UNIVERSITY OF ALBERTA

MECHANISMS OF T CELL-MEDIATED CYTOTOXICITY

by

ALISON J. DARMON

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Without your constant support and encouragement,
none of this would have been possible.

ABSTRACT

Cytotoxic T lymphocytes (CTLs) are the body's primary defense against viral-infected and tumourigenic cells, and are involved in autoimmune disease and transplant rejection. CTLs possess two main effector mechanisms through which they induce target cell death. In granule-based killing, lytic granules are vectorally exocytosed towards a target cell bearing a foreign antigen. Perforin then forms a pore in the target cell membrane which allows entry of the granzymes, a family of serine proteases, into the target where they induce cell death. In contrast, Fas-based cytotoxicity involves ligation of a cell surface receptor (Fas) on the target cell by its ligand on the CTL. Fas then transduces an apoptotic signal to the target cell through a number of associated proteins.

Granzyme B has been shown to play a key role in the induction of target cell DNA fragmentation, although at the start of this work, its mechanism of action was undefined. Here, an intracellular substrate for granzyme B is identified as the precursor of a cysteine protease related to Interleukin-1β Converting Enzyme (ICE). This protease, called CPP32, is shown to be involved in initiating the nuclear events of apoptosis and failure to activate this protease (and related proteases) can account for the inefficiency of DNA fragmentation induced by granzyme B-deficient CTLs.

The final part of the work presented here examines the role of CPP32, and other proteases related to ICE, in Fas-mediated killing. In contrast to granule-mediated cytotoxicity, CPP32 does not appear to play a central role in Fas-induced apoptosis in all cell types. Rather, there exists a central ICE-like protease which is responsible for initiating both the nuclear and cytoplasmic events of apoptosis.

These studies were the first to demonstrate that a CTL initiates target cell death by utilizing a cell's endogenous death pathway, and have vastly expanded our understanding of CTL-mediated cytotoxicity.

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abbreviations	
2,6-BTBz	2,6-bis-(triflouromethyl)benzoyl
2,6-DMBz	2,6-dimethylbenzoyl
2,4,6-TMB	2,4,6-trimethylbenzoyl
APC	antigen presenting cell
ARAM	antigen recognition activation motif
aSMase	acidic sphingomyelinase
ATP	adenosine triphosphate
BCA	bicinchoninic acid
CAP	cytotoxicity-dependent APO-1-associated protein
cDNA	complementary DNA
CHAPS	3-[(3-cholamidopropyl)dimethylammonio]-1-propane-sulfonate
ConA	concanavalin A
CrmA	cytokine response modifier A
CTL	Cytotoxic T lymphocyte
DAG	diacylglycerol
DEAE	diethylaminoethyl
DISC	death-inducing signaling complex
DMSO	dimethyl sulfoxide
DNA-PK$_{(CS)}$	DNA-dependent protein kinase (catalytic subunit)
dNTPs	deoxynucleotide triphosphates (dATP, dCTP, dGTP, dTTP)
DPPI	dipeptidyl peptidase I
DSB	double strand break
DTNB	5,5'-dithio-bis (2-nitrobenzoic acid)
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
EGTA	ethylene glycol-bis(β-aminoethyl) ether
EM	electron microscopy
ER	endoplasmic reticulum
FAP	Fas-associated phosphatase
FasL	Fas ligand
FAST	Fas-activated serine/threonine kinase
FPLC	fast protein liquid chromatography
GB KO	granzyme B$^{-/-}$ cells

GB WT	granzyme $B^{+/+}$ cells
gld	generalized lymphoproliferative disorder
HCP	hematopoietic cell protein tyrosine phosphatase
HEL	hen egg-white lysozyme
HEPES	N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]
ICAM	intercellular adhesion molecule
ICE	Interleukin-1β converting enzyme
Ig(E/G/M)	immunoglobulin (E/G/M)
IL-1β	Interleukin-1β
IP_3	inositol 1,4,5-trisphosphate
Ku70	70-kDa subunit of Ku protein
Ku80	80-kDa subunit of Ku protein
L1210-Fas	L1210 cells stably transfected with murine Fas cDNA
LAK	lymphokine-activated killer
LFA	lymphocyte function-associated
lpr	lymphoproliferation
MES	2-(N-morpholino)ethanesulfonic acid
MHC	Major Histocompatibility Complex
mICE	murine Interleukin-1β converting enzyme
NAD	nicotinamide adenine dinucleotide
NGF	nerve growth factor
NK	natural killer
nSMase	neutral sphingomyelinase
NuMA	nuclear-mitotic apparatus
PARP	poly(ADP-ribose) polymerase
PBS	phosphate-buffered saline
PC-PLC	phosphatidylcholine-specific phospholipase C
PCR	polymerase chain reaction
PI-PLCγ1	phosphatidylinositol-specific phospholipase C γ1 isoform
PIP_2	phosphatidylinositol 4,5-bisphosphate
PIPES	piperazine-N,N'-bis[2-ethanesulfonic acid]
PKC	protein kinase C
PLA_2	phospholipase A_2
PMA	phorbol myristate acetate
pro-CPP32	CPP32 precursor
pro-mICE	murine ICE precursor

PTK	protein tyrosine kinase
RBL	rat basophilic leukemia (cell line)
ROS	reactive oxygen species
S183A	serine-alanine mutant of murine granzyme B
SCID	severe combined immunodeficiency
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SH2	src homology 2
SR	serine/arginine rich
SREBP	sterol regulatory element binding protein
TAP	transporter in antigen processing
TBS	tris-buffered saline
TCR	T Cell receptor
Th	T Helper
TNF(α)	tumour necrosis factor (α)
TNFRI	TNF receptor type I
TNFRI	tumour necrosis factor receptor type I
U1-70kDa	70-kDa protein component of small nuclear ribonucleoprotein
UV	ultraviolet
VCAM	vascular cell adhesion molecule

peptides

Ac-DEVD-CHO	acetyl-Asp-Glu-Val-Asp-aldehyde
Ac-YVAD-CHO	acetyl-Tyr-Val-Ala-Asp-aldehyde
Ac-YVAD-cmk	acetyl-Tyr-Val-Ala-Asp-chloromethylketone
BAADT	*tert*-butyloxycarbonyl-Ala-Ala-Asp-thiobenzyl ester
BLT	Nα-carbobenzoxy-L-Lys-thiobenzyl ester
Succ-AAPF-Bz	succinyl-Ala-Ala-Pro-Phe-thiobenzyl ester
YVAD-AFC	benzyloxycarbonyl-Tyr-Val-Ala-Asp-7-amino-4-triflouromethyl coumarin

amino acids

three letter code	amino acid name	single letter code
Ala	alanine	A
Arg	arginine	R
Asn	asparagine	N
Asp	aspartic acid	D
Cys	cysteine	C
Gln	glutamine	Q
Glu	glutamic acid	E
Gly	glycine	G
His	histidine	H
Ile	isoleucine	I
Leu	leucine	L
Lys	lysine	K
Met	methionine	M
Phe	phenylalanine	F
Pro	proline	P
Ser	serine	S
Thr	threonine	T
Trp	tryptophan	W
Tyr	tyrosine	Y
Val	valine	V

Greek letters

α	alpha
β	beta
γ	gamma
δ	delta
ε	epsilon
ζ	zeta
η	eta
κ	kappa

CHAPTER I - INTRODUCTION

CHAPTER I - INTRODUCTION

An organism's immune system is essential for protection against disease-causing microorganisms, viruses, parasites, and fungi, as well as against self cells which have become tumourigenic (for a basic review see Abbas *et al.*, 1994). This vital function has been spectacularly underlined by the advent of AIDS, in which the host's immune system is disabled rendering an individual susceptible to a wide-ranging number of opportunistic infections and cancers.

Immunity itself can be divided into two types: natural and acquired. Natural immunity involves defense mechanisms which are consistently present within the body prior to exposure to foreign matter, such as physical barriers, the phagocytic cells, and various molecules found in the blood, such as the complement proteins. Acquired immunity is induced in response to a specific stimulus, such as an infection. While natural immunity is non-discriminatory, acquired immunity is exquisitely specific.

Acquired immunity is mediated primarily by the lymphocytes and can itself be further subdivided into two categories. Humoral immunity is mediated by antibodies produced by the B lymphocytes (so-called because they mature in the bone marrow) and is most effective against extracellular bacterial infections. The antibodies generally recognize structural determinants in their cognate antigens and can therefore recognize antigen on the surface of cells or in solution. In contrast, cellular immunity is mediated primarily by the cytotoxic T lymphocytes (CTLs, T because they mature in the thymus) and the natural killer (NK) cells, which lyse intracellularly infected cells and tumourigenic cells. CTLs are also responsible for autoimmune disease and transplant rejection. Another subset of T cells, the T helper (Th) lymphocytes, produce cytokines which regulate the immune response. Both types of T cells recognize linear determinants in their cognate antigens, through the T cell receptor (TCR), and are only able to recognize antigen presented on the surface of another cell (the antigen presenting cell or APC) along with the major histocompatibility complex (MHC) proteins.

I. T LYMPHOCYTES

Although exceptions do exist, the two T cell subsets, CTLs and Th cells, can be generally distinguished at a number of levels. Firstly, function - CTLs are

effectors of a cytolytic response while Th cells produce cytokines to regulate the functions of other immune system cells, including the CTLs.

These cells can also be distinguished on the basis of cell surface markers. Th cells express the CD4 protein while CTLs generally possess CD8, although $CD4^+$ CTLs have been found. The CD4 and CD8 proteins are both involved in antigen recognition and are therefore called coreceptors.

As mentioned above, the TCR recognizes peptides derived from intracellular proteins presented on the surface of the APC in the context of the MHC proteins. The MHC proteins are highly polymorphic and contain both constant and variant regions. Two classes of MHC proteins have been identified: Class I, a 44-kDa transmembrane protein which is found on nearly every nucleated vertebrate cell in association with β_2-microglobulin, although expression levels vary; and Class II, consisting of a 33-kDa α and a 28-kDa β subunit, which is found on only a limited number of cells including B cells, macrophages, monocytes, dendritic cells and the endothelium. Each T cell recognizes only one class of MHC proteins and this specificity is determined by the coreceptors - the CD4 protein recognizes constant regions of the MHC Class II molecule (and Th cells are therefore MHC Class II-restricted) while the CD8 protein recognizes invariant regions of the MHC Class I molecule. Therefore, T cell specificity for the MHC proteins is not determined by the TCR itself, but rather by the coreceptor molecules. Interestingly, T cells can only recognize peptide antigens presented in the context of self MHC molecules so are therefore self-restricted as well as class-restricted.

A. T CELL DEVELOPMENT

Both T cell subsets originate in the bone marrow (or fetal liver) from hematopoietic stem cells (reviewed by Shortman and Wu, 1996; Weissman, 1994). These T cell precursors lack functional TCRs and are therefore unresponsive to antigen. Once released from the bone marrow, the precursors migrate to the thymic cortex to undergo further maturation. Selection of the final T cell repertoire begins as the thymocytes rearrange the genes encoding the TCR subunits from the nonfunctional germline configuration. Following rearrangement, via a mechanism termed V(D)J recombination, the TCR contains randomly rearranged and junctionally modified TCR component chains. Most T cells utilize a TCR consisting of a heterodimer of α and β subunits, although TCRs utilizing a $\gamma\delta$ heterodimer also exist. The function of these T cells is unknown although a role in antimicrobial immunity has been established (Kaufmann, 1996). Prior to TCR expression, other cell surface

proteins which act in conjunction with the TCR are expressed. These include the CD3 proteins (γ, δ, and ε chains) as well as the ζ and/or η chains. As described below, the expression of all of these proteins is required for expression of a functional TCR. In addition, the T cell coreceptors CD4 and CD8 are expressed. As a result of this process, the $TCR^{-}CD4^{-}CD8^{-}$ (double negative) cells that entered the thymic cortex are now $TCR\alpha\beta^{low}CD4^{+}CD8^{+}$ (double positive).

Because of the random nature of TCR generation, these cells are capable of recognizing both self and foreign MHC presenting both self and foreign peptides. These cells must therefore undergo processes of both positive and negative selection to select T cells which are self MHC-restricted and recognize only foreign peptides. Positive selection is the process whereby T cells expressing a TCR which recognizes self MHC are stimulated to survive while T cells expressing no TCR, or a TCR which does not recognize self MHC are eliminated (reviewed by Jameson *et al.*, 1995; Robey and Fowlkes, 1994; von Boehmer, 1994). This selected set of T cells must then undergo negative selection where cells expressing TCR recognizing self antigen-self MHC complexes are eliminated (clonal deletion) or inactivated (clonal anergy). Other cells (which recognize foreign antigen-self MHC) are allowed to survive (reviewed by Nossal, 1994). Following these selection processes, the TCR repertoire has been determined.

During the selection process the T cells also become committed to one of the T cell lineages, with the end result that T cells which are either $TCR\alpha\beta^{+}CD4^{+}CD8^{-}$ or $TCR\alpha\beta^{+}CD4^{-}CD8^{+}$ are released into the peripheral blood.

B. ANTIGEN PRESENTATION

As outlined briefly above, antigenic peptides are presented to the T cell through association with an MHC molecule on the surface of an APC. The class of MHC that presents the peptide and the mechanism by which a peptide associates with the MHC molecules is determined by the origin of the peptide - MHC Class I molecules present peptides derived from endogenously synthesized proteins while MHC Class II molecules present extracellula y synthesized sequences (reviewed by Germain, 1994; Germain and Margulies, 1993).

1. Association With MHC Class I Molecules

For presentation in association with the MHC Class I, endogenously synthesized proteins must be proteolytically processed in the cell's cytoplasm. This is part of the normal cellular protein turnover and is carried out by the proteasome, a

large molecular weight (650-kDa) complex of up to 24 subunits. The peptides generated by protein degradation in the cell's cytoplasm are then transported in an ATP-dependent manner into the endoplasmic reticulum (ER) by the protein products of the transporter in antigen processing *TAP1* and *TAP2* genes. In the ER, the peptides associate with the newly-synthesized MHC class I proteins and the complexes are then transported through the Golgi to the cell surface for presentation. Any cytoplasmically synthesized protein is therefore presented on a cell's surface for immune surveillance. If a cell becomes intracellularly infected or transformed, non-self peptides are presented on the cell's surface resulting in recognition of the cell as foreign. In this manner the immune system constantly monitors the body's cells. Since MHC Class I molecules are expressed on the surface of nearly every cell in the body, this enables the CTLs to recognize and lyse any cell which becomes infected or tumourigenic, regardless of cell type.

2. Association with MHC Class II Molecules

The mechanism of association of peptides with MHC class II is quite different because these peptides are derived from extracellularly-synthesized proteins. These peptides are the breakdown products of matter which has been taken up into the APC, probably by receptor-mediated endocytosis or phagocytosis, and has been degraded in the APC's lysosomes (reviewed by Wolf and Ploegh, 1995). At some point, an endocytic vesicle containing MHC class II α and β subunits fuses with the endosome containing the breakdown products, allowing the peptides and MHC molecules to come together. This newly-formed vesicle then travels to the plasma membrane where it fuses, resulting in expression of MHC Class II-peptide complexes on the cell surface.

The peptides which associate with the MHC proteins in both of the above mechanisms are determined by the sequence of the MHC molecule itself (reviewed by Barber and Parham, 1993). Peptides bind a groove in the MHC proteins which requires the presence of critical anchor residues within the target peptide. Most of the polymorphic residues of the MHC proteins are found within this peptide-binding region so that MHC molecules from different individuals bind different peptides derived from the same protein. In addition, not all portions of a protein are presented equally to T cells. Cibotti *et al.* (1992) have demonstrated using transgenic mice expressing hen egg-white lysozyme (HEL) that these mice are tolerant to the immunodominant peptides of HEL, but not to subdominant peptides, and tolerance

levels depend upon the serum levels of HEL. Thus, when proteins are degraded for presentation, not all the peptides generated are presented to T cells.

These findings also have relevance to studies of autoimmunity. When the TCR repertoire is being shaped in the thymus, the developing T cells are generally only exposed to immunodominant peptides presented to them by the thymic APCs. It has now been shown that some T cells escape tolerance induction (that is, are not deleted because they recognize self antigen) if they recognize a subdominant or cryptic determinant of a self protein (Gammon and Sercarz, 1989; Lipham *et al.*, 1991; Cibotti *et al.*, 1992). Generally this has no consequence to an individual since the target of the TCR is not efficiently presented by the body's APCs. However, under certain circumstances, the cryptic determinant may become more easily presented due to protein modification events. In this situation, T cells which escaped deletion in the thymus may become activated and initiate an immune response. As the immune response develops, it can spread from the initial cryptic determinant to any determinant of the self protein (Mamula, 1993; Mamula *et al.*, 1994) thereby initiating autoimmune disease. This point will be returned to later in the discussion of ICE/Ced-3 proteases.

C. ANTIGEN RECOGNITION AND T CELL ACTIