltransferase, core 2  $\beta$ 1,6-N-acetylglucosaminyltransferase, regulates cell surface sialyl-Lex expression level in human pre-B lymphocytic leukemia cell line KM3 treated with phorbol ester. *J. Biol. Chem.* **273**: 26779-26789.

Nash, P., Barrett, J., Cao, J., Hota-Mitchell, S., Lalani, A., Everett, H., Xu, X., Robichaud, J., Hnatiuk, S., Ainslie, C., Seet, B., and McFadden, G. (1999). Immunomodulation by viruses: the myxoma virus story. *Immunological Reviews*: (in press).

Nash, P., Lucas, A., and McFadden, G. (1997). SERP-1, a poxvirus-encoded serpin, is expressed as a secreted glycoprotein that inhibits the inflammatory response to myxoma virus infection. In *Chemistry and Biology of Serpins*, F. C. Church, D. D. Cunningham, D. Ginsburg, M. Hoffman, S. R. Stone and D. M. Tollefsen, eds. (New York: Oxford University Press), pp. 195-205.

Nash, P., Whitty, A., Handwerker, J., Macen, J., and McFadden, G. (1998). Inhibitory specificity of the anti-inflammatory myxoma virus serpin, Serp-1. *J. Biol. Chem.* **273**: 20982-20991.

Parodi, A. J. (1999). Reglucosylation of glycoproteins and quality control of glycoprotein folding in the endoplasmic reticulum of yeast cells. *Biochim. Biophys. Acta* **1426**: 287-95.

Powell, L. D., and Varki, A. (1995). I-type lectins. *J. Biol. Chem.* **270**: 14243-14246.

Ritchie, H., and Booth, N. A. (1998). Secretion of plasminogen activator inhibitor 2 by human peripheral blood monocytes occurs via an endoplasmic reticulum-golgi-independent pathway. *Exp. Cell. Res.* **242**: 439-50.

Rubin, H. (1996). Serine protease inhibitors (SERPINS): where mechanism meets medicine. *Nature Medicine* **2**: 632-633.

Sakaguchi, M. (1997). Eukaryotic protein secretion. *Curr. Opin. Biotechnol.* **8**: 595-601.

Schauer, R., Kelm, S., Reuter, G., Roggentin, P., and Shaw, L. (1995). . In *Biology of the sialic acids*, A. Rosenburg, ed. (New York: Plenum Press), pp. 7-67.

Schreiber, M., Rajarathnam, K., and McFadden, G. (1996). Mxyoma virus T2 protein, a tumor necrosis factor (TNF) receptor homolog, is secreted as a monomer and dimer that each bind rabbit TNF $\alpha$ , but the dimer is a more potent TNF inhibitor. *J. Biol. Chem.* **271**: 13333-13341.

Spriggs, M. K. (1994). Cytokine and cytokine receptor genes 'captured' by viruses. *Curr Opin Immunol* **6**: 526-529.

- Stone, S. R., and Hermans, J. M. (1995). Inhibitory mechanism of serpins. Interaction of thrombin with antithrombin and protease nexin 1. *Biochemistry* **34**: 5164-72.
- Tedder, T. F., Steeber, D. A., Chen, A., and Engel, P. (1995). The selectins: vascular adhesion molecules. *FASEB J.* **9**: 866-873.
- Timmis, K. N., Boulnois, G. J., Bitter, S. D., and Cabello, F. C. (1985). *Curr. Top. Microbiol. Immunol.* **118**: 197-218.
- Traving, C., and Schauer, R. (1998). Structure, function and metabolism of sialic acids. *Cellular and Molecular Life Sciences* **54**: 1330-1349.
- Turner, P. C., Musy, P. W., and Moyer, R. W. (1995). Poxvirus serpins. In *Viroceptors, Virokines and Related Immune Modulators Encoded By DNA Viruses*, G. McFadden, ed. (Austin, TX: R.G. Landes Company), pp. 67-88.
- Upton, C., Macen, J. L., Wishart, D. S., and McFadden, G. (1990). Myxoma virus and malignant rabbit fibroma virus encode a serpin-like protein important for virus virulence. *Virology* **179**: 618-631.
- Varki, A. (1993). Biological roles of oligosaccharides: all the theories are correct. *Glycobiology* **3**: 97-130.

Varki, A. (1997). Sialic acids as ligands in recognition phenomena. *FASEB Journal* **11**: 248-255.

Zhang, J. X., Braakman, I., Matlack, K. E., and Helenius, A. (1997). Quality control in the secretory pathway: the role of calreticulin, calnexin and BiP in the retention of glycoproteins with C-terminal truncations. *Mol. Biol. Cell.* **8**: 1943-1954.

Zinkernagel, R. M. (1996). Immunology taught by viruses. *Science* **271**: 173-178.



## **CHAPTER V**

### **GENERAL DISCUSSION**

**"The important thing is not to stop questioning."**

*-Albert Einstein*

*Nunc Est Bibendum!*

Eons of evolution have required that DNA viruses become endowed with an array of proteins that target critical aspects of the host innate and acquired immune response. In essence, these viruses have learned from, and adapted to survive and propagate within, an environment of complex anti-viral challenges. The result, in the case of myxoma virus, is that the viral genome encodes a number of proteins that, while not essential for virus replication in tissue culture, are critical elements in establishing virulence in an immunocompetent rabbit host. As we study these virulence factors and begin to understand how they function both at a molecular level *in vitro*, and in the complex biology of infected tissue, we gain important insight into the elaborate workings of the hosts' response to infection. The study of viruses not only increases our knowledge of pathogenesis and allows a better control of viral diseases, but also facilitates a greater understanding of immunology and other related fields. Thus, the study of viral immuno-modulators has been compared to the use of the Rosetta stone to decode the complexities of Egyptian hieroglyphics (Barinaga, 1992).

The work presented in this thesis provides a detailed biochemical analysis of the myxoma virus encoded SERP-1 protein. This not only provides a much greater understanding of SERP-1 both as a serine proteinase inhibitor and glycoprotein, but also brings us closer to understanding the functions of SERP-1 *in vivo*, and provides a foundation upon which future studies may be based. The important findings and conclusions from the studies of SERP-1 are summarized below, and the implications of this work for future study are outlined.

As the last open reading frame in the TIR region of the myxoma virus genome, the SERP-1 gene was initially identified as encoding a secreted glycoprotein with homology to members of the serpin superfamily of serine proteinase inhibitors (Upton et al., 1990). This was followed up by studies that examined the effect of SERP-1 on viral virulence using a myxoma virus that contained a targeted disruption of the SERP-1 gene (Macen et al., 1993; Upton et al., 1990). While SERP-1 proved dispensable for virus replication in tissue culture, the resulting virus was attenuated *in vivo* (Macen et al., 1993). Histological analysis suggested that SERP-1 was interfering in inflammation, setting the stage for the use of this protein as an anti-inflammatory compound.

The work presented in Chapter II represents the complete analysis of the kinetics of inhibition of a panel of serine proteinases by SERP-1. In order to study SERP-1 effectively, a vaccinia virus expression system was employed to produce protein in sufficient quantities to allow purification by FPLC. Since serpins form covalent complexes with their target proteinases, we assessed the ability of SERP-1 to form tight SDS-stable complexes with a panel of known proteinases. Of the more than twenty proteinases tested, SERP-1 was observed to form complexes with uPA, tPA, plasmin, thrombin, factor Xa and C1s (Lomas et al., 1993; Nash et al., 1998). The interaction between SERP-1 and inhibited proteinases was found to take place at the P1-P1' residues of Arg<sup>319</sup>-Asn<sup>320</sup>, and mutation of these residues to Ala-Ala abolished all activity (Nash et al., 1998). By utilizing slow-binding kinetic analysis,

constants for the inhibition of uPA, tPA, plasmin, thrombin, factor Xa and C1s were determined. Kinetic analysis of the wild-type SERP-1 protein showed second-order association rate constants ( $k_{inh}$ ) that varied from  $6 \times 10^2 \text{ M}^{-1}\text{s}^{-1}$  to  $3.4 \times 10^5$ , and steady state inhibition constants ( $K_I$ ) that varied from 1.3 nM to 10 pM (Nash et al., 1998). Stoichiometries of inhibition were determined experimentally and varied from 1.4 for uPA to 13 for thrombin (Nash et al., 1998). These studies confirmed the initial prediction that the SERP-1 gene encodes a functional serpin-type proteinase inhibitor. While it is unknown whether any of these enzymes is the true target of SERP-1 in virus infected lesions, the biochemical analysis has provided considerable insight into the possible nature of the target. SERP-1 inhibits proteinases having preferences for cleaving after basic amino acids. Analysis of the variations in the kinetics of inhibition tells us about the nature of viral serpins as immunomodulatory proteins. When serpins act at low concentrations, as appears likely for SERP-1 *in vivo*, inhibitory specificity becomes less dominated by the on-rate ( $k_{inh}$ ) and is increasingly dependent upon the partitioning within the branched pathway mechanism and on the lifetime of the inhibited complex (Nash et al., 1998).

The comprehensive examination of the ability of SERP-1 to inhibit an extensive panel of proteinases provided a unique opportunity to evaluate the serpin mechanism. This was undertaken in Appendix A. Using the powerful tool of linear free energy relationships to analyze the data for SERP-1 inhibition detailed in Chapter II, we were able to ascertain a relationship between  $\log k_{inh}$  and  $\log K_I$ . This

implies that the enzyme-serpin interactions and other factors that are responsible for the observed variations in  $k_{inh}$  are retained in the final inhibited complex. If the factors affecting the rates of inhibition were substantially different from those affecting  $K_i$ , no such relationship would be expected, suggesting that the rate-limiting transition state for inhibition is closely related to the final inhibited complex. Examining the serpin reaction in terms of free energy profiles, the appearance of a relationship between  $\log k_{inh}$  and  $\log K_i$  implies that  $k_{diss} = k_{-4}$ , and therefore that  $K_i = k_{-4}/k_{inh}$ . The observation of this thermodynamic link suggests that the serpin-proteinase complex is at least partially reversible, which is in opposition to the current model of serpins as irreversible suicide-substrate inhibitors. The application of this analysis to other serpin-proteinase cases will be required to determine whether this is a universal phenomena, or is a unique characteristic of SERP-1. Unfortunately such a complete data set for another serpin with a panel of proteinases does not currently exist in the literature.

The binding of a proteinase to the RCL of a serpin encompasses a wider region than the P1-P1' bond (O' Malley et al., 1997; Wilczynska et al., 1997). This extended substrate binding site means that residues in the RCL apart from the P1-P1' residues play roles in determining the specificity and effectiveness of a given serpin. The studies detailed in Chapter III were undertaken with two objectives in mind. A panel of SERP-1 RCL chimeras were constructed and purified from a recombinant vaccinia virus expression system in order to identify the key RCL residues that

conferred inhibitory specificity and affected the kinetic constants of inhibition. Identifying these key residues would enhance our understanding of SERP-1 function at a basic biochemical level. The second objective was to examine the SERP-1 RCL chimeras with altered inhibitory profiles, in an attempt to understand the nature of the interactions that confer anti-inflammatory activity upon SERP-1 better. By testing SERP-1 mutants in the primary restenosis model of arterial inflammation following injury, it should be possible to ascertain what specific characteristics make SERP-1 such a powerfully anti-inflammatory molecule.

The failure of any of the SERP-1 RCL chimeras to act as effective inhibitors *in vitro* suggests that the SERP-1 scaffold is inherently incompatible with RCL mutations or replacements. There are no obvious structural reasons why this would be the case. It may be that SERP-1 evolved within myxoma virus from an ancestral serpin pirated by myxoma virus, and that the accelerated process of evolution that occurs within the virus has created a serpin that is almost perfect in its role as an inhibitor of a key inflammation mediating proteinase. The observed inhibition by SERP-1 of proteinases within the plasminogen and thrombotic pathways likely results from similarities between these enzymes and the true SERP-1 target. If SERP-1 is already an almost perfectly adapted serpin, then any changes made within the RCL could only decrease its inhibitory specificity. In order to increase the ability of SERP-1 to inhibit the enzymes tested in Chapter III, it may be necessary to alter the structure of the RCL in a more drastic manner. This could be accomplished by altering the length of the RCL to increase flexibility and decrease the time required

for loop insertion upon formation of the acyl-enzyme intermediate (Bottomley and Chang, 1997; Bottomley and Stone, 1998; Lawrence et al., 1994). Changes could also be made in two other critical regions of SERP-1. The “hinge” region that connects the RCL to the serpin scaffold and directs insertion of the RCL into  $\beta$ -sheet A is a critical determinant of both the stoichiometry of inhibition and the second order rate constant of inhibition (Hopkins et al., 1993). This region spans approximately P16 to P10, with the P14 threonine residue being essential in most cases (Huntington et al., 1997). Certain residues within  $\beta$ -sheet A are also critical determinants of the ability of the RCL to fold into the stable anti-parallel  $\beta$ -sheet in order to convert the acyl-enzyme intermediate into a stable inhibited complex. This region is now beginning to be explored in other serpins, and as our understanding of the structural and mechanistic elements in this region that contribute to serpin activity improve, it may be possible to target specific residues within SERP-1 in order to alter or improve its inhibition profile. Until we have determined the target of SERP-1 *in vivo*, mutagenesis of wider regions of SERP-1 is not likely to prove informative.

Although SERP-1 is known to be a secreted glycoprotein, little attention has been paid to the nature of the glycosylation, secretion pathway, or importance of glycosylation. In Chapter IV, the glycosylation of SERP-1 was examined in order to determine which potential N-linked sites were used, the importance of these sites in the secretion and trafficking, whether SERP-1 was a target of the myxoma virus

encoded sialyltransferase, and what effect glycosylation had on SERP-1 proteinase inhibition function *in vitro*. To determine at which sites SERP-1 was glycosylated, the three potential N-linked glycosylation sites were mutated and the protein expressed in vaccinia virus. All three sites proved to be used for glycosylation, which proved essential for SERP-1 secretion. Mutation of Asn<sup>172</sup> blocked all SERP-1 secretion and led to the rapid degradation of intracellular SERP-1, whereas mutation of Asn<sup>99</sup> resulted in decreased SERP-1 secretion. The use of glycosylation state as a marker for the correct folding has been documented for at least one other serpin (Liu et al., 1999), and may be a general feature of serpins that must fold into a metastable state. This is consistent with the inability to express SERP-1 from bacterial systems, another feature in common with the serpins family. In the case of antitrypsin, glycosylation abnormalities are recognized by calnexin and the protein is targeted for degradation by the proteasome (Liu et al., 1999). This may also be the case for the SERP-1 glycosylation mutants, and the precise method of intracellular degradation can be examined in future studies. By utilizing the proteasome specific inhibitor, lactacystin, in conjunction with more general proteinase inhibitors such as the membrane-permeable peptide aldehydes *N*-acetyl-leu-leu-norleucinal and *N*-acetyl-leu-leu-methioninal, it would be possible to determine whether SERP-1 glycosylation site mutants are actively targeted to the proteasome. As well, by using the monoclonal antibodies now available against SERP-1, and commercially available antibodies directed against calnexin and other ER chaperones, it would be possible to test whether SERP-1 and the SERP-1



glycosylation mutants are sequestered by calnexin in the endoplasmic reticulum prior to degradation.

Using a myxoma virus strain devoid of expression of the MST3N sialyltransferase, we have demonstrated for the first time that a secreted viral protein is actively modified by a virally encoded glycosyltransferase. While SERP-1 is also sialylated by endogenous cellular sialyltransferases, the MST3N gene product modifies SERP-1 to yield a species with a distinct net negative charge that can be separated on the basis of its isoelectric point. It is not clear at this point what effect MST3N-directed sialylation of SERP-1 has, though reducing antigenicity and improving localization to the surface of target cells are both reasonable hypotheses. Future work on the role of the MST3N sialyltransferase modifications of SERP-1 and other myxoma virus virulence factors will be needed to answer this question. Studies are now underway to compare the anti-inflammatory properties of SERP-1 purified from wild-type myxoma with SERP-1 from the MST3N knock-out virus in the animal model of restenosis following primary balloon angioplasty. While we expect SERP-1 to be an effective anti-inflammatory protein in both cases, the dose-response curve may be different for wild-type SERP-1 than for MST3N knock-out virus derived SERP-1 if MST3N sialylation plays a role in targeting SERP-1 to its site of action.

The single most important unanswered question regarding SERP-1 is the mechanism by which it achieves such a potentially anti-inflammatory effect *in vivo*.

Specifically, what is the target proteinase that is responsible for the biological effects of SERP-1. As a viral protein, SERP-1 appears to function by inhibiting key proteinases and thereby obstructing the inflammatory response. By doing this, SERP-1 may block the downstream production of pro-inflammatory cytokines and chemokines that act to recruit immune cells such as those of the lymphocytic and monocytic lineages into the infected tissue. In this manner SERP-1 assures that virus infected cells avoid a full scale assault by the cellular arm of the immune system. If SERP-1 does indeed act upstream of cytokine and chemokine activation, it may explain why SERP-1 is produced in relatively low abundance compared to viral proteins aimed at blocking the action of key cytokines and chemokines directly such as the IFN- $\gamma$  binding protein (M-T7) and the chemokine binding protein (M-T1). Away from the virus, SERP-1 retains powerfully anti-inflammatory effects in models of restenosis, arthritis and transplatastion (Christov et al., 1999; Lucas et al., 1996; Maksymowych et al., 1996; Miller et al., 1999). In these model systems, SERP-1 functions at very low concentrations, suggesting that the target is a rare proteinase that acts at a critical early stage in the inflammatory response. These studies have expanded a new field with the investigation of viral proteins as therapeutic agents.

This thesis has presented a detailed biochemical examination of SERP-1 as a serpin and a viral glycoprotein. Additional *in vivo* studies have proven the effectiveness of SERP-1 as an anti-inflammatory agent. As of yet, the link between the *in vitro* and *in vivo* data remains elusive. As the work presented herein has been compiled, a hint of a possible link between the biochemical inhibitory and the anti-

inflammatory functions of SERP-1 has begun to develop. Recent work has demonstrated that SERP-1 is capable of blocking the release of TNF $\alpha$  from an LPS stimulated THP-1 monocyte-derived cell line (unpublished data). While this is still preliminary work, it may be a link that relates the biochemistry to the biology of SERP-1. TNF $\alpha$  is a key inflammatory cytokine, and has been described as the primary cytokine relevant in antigen-induced arthritis (Badolato and Oppenheim, 1996; Feldmann et al., 1996; Maini et al., 1995; Piguet et al., 1992), a system in which SERP-1 has shown excellent efficacy (Maksymowych et al., 1996). The release of TNF $\alpha$  from stimulated cells is dependent upon the proteinase TNF $\alpha$  converting enzyme (TACE). While our biochemical data suggests that TACE is unlikely to be a target of SERP-1, TACE must itself be activated by proteolytic cleavage (Moss et al., 1997). Among the proteinases reported to activate TACE is uPA and uPA-like proteinases that are potential targets for SERP-1 inhibition. Much work remains to be done to elucidate the role that SERP-1 is playing in blocking TNF $\alpha$  release from stimulated THP-1 cells, but this remains a promising lead for discovering the target of SERP-1.

SERP-1 has proven to be an effective drug, a tool for studying the mechanism of serpin function, and the first reported virally sialylated glycoprotein. A few years ago the concept of using viral proteins to probe the immune system and improve our understanding of the complex biology of inflammation was introduced (Barinaga, 1992; Davis-Poynter and Farrell, 1996; Gooding, 1992; Gooding, 1992; Isaacs and Moss, 1995; McFadden, 1995; McFadden and Graham, 1994; McFadden

et al., 1996; McFadden et al., 1995; Mossman et al., 1995; Ploegh, 1998; Smith, 1993; Smith et al., 1997). The detailed investigation of SERP-1 on many fronts places it as a prime example of this concept.

Members of the poxvirus family are the first examples of viruses that encode serpins. The fact that poxviruses have evolved to utilize multiple serpins as part of their concerted anti-immune strategies underscores their importance in regulating key steps in the host's anti-viral immune response. The ability of SERP-1 to dampen the inflammatory response to the virus infection offers clues to the underlying role that serine proteinases may be playing in these early processes. A better understanding of the underlying mechanisms may help in the development of anti-inflammatory drugs based on serpins and small molecule proteinase inhibitors. As SERP-1 has now been shown to have therapeutic potential in diseases of excessive inflammatory responses, other proteinase inhibitors which target related pathways are also likely to be effective at low pharmacological doses. The observation that Spi-2/crmA inhibits ICE-type proteinases, and in doing so blocks apoptosis, has already contributed to our understanding of programmed cell death. As a cross-class inhibitor, Spi-2/crmA is further evidence of the diversity of roles that serpins can play to modulate the host immune responses to favor virus survival.

Viruses have co-evolved with the increasingly complex immune and cellular systems of eukaryotes. While we have been studying such matters for a mere hundred years or so, viruses have been actively engaging these same immune

pathways for millennia. It is not surprising then, that we have much to learn from such seemingly simple organisms. Work on poxviral serpins has only begun and we have yet to identify the "true" targets of SERP-1 in infected tissues and the role these targets play in the regulation of inflammation. Meanwhile, the remaining poxviral serpins remain largely uncharacterized. The tantalizing clues already uncovered suggest that there is still a great deal that viruses can teach us about the immune system in general.

**REFERENCES**

- Badolato, R., and Oppenheim, J. J. (1996). Role of cytokines, acute-phase proteins, and chemokines in the progression of rheumatoid arthritis. *Semin Arthritis Rheum* **26**: 526-38.
- Barinaga, M. (1992). Viruses launch their own "Star Wars". *Science* **258**: 1730-1731.
- Bottomley, S., and Chang, W. (1997). The effects of reactive centre loop length upon serpin polymerization. *Biochem. Biophys. Res. Commun.* **241**: 264-269.
- Bottomley, S. P., and Stone, S. R. (1998). Protein engineering of chimeric serpins: an investigation into effects of the serpin scaffold and reactive centre loop length. *Prot. Eng.* **11**: 1243-1247.
- Christov, A., Dai, E., Liu, L., Miller, L. W., Nash, P., Lalani, A., McFadden, G., Nation, P. N., Tulip, J., and Lucas, A. (1999). Detection of transplant vasculopathy in a rat aortic allograft model by fluorescence spectroscopic optical analysis. *Lasers in Surgery and Medicine*: (submitted).
- Davis-Poynter, N. J., and Farrell, H. E. (1996). Masters of deception: A review of herpesvirus immune evasion strategies. *Immunology and Cell Biology* **74**: 513-522.

- Feldmann, M., Brennan, F. M., and Maini, R. N. (1996). Role of cytokines in rheumatoid arthritis. *Annu Rev Immunol* **14**: 397-440.
- Gooding, L. R. (1992). Virus proteins that counteract host immune defences. *Cell* **61**: 5-7.
- Gooding, L. R. (1992). Virus proteins that counteract host immune defenses. *Cell* **71**: 5-7.
- Hopkins, P. C., Carrell, R. W., and Stone, S. R. (1993). Effects of mutations in the hinge region of serpins. *Biochemistry* **32**: 7650-7.
- Huntington, J. A., Fan, B., Karlsson, K. E., Deinum, J., Lawrence, D. A., and Gettins, P. G. (1997). Serpin conformational change in ovalbumin, enhanced reactive center loop insertion through hinge region mutations. *Biochemistry* **36**: 5432-5440.
- Isaacs, S. N., and Moss, B. (1995). Viroceptors, Virokines and Related Immune Modulators Encoded by DNA Viruses. In Inhibition of complement activation by vaccinia virus, G. McFadden, ed. (Austin, TX: R.G. Landes Company), pp. 55-66.
- Lawrence, D. A., Olson, S. T., Palaniappan, S., and Ginsburg, D. (1994). Serpin reactive center loop mobility is required for inhibitor function but not for enzyme recognition. *J. Biol. Chem.* **269**: 27657-62.

Liu, Y., Choudhury, P., Cabral, C. M., and Sifers, R. N. (1999). Oligosaccharide modification in the early secretory pathway directs the selection of a misfolded glycoprotein for degradation by the proteasome. *J. Biol. Chem* **274**: 5861-5867.

Lomas, D. A., Evans, D. L., Upton, C., McFadden, G., and Carrell, R. W. (1993). Inhibition of plasmin, urokinase, tissue plasminogen activator, and C1S by a myxoma virus serine proteinase inhibitor. *J. Biol. Chem.* **268**: 516-21.

Lucas, A., Liu, L., Macen, J. L., Nash, P. D., Dai, E., Stewart, M., Yan, W., Graham, K., Etches, W., Boshkov, L., Nation, P. N., Humen, D., Hobman, M., and McFadden, G. (1996). Virus-encoded serine proteinase inhibitor SERP-1 inhibits atherosclerotic plaque development following balloon angioplasty. *Circulation* **94**: 2890-2900.

Macen, J. L., Upton, C., Nation, N., and McFadden, G. (1993). SERP-1, a serine proteinase inhibitor encoded by myxoma virus, is a secreted glycoprotein that interferes with inflammation. *Virology* **195**: 348-363.

Maini, R. N., Elliott, M. J., Brennan, F. M., and Feldmann, M. (1995). Beneficial effects of tumour necrosis factor-alpha (TNF-alpha) blockade in rheumatoid arthritis (RA). *Clin Exp Immunol* **101**: 207-12.

Maksymowych, W. P., Nation, N., Nash, P. D., Macen, J., Lucas, A., McFadden, G., and Russell, A. S. (1996). Amelioration of antigen-induced arthritis in rabbits treated with a secreted viral serine proteinase inhibitor. *J. Rheumatol.* **23**: 878-882.



McFadden, G. (1995). **Viroceptors, virokines and related immune modulators encoded by DNA Viruses** (Austin, Texas: R. G. Landes Co.).

McFadden, G., and Graham, K. (1994). Modulation of cytokine networks by poxviruses: The myxoma virus model. *Semin. Virology* **5**: 421-429.

McFadden, G., Graham, K., and Barry, M. (1996). New strategies of immune modulation by DNA viruses. *Transplantation Proceedings* **28**: 2085-2088.

McFadden, G., Graham, K., Ellison, K., Barry, M., Macen, J., Schreiber, M., Mossman, K., Nash, P., Lalani, A., and Everett, H. (1995). Interruption of cytokine networks by poxviruses: lessons from myxoma virus. *J. Leukoc. Biol.* **57**: 731-8.

Miller, L., Dai, E., Nash, P., Jiu, L., Nation, P., Icton, C., Klironomous, D., Fan, L., Ling, L., McFadden, G., and Lucas, A. (1999). Inhibition of transplant vasculopathy in a rat aortic allograft model after infusion of a viral serpin with anti-inflammatory activity. *Cirulation*: (submitted).

Moss, M. L., Jin, S. L., Becherer, J. D., Bickett, D. M., Burkhart, W., Chen, W. J., Hassler, D., Leesnitzer, M. T., McGeehan, G., Milla, M., Moyer, M., Rocque, W., Seaton, T., Schoenen, F., Warner, J., and Willard, D. (1997). Structural features and biochemical properties of TNF-alpha converting enzyme (TACE). *J Neuroimmunol* **72**: 127-9.

Mossman, K., Barry, M., and McFadden, G. (1995). *Viroceptors, Virokines and Related Immune Modulators Encoded by DNA Viruses*, G. McFadden, ed. (Austin: R.G. Landes Company).

Nash, P., Whitty, A., Handwerker, J., Macen, J., and McFadden, G. (1998). Inhibitory specificity of the anti-inflammatory myxoma virus serpin, Serp-1. *J. Biol. Chem.* **273**: 20982-20991.

O' Malley, K., Nair, S., Rubin, H., and Cooperman, B. (1997). The kinetic mechanism of serpin-proteinase complex formation. *J. Biol. Chem.* **272**: 5354-5359.

Piguat, P. F., Grau, G. E., Vesin, C., Loetscher, H., Gentz, R., and Lesslauer, W. (1992). Evolution of collagen arthritis in mice is arrested by treatment with anti-tumour necrosis factor (TNF) antibody or a recombinant soluble TNF receptor. *Immunology* **77**: 510-514.

Ploegh, H. L. (1998). Viral strategies of immune evasion. *Science* **280**: 248-253.

Smith, G. L. (1993). Vaccinia virus glycoproteins and immune evasion. *J. Gen. Virol.* **74**: 1725-1740.

Smith, G. L., Symons, J. A., Khanna, A., Vanderplasschen, A., and Alcamí, A. (1997). Vaccinia virus immune evasion. *Immunological Reviews* **159**: 137-154.

Upton, C., Macen, J. L., Wishart, D. S., and McFadden, G. (1990). Myxoma virus and malignant rabbit fibroma virus encode a serpin-like protein important for virus virulence. *Virology* **179**: 618-631.

Wilczynska, M., Fa, M., Karolin, J., Ohlsson, P.-I., Johansson, L., and Ny, T. (1997). Structural insights into serpin-protease complexes reveal the inhibitory mechanism of serpins. *Nat. Struct. Biol.* **4**: 354-356.

## **APPENDIX A**

### **THE APPLICATION OF LINEAR FREE ENERGY RELATIONSHIPS TO SERPIN MECHANISM:**

*Kinetic and Thermodynamic Determinants of the Inhibitory Specificity of SERP-1*

## INTRODUCTION

Serine proteinases inhibitors of the serpins family are structurally homologous proteins whose members play central roles in the regulation of a wide variety of physiological processes including fibrinolysis, coagulation, inflammation, fertilization, malignancy, neuromuscular patterning and development (Rubin, 1996). While most in the field now agree on a basic kinetic and mechanistic model, certain details of the mechanism by which serpins inhibit their target proteinases remain a hotly debated topic. One of the most powerful tools in the search to understand the relationship between molecular structures and activity is the linear free energy relationship (LFER). LFERs link the changes in rate constants for a reaction to changes in the equilibrium caused by alterations in structure (Exner, 1988). They have primarily been used in the analysis of chemical reactions, but have increasingly found applications in enzymology with their use in determining the mechanism of GTP hydrolysis by GTP-binding proteins (Schweins et al., 1996; Schweins et al., 1995; Schweins and Warshel, 1996), the evaluation of cytochrome P450 mechanism (Guengerich et al., 1996; Higgins et al., 1998; Vaz and Coon, 1994), and energy coupling in the  $F_0F_1$ -ATP synthase (Al-Shawi and Nakamoto, 1997). LFERs have also proven to be an invaluable tool in the analysis of protein folding (Matouschek

and Fersht, 1993). To date, they have not been used in the analysis of serpin mechanism, largely because of the complexity of the mechanism and the difficulty of obtaining sufficiently complete sets of kinetic data. In the analysis of serpin mechanism, LFERs offer a unique glimpse at events occurring in the transition states of the reaction. They may also allow us to address certain key questions of the serpin inhibition mechanism. This chapter presents the first demonstration that a linear energy relationship exists between  $k_{inh}$  and  $K_i$  for a serpin inhibiting a panel of serine proteinases, suggesting that the serpin mechanism may be reversible. The serpin mechanism is considered in terms of free energy profiles in order to examine the implications of this relationship to serpin mechanism.

## THE SERPIN MECHANISM

The mechanism for the formation of the final inhibited serpin-proteinase complex,  $EI'$ , is incompletely understood, and remains a subject of controversy. Kinetic evidence exists that formation of the inhibited acyl enzyme 2 ( $EI'$ ) proceeds via a Michaelis complex ( $EI$ ) and so involves at least two steps (scheme A-1). The basic mechanism, generally accepted as a starting point for serpin debate is outlined as scheme 1A. In contrast to small molecule inhibitors, serpins undergo several steps

between the formation of the initial complex and the eventual highly stable, covalent serpin-proteinase complex. The structures of the various species indicated in scheme A-1 have been modeled and are presented in some detail elsewhere (Stratikos and Gettins, 1997; Whisstock et al., 1998; Wright and Scarsdale, 1994).

According to scheme A-1A, the steps with rate constants  $k_2$  and  $k_4$  are irreversible leading down a one-way path to the stable complex, and from there to cleaved serpin and active enzyme (with rate constant  $k_5$ ). Kinetic evidence has existed for some time that for at least some serpin-proteinase pairs, complex formation is reversible (Longstaff and Gaffney, 1991; Shieh et al., 1989; Stone and Hermans, 1995). This leads to the consideration of a more general reaction mechanism outlined in scheme A-1B. According to this mechanism, all of the steps are potentially reversible, though the rate constants could still heavily bias the reaction in a forward direction.

While the kinetic and mechanistic model in scheme A-1 serves as an excellent basis for the analysis of serpin-proteinase interactions, it is not possible in practice to directly measure the kinetic constants of each individual step in this mechanism. Instead, we are limited to deriving constants that measure the reaction as a whole. The apparent second order rate constant  $k_{app}$ , is a measure of the overall rate at which a serpin forms the inhibited complex  $EI'$ . The rate at which the inhibited

complex ( $EI'$ ) releases active enzyme (E) can be determined directly as  $k_{diss}$ , however, this is the sum of  $k_{-4}$  and  $k_5$ . The steady state inhibition constant,  $K_{i(app)}$ , can also be determined experimentally, though the interpretation of its relevance to the mechanism in scheme A-1 depends upon the relative values of the rate constants  $k_{-4}$  and  $k_5$  (see below). The branched nature of the mechanism further complicates matters. The ratio of the rate constants  $k_3$  and  $k_4$  can be determined by measuring the stoichiometry of inhibition (SI), where  $SI = 1 + (k_3/k_4)$ . This allows us to correct  $k_{app}$  and  $K_{i(app)}$  for the effect of partitioning (Hood et al., 1994). Multiplying  $k_{app}$  by SI compensates for the fact that for an SI of 10 only 1 out of every 10 reactions between enzyme and serpin leads to the formation of the inhibited complex,  $EI'$ . Because of this effect, the true second order rate constant,  $k_{inh}$ , in such a case is 10 times higher than the apparent rate constant for the formation of  $EI'$  that is given by  $k_{app}$ . The apparent steady-state  $K_{i(app)}$  values must also be corrected for the effect of partitioning, in this case by dividing by a factor of SI, if they are to reflect the balance between the true rate constants for the formation and decomposition of  $EI'$  (Hood et al., 1994).

## APPLICATION OF LFER TO SERP-1

In order for us to consider the implications of kinetic values on the serpin mechanism described in scheme 1, it is necessary to have a complete set of data for a single serpin with a number of proteinases. Such an unusually complete data set is available for the myxoma virus serpin, SERP-1 (Nash et al., 1998). The values for  $k_{app}$ ,  $K_{I(app)}$ ,  $k_{diss}$ ,  $SI$ ,  $k_{inh}$  and  $K_I$  for the interaction of SERP-1 with urokinase (uPA), tissue-type plasminogen activator (tPA), plasmin, thrombin, factor Xa, and C1s are presented in Table A-1. SERP-1 is one of many immunomodulatory proteins produced by myxoma virus (Nash et al., 1999), but has the distinction of being the only known virus-encoded secreted serpin. SERP-1 serves to dampen the *in vivo* inflammatory response to virus infection (Macen et al., 1993). Biochemically, SERP-1 has been shown to inhibit enzymes of the plasminogen cascade such as tissue-type plasminogen activator (t-PA), urokinase (u-PA) and plasmin (Nash et al., 1998). It is not clear yet whether these are in fact the biological targets, or whether SERP-1 is targeting other yet unidentified proteinases. Purified SERP-1 protein has also been employed as an effective anti-inflammatory agent in model systems relevant to human disease (Lucas et al., 1996; Maksymowych et al., 1996). For the purposes of



this discussion, SERP-1 represents the most complete set of kinetic data for a single serpin inhibiting multiple proteinases.

Figure A-1 shows that a plot of  $\log k_{\text{inh}}$  against  $\log K_i$  reveals the existence of a linear free energy relationship (LFER) of slope  $\beta = -0.55$ . The observation of such a relationship is somewhat surprising, and implies that the enzyme-serpin interactions and other factors that are responsible for the observed variations in  $k_{\text{inh}}$  are retained in the final inhibited complex. The slope of  $\beta = -0.55$  implies that these interactions are approximately half formed in the transition state for the conformational rearrangement of [EI] relative to their final state in EI' (Eaton et al., 1991; Fersht and Wells, 1991). If the factors affecting the rates of inhibition were substantially different from those affecting  $K_i$ , no such relationship would be expected. This result therefore suggests that the rate-limiting transition state for inhibition is closely related to the final inhibited complex, at least in regard to that subset of serpin-proteinase interactions and other factors that are responsible for the discrimination that SERP-1 displays among the proteinases listed in Table A-1. In contrast, the wide variations in the relative magnitudes of  $k_3$  and  $k_4$  among the enzymes listed in Table A-1, and the lack of any relationship between  $k_3/k_4$  and  $k_{\text{inh}}$  or  $K_i$ , implies that the interactions responsible for discrimination between the enzymes in the transition state for the  $k_4$  step are not related to the interactions leading to variations among the

rates of [EI] deacylation in the  $k_3$  step. This conclusion is consistent with the belief that the steps with rate constants  $k_3$  and  $k_4$  represent quite distinct processes: in one case, the enzyme-catalyzed deacylation of [EI] to release cleaved serpin; in the other, a conformational rearrangement involving insertion of the remaining uninserted portion of the P1-P14 stretch of serpin sequence into sheet A of the serpin structure, with concomitant loss of interaction between the enzyme active site and serpin residues on the P1' (N-terminal) side of the cleavage site within the RCL, and a substantial change in the geometry of interaction between the enzyme and the P1-P $n$  residues on the C-terminal side of the cleavage site (Wright and Scarsdale, 1994) and possibly also a distortion of enzyme active site residues themselves (Plotnick et al., 1996).

### **FREE ENERGY PROFILES FOR THE SERPIN-PROTEINASE REACTION**

In order to visualize the effect that the variations in kinetic behavior observed for the inhibited proteinases, free energy profiles can be employed. The LFER observed in Figure A-1 suggests that the stable serpin-proteinase complex EI\* is reversible. To examine this in more detail we can interpret the variations in terms of

the serpin inhibition mechanism shown in scheme 1 under two different sets of assumptions, depending on whether the formation of the final inhibited complex ( $EI'$ ) from acyl-enzyme 1 is considered to be irreversible (scheme A-1A) or reversible (scheme A-1B). Under case A, the conversion of acyl-enzyme 1 [ $EI$ ] to acyl-enzyme 2 ( $EI'$ ) is assumed to be essentially irreversible (i.e.  $k_5 \gg k_{-4}$ ), the predominant pathway for the decomposition of acyl-enzyme 2 will be through direct hydrolysis via the  $k_5$  pathway to give  $E + I'$  (scheme A-1A). Partitioning of acyl-enzyme 1 through the inhibition and substrate pathways is assumed to be fast compared to its formation, and the relatively low values observed for  $k_{inh}$  are taken to indicate that the chemical step, and not the diffusional encounter of serpin with the proteinase, is the rate-limiting step in the formation of acyl-enzyme 1. Adoption of these case A assumptions corresponds to a free energy profile such as that shown in Figure A-2A. Application of these assumptions to the kinetic data in Table A-1 allows us to compare the reactions with the various inhibited enzymes by means of the overlaid free energy profiles shown in Figure A-2B.

Under case B, the conversion of acyl-enzyme 1 [ $EI$ ] to acyl-enzyme 2 ( $EI'$ ) is considered to be reversible, and the hydrolysis of acyl-enzyme 2 is considered to be very slow ( $k_5 \ll k_{-4}$ ), the predominant pathway for the decomposition of acyl-enzyme 2 will be through the reverse of its formation (pathway with rate

constant  $k_{-4}$ ) leading to the release of active serpin. Evidence exists for several enzyme serpin systems to suggest that this description is accurate in at least some cases (Griffith and Lundblad, 1981; Shieh et al., 1989; Stone and Hermans, 1995). Under these postulates, for interactions where the stoichiometry of inhibition is less than two ( $SI < 2$ ), the formation of acyl-enzyme 1 must be fast compared to its partitioning through the inhibition and substrate pathways, and the rate-limiting step in the formation of acyl-enzyme 2 will therefore be  $k_4$ . Adoption of case B assumptions corresponds to a free energy profile such as that shown in Figure A-3A. Application of these assumptions to the kinetic data in Table A-1 produces the overlaid free energy profiles shown in Figure A-3B.

The differences in the specificity of SERP-1 for the six inhibited proteinases shown in Table A-1 are manifested as variations in the magnitudes of the constants  $k_{inh}$ ,  $k_{diss}$ ,  $K_i$  and  $SI$ . Because we do not yet know whether case A or case B best describes the reactions of SERP-1, we can derive two distinct interpretations of the data. If case A is correct (i.e.  $k_5 \gg k_{-4}$ ), then variations in the rate of formation of the final inhibited complex, acyl-enzyme 2 ( $EI'$ ), arise from differences in the interactions between the enzyme and SERP-1 in the transition state for the formation of acyl-enzyme 1. This transition state is presumably that for the chemical step of acylation of the enzyme by SERP-1. The rapidly-inactivated enzymes uPA, tPA,

plasmin, and thrombin therefor have more favorable binding interactions with SERP-1 in this transition state than do the more slowly inhibited factor Xa and C1s. Under the case A assumptions, the variations in  $k_{\text{diss}}$  reflect differences in the activation barrier for the deacylation of acyl-enzyme 2 ( $E + I'$ ) via the pathway with rate constant  $k_5$ . These differences arise from some combination of variations in the ground state and transition state energies. The variations in  $K_i$  reflect the balance between these two independent effects, while  $K_i$  has no intrinsic thermodynamic significance.

Under the case B assumptions, variations in the rate of the formation of the final inhibited complex, acyl-enzyme 2 ( $EI'$ ), arise from differences in the interactions between the enzyme and SERP-1 in the transition state for the conformational change in which acyl-enzyme 1 is converted to acyl-enzyme 2. Thus, variations in  $K_i$  have a more direct interpretation than was the instance in case A.  $K_i$  can now be seen as directly reflecting differences in the thermodynamic stability of the final inhibited complex, with stabilities of the final inhibited complexes varying over some 5.4 kcal/mol in the order thrombin > tPA  $\approx$  plasmin  $\approx$  uPA > Xa > C1s (Figure A-3B).

Under both case A and case B assumptions, the variations in SI reflect variations in the partitioning of acyl-enzyme 1 between inhibition and substrate

pathways, where deacylation of acyl-enzyme 1 is much slower, relative to conversion to acyl enzyme 2, for uPA than it is for thrombin.

## CONCLUSIONS AND FUTURE DIRECTIONS

Although, in principle, it is possible to distinguish experimentally whether the step with rate constant  $k_{-4}$  or the step with rate constant  $k_5$  represents the predominant pathways for the breakdown of  $EI^*$ , and such measurements have been made in a few cases (Stone and Hermans, 1995), in practice such experimental tests are quite difficult and, even if successful, may not be definitive. In kinetic terms we can attempt to answer this question by looking at the relationship between  $k_{inh}$  and  $K_I$ . According to case A (i.e.  $k_5 \gg k_{-4}$ ), we would not expect any relationship between  $k_{inh}$  and  $K_I$ . If, however, case B (reversible  $k_{-4} \gg k_5$ ) more closely models the data, then  $k_{inh}$  and  $K_I$  are linked thermodynamically. Thus, the observation of the linear correlation shown in Figure A-1 itself provides support for the assumption that  $k_{diss} = k_{-4}$ , and therefore that  $K_I = k_{-4}/k_{inh}$ , since no correlation between  $k_{inh}$  and  $K_I$  would be expected if  $K_I = k_5/k_{inh}$ . Despite this, the observation of a thermodynamic link between  $k_{inh}$  and  $K_I$  is not conclusive evidence that  $k_{-4} \gg k_5$ , and we therefore

regard the nature of the dissociation pathway in the reaction to remain an open question

Regardless of which interpretation on this point is correct, our results show unequivocally that, although the inhibitory specificity of SERP-1 is substantially influenced by variations in the interactions between enzyme and serpin in the transition state of the rate-limiting step in the formation of  $EI^*$ , specific binding interactions in ground states or transition states that occur after the rate-limiting step for inhibition may also play a significant role. The strength of the interaction between enzyme and serpin in these later complexes, and also the partitioning between inhibition and substrate pathways, may therefore contribute to the inhibitory specificity of serpins that act at concentrations close to their  $IC_{50}$  for interaction with a given proteinase, or that are not in significant excess over their proteinase targets. These conditions may pertain to the anti-inflammatory role of SERP-1, as well as to situations such as the inhibition of intracellular caspases by crmA or other serpin inhibitors of apoptosis, where the serpin may be acting at relatively low concentrations.

TABLE A-1

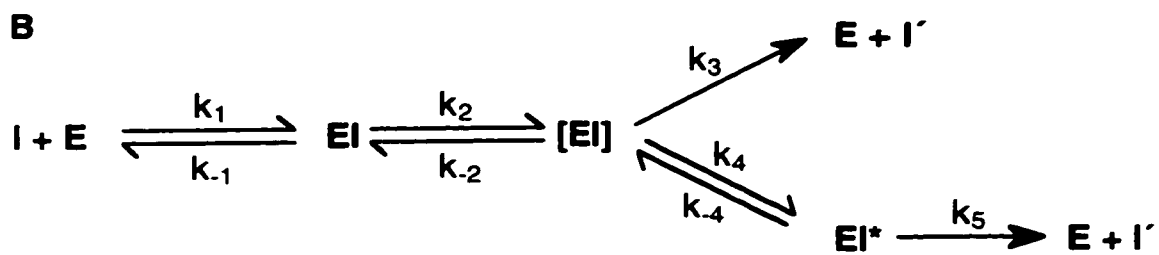
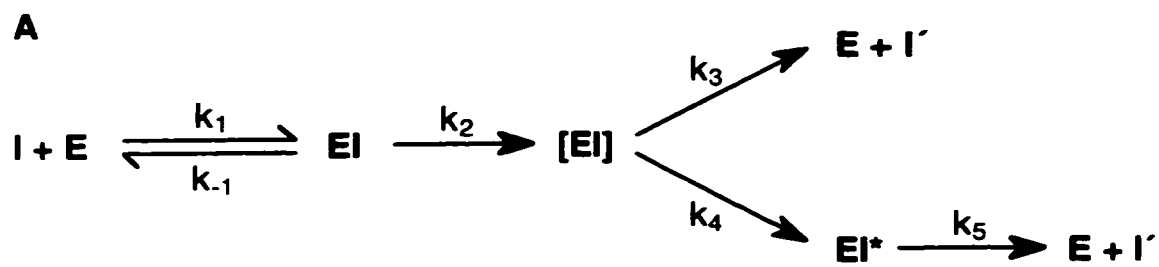
## SUMMARY OF SERP-1 INHIBITION KINETICS

| Enzyme          | $K_{I(app)}$ | $k_{app}$         | $kd_{iss}$         | $SI$ | $K_I$     | $k_{inh}$         |
|-----------------|--------------|-------------------|--------------------|------|-----------|-------------------|
|                 | <i>nM</i>    | $M^{-1}s^{-1}$    | $s^{-1}$           |      | <i>nM</i> | $M^{-1}s^{-1}$    |
| uPA             | 0.16         | $5.0 \times 10^4$ | $8 \times 10^{-6}$ | 1.4  | 0.11      | $7.0 \times 10^4$ |
| tPA             | 0.14         | $4.3 \times 10^4$ | $7 \times 10^{-6}$ | ~2   | 0.07      | $8.6 \times 10^4$ |
| Plasmin         | 0.44         | $4.8 \times 10^4$ | $2 \times 10^{-5}$ | 2.0  | 0.22      | $9.6 \times 10^4$ |
| Thrombin        | 0.13         | $2.6 \times 10^4$ | $3 \times 10^{-6}$ | 13   | 0.01      | $3.4 \times 10^5$ |
| Factor Xa       | 4.3          | $1.7 \times 10^3$ | $7 \times 10^{-6}$ | 3.2  | 1.3       | $5.4 \times 10^3$ |
| Cl <sub>s</sub> | 200          | $3 \times 10^2$   | $6 \times 10^{-5}$ | ND   | ~100      | ~ $6 \times 10^2$ |

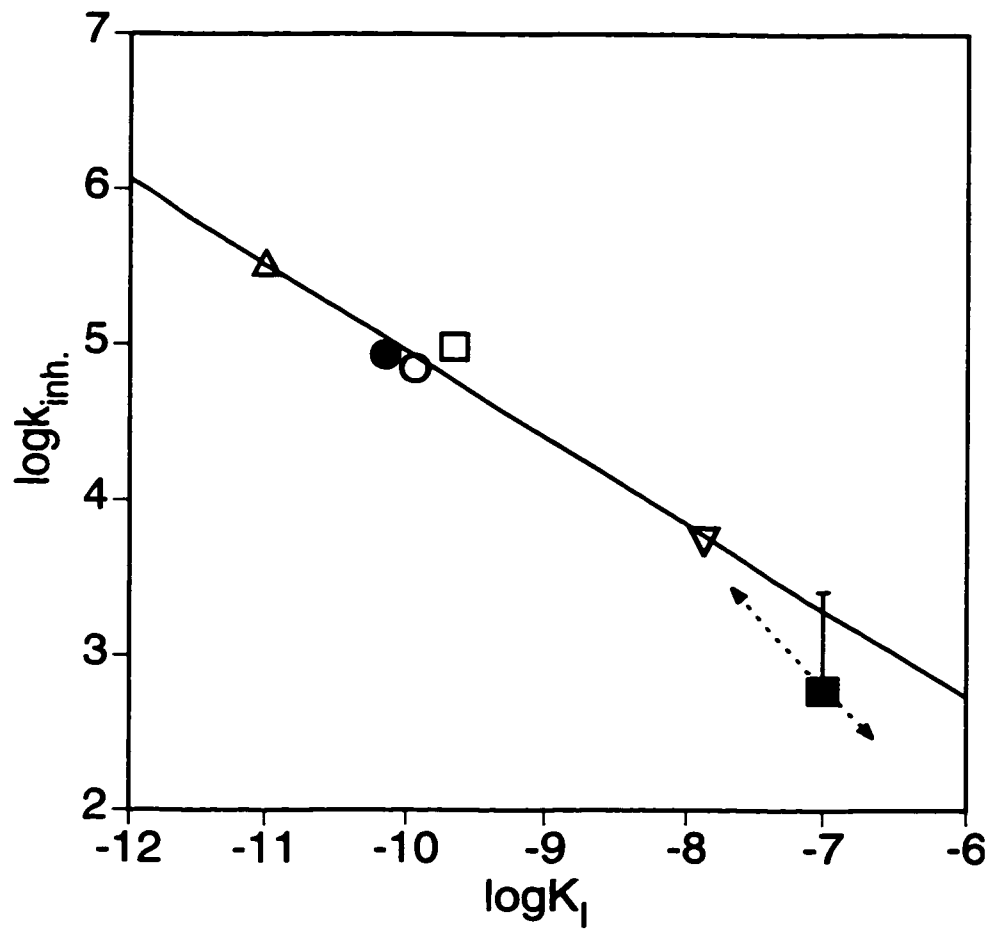
Kinetic parameters for the reaction of SERP-1 with six inhibited proteinases before and after correction for the effects of partitioning in the branched kinetic mechanism. The values are as reported in Nash *et al.* (Nash et al., 1998).



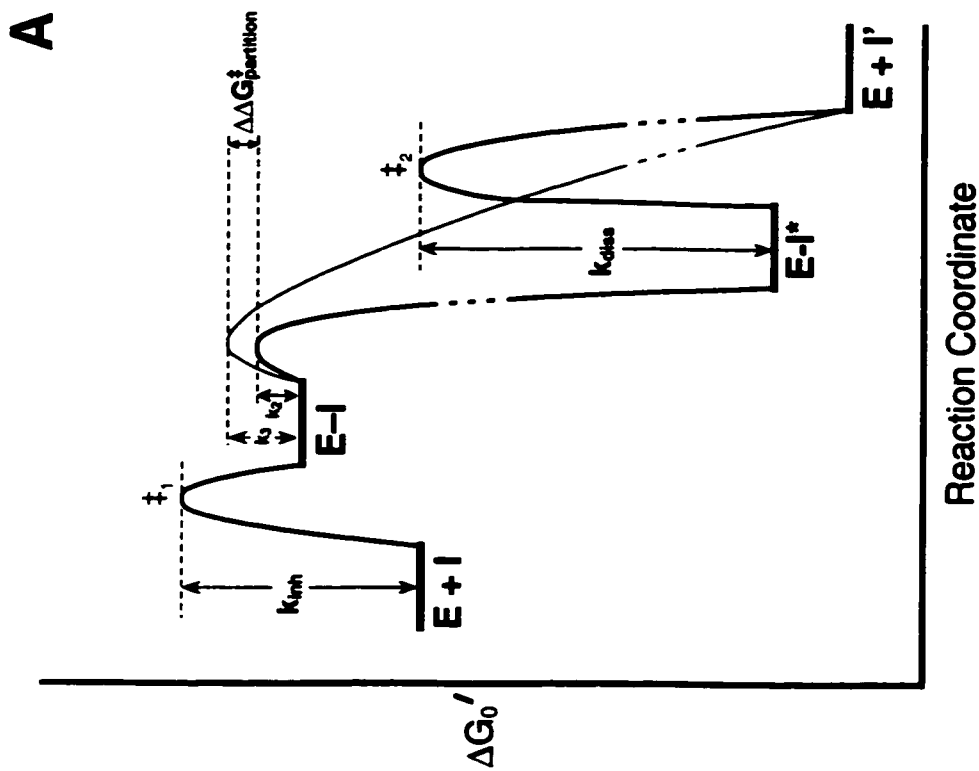
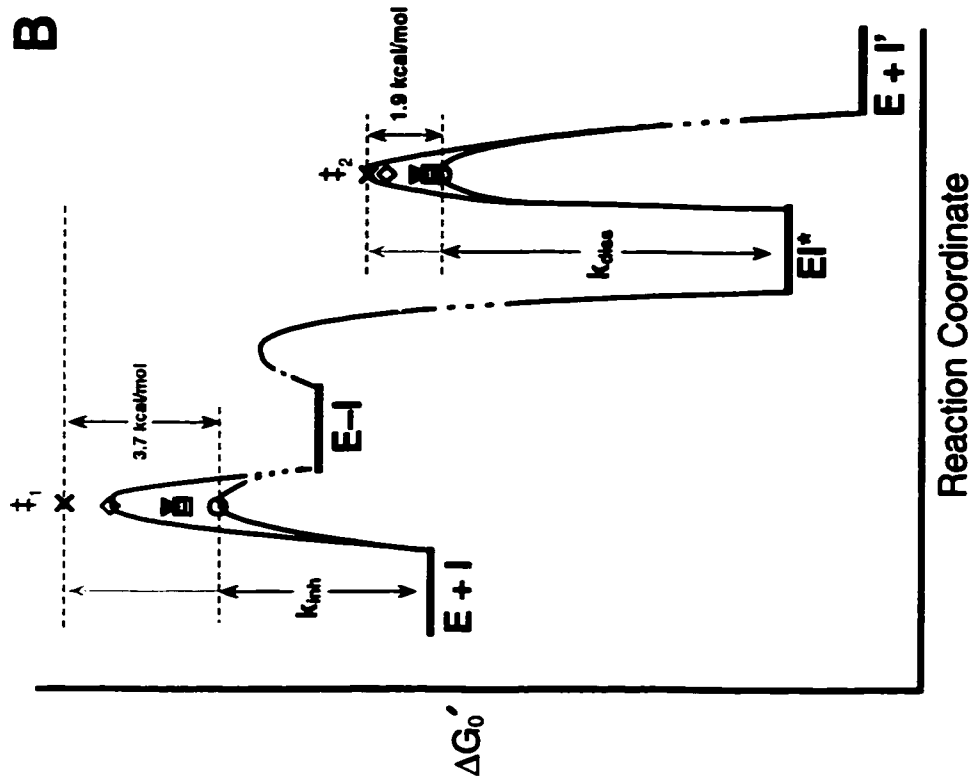
## SCHEME A-1



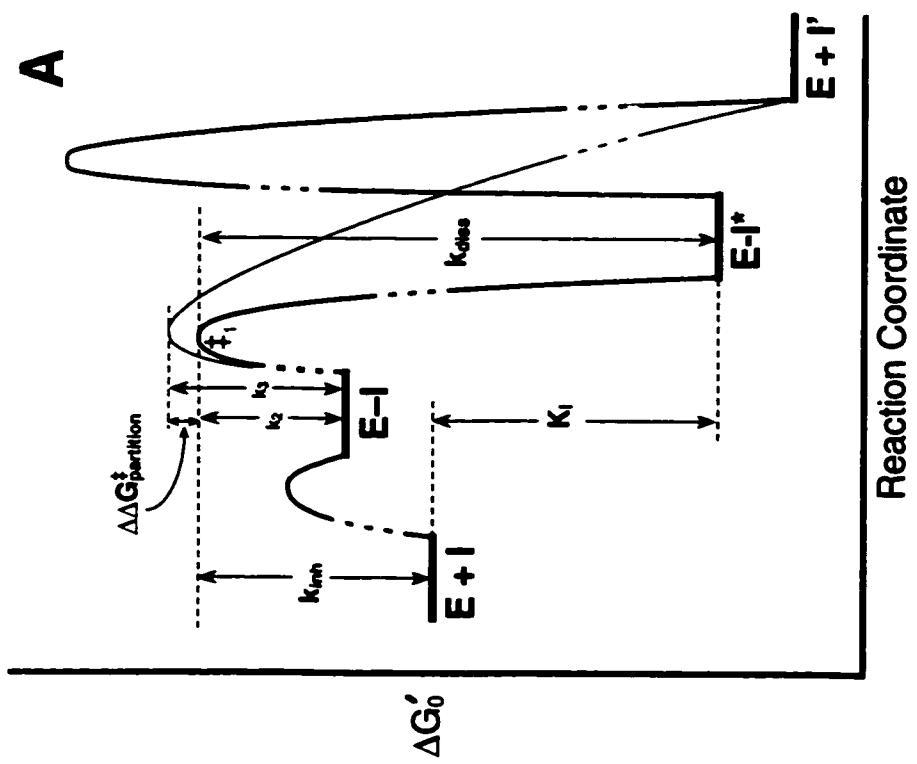
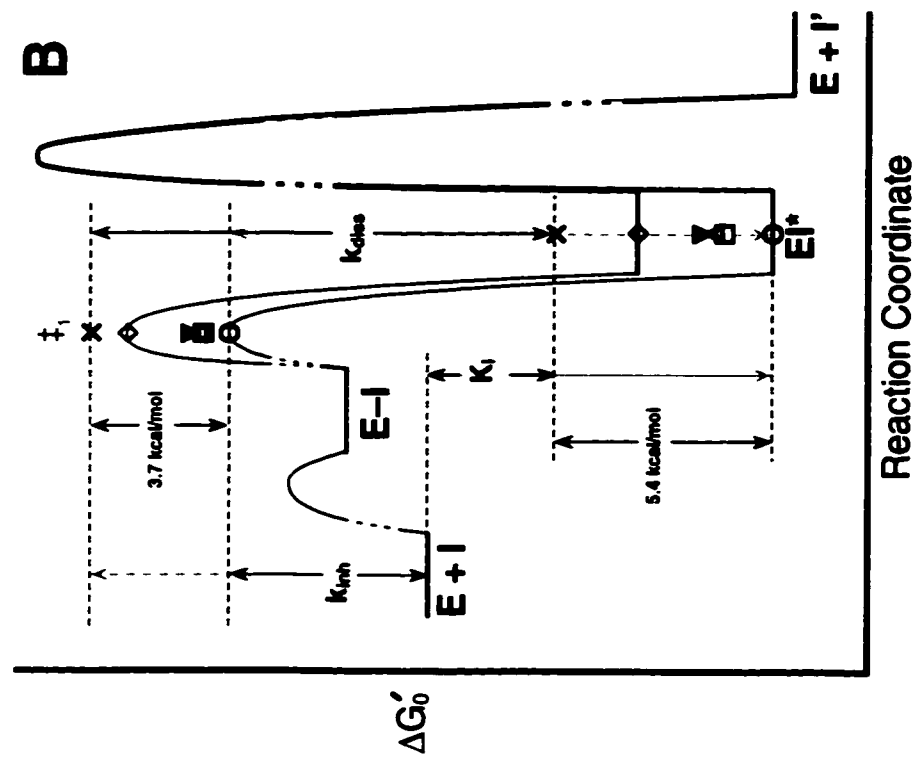
**Figure A-1. Linear Free Energy Relationship for SERP-1: Linear Free Energy Relationship between  $\log k_{inh}$  and  $\log K_i$  based on data from Table A-1. The solid line represents the best fit to the data for Plasmin, urokinase, tPA, thrombin and Xa, and has a slope of -0.55. The error bar on the data point for C1s indicates the higher position it would occupy if it were plotted based on the higher value of  $k_{app} = 1300 \text{ M}^{-1}\text{s}^{-1}$  reported by Lomas *et al.* (1993). Data for C1s were estimated based on an assumed SI of 2, to give  $k_{inh} = 600 \text{ M}^{-1}\text{s}^{-1}$ , and  $K_i = 100 \text{ nM}$ ; the dashed arrow indicates how the location of the data point for C1s would change for SI values from 1 (bottom right) to 10 (upper left), which span the likely range for this enzyme (see text).**



**Figure A-2.** Free energy profiles for the inhibition of serine proteases by SERP-1 based on the mechanism in Scheme A-1A. **A.** Free energy profile for the reaction of a protease with SERP-1 for the case where the predominant pathway for the breakdown of the final inhibited complex at concentrations below  $K_i$  is through the release of cleaved serpin (i.e.  $k_5 \gg k_{-4}$ ; see Scheme A-1). Under these assumptions, variations in  $k_{diss}$  reflect differences in the activation barrier for the deacylation of acyl-enzyme 2 ( $E + I'$ ) via the pathway with rate constant  $k_5$ . The variations in  $K_i$  reflect the balance between these two independent effects, while  $K_i$  has no intrinsic thermodynamic significance. (see text). **B.** Experimental data for the reactions of SERP-1 with thrombin (○), tPA (□), uPA (△), plasmin (▼), Xa (◇) and C1s (⊗) mapped onto a free energy profile of the form shown in (A). For clarity, the  $k_3$  pathway, which is shown in (A), has been omitted from (B). Free energies were calculated from the data in Table A-1 using the relations  $\Delta\Delta G^\ddagger = -RT\ln[k_{inh.}(1)/k_{inh.}(2)]$  and  $\Delta\Delta G = -RT\ln[k_{diss}(1)/k_{diss}(2)]$ .



**Figure A-3.** Free energy profiles for the inhibition of serine proteases by SERP-1 based on the mechanism in Scheme A-1B. **A.** Free energy profile for the reaction of a protease with SERP-1 for the case where the predominant pathway for the breakdown of the final inhibited complex at concentrations below  $K_i$  is through the release of active serpin (i.e.  $k_5 \ll k_{-4}$ , and  $k_3 \ll k_2$ ; see Scheme A-1). Under these assumptions, variations in  $k_{inh.}$  represent variations in the stability of the transition state for the  $k_2$  step, and variations in  $K_i$  reflect variations in the stability of the final inhibited complex, both with respect to the  $E + I$  ground state (see text). **B.** Experimental data for the reactions of SERP-1 with thrombin (O), tPA (□), uPA (Δ), plasmin (▼), Xa (◇) and C1s (⊠) mapped onto a free energy profile of the form shown in (A). For clarity, the  $k_3$  pathway, which is shown in (A), has been omitted from (B). Free energies were calculated from the data in Table A-1 using the relations  $\Delta\Delta G^\ddagger = -RT\ln[k_{inh.}(1)/k_{inh.}(2)]$  and  $\Delta\Delta G = -RT\ln[K_i(1)/K_i(2)]$ .



**REFERENCES:**

Al-Shawi, M., and Nakamoto, R. K. (1997). Mechanism of energy coupling in the  $F_0F_1$ -ATP synthase: The uncoupling mutation,  $\gamma$ M23K, disrupts the use of binding energy to drive catalysis. *Biochemistry* **36**: 12854-12960.

Eaton, W. A., Henry, E. R., and Hofrichter, J. (1991). Application of linear free energy relations to protein conformational changes: the quaternary structural changes of hemoglobin. *Proc. Natl. Acad. Sci. USA* **88**: 4472-4475.

Exner, O. (1988). Correlation analysis of chemical data (New York: Plenum Press).

Fersht, A. R., and Wells, T. N. C. (1991). *Prot. Eng.* **4**: 229-231.

Griffith, M. J., and Lundblad, R. L. (1981). *Biochemistry* **20**: 105-110.

Guengerich, F. P., Yun, C.-H., and Macdonald, T. L. (1996). Evidence for a 1-electron oxidation mechanism in *N*-dealkylation on *N,N*-dialkylanilines by cytochrome P450 2B1. *J. Biol. Chem.* **271**: 27321-27329.

Higgins, L., Bennett, G. A., Shimoji, M., and Jones, J. P. (1998). Evaluation of cytochrome P450 mechanism and kinetics using kinetic deuterium isotope effects. *Biochemistry* **37**: 7039-7046.



Hood, D. B., Huntington, J. A., and Gettins, P. G. (1994). Alpha 1-proteinase inhibitor variant T345R. Influence of P14 residue on substrate and inhibitory pathways. *Biochemistry* **33**: 8538-47.

Longstaff, C., and Gaffney, P. J. (1991). Serpin-serine protease binding kinetics: alpha 2-antiplasmin as a model inhibitor. *Biochemistry* **30**: 979-86.

Lucas, A., Liu, L., Macen, J. L., Nash, P. D., Dai, E., Stewart, M., Yan, W., Graham, K., Etches, W., Boshkov, L., Nation, P. N., Humen, D., Hobman, M., and McFadden, G. (1996). Virus-encoded serine proteinase inhibitor SERP-1 inhibits atherosclerotic plaque development following balloon angioplasty. *Circulation* **94**: 2890-2900.

Macen, J. L., Upton, C., Nation, N., and McFadden, G. (1993). SERP1, a serine proteinase inhibitor encoded by myxoma virus, is a secreted glycoprotein that interferes with inflammation. *Virology* **195**: 348-63.

Maksymowych, W. P., Nation, N., Nash, P. D., Macen, J., Lucas, A., McFadden, G., and Russell, A. S. (1996). Amelioration of antigen-induced arthritis in rabbits treated with a secreted viral serine proteinase inhibitor. *J. Rheumatol.* **23**: 878-882.

Matouschek, A., and Fersht, A. (1993). Application of physical organic chemistry to engineered mutants of proteins: Hammond postulate behaviour in the transition state of protein folding. *Proc. Natl. Acad. Sci. USA* **90**: 7814-7818.

Nash, P., Barrett, J., Cao, J., Hota-Mitchell, S., Lalani, A., Everett, H., Xu, X., Robichaud, J., Hnatiuk, S., Ainslie, C., Seet, B., and McFadden, G. (1999). Immunomodulation by viruses: the myxoma virus story. *Immunological Reviews*: (in press).

Nash, P., Whitty, A., Handwerker, J., Macen, J., and McFadden, G. (1998). Inhibitory specificity of the anti-inflammatory myxoma virus serpin, Serp-1. *J. Biol. Chem.* **273**: 20982-20991.

Plotnick, M. I., Mayne, L., Schechter, N. M., and Rubin, H. (1996). Distortion of the active site of chymotrypsin complexed with a serpin. *Biochemistry* **35**: 7586-90.

Rubin, H. (1996). Serine protease inhibitors (SERPINS): where mechanism meets medicine. *Nature Medicine* **2**: 632-633.

Schweins, T., Geyer, M., Kalbitzer, H. R., Wittinghofer, A., and Warshel, A. (1996). Linear free energy relationships in the intrinsic and GTPase activating protein-stimulated guanosine 5'-triphosphate hydrolysis of p21<sup>ras</sup>. *Biochemistry* **35**: 14225-14231.

Schweins, T., Geyer, M., Scheffzek, K., Warshel, A., Kalbitzer, H. R., and Wittinghofer, A. (1995). Substrate-assisted catalysis as a mechanism for GTP hydrolysis of p21<sup>ras</sup> and other GTP-binding proteins. *Nat. Struct. Biol.* **2**: 36-44.

Schweins, T., and Warshel, A. (1996). Mechanistic analysis of the observed linear free energy relationships in p21<sup>ras</sup> and related systems. *Biochemistry* **35**: 14232-14243.

Shieh, B., Potempa, J., and Travis, J. (1989). *J. Biol. Chem.* **264**: 13420-13424.

Stone, S. R., and Hermans, J. M. (1995). Inhibitory mechanism of serpins. Interaction of thrombin with antithrombin and protease nexin 1. *Biochemistry* **34**: 5164-72.

Stratikos, E., and Gettins, P. G. (1997). Major proteinase movement upon stable serpin-proteinase complex formation. *Proc. Natl. Acad. Sci. USA* **94**: 453-458.

Vaz, A. D. N., and Coon, M. J. (1994). On the mechanism of action of cytochrome P450: Evaluation of hydrogen abstraction in oxygen-dependent alcohol oxidation. *Biochemistry* **33**: 6442-6449.

Whisstock, J., Skinner, R., and Lesk, A. M. (1998). An atlas of serpin conformations. *Trends Biochem. Sci.* **23**: 63-67.

Wright, H. T., and Scarsdale, J. N. (1994). Structural basis for serpin inhibitor activity. *Proteins: Structure, Function and Genetics* **22**: 210-225.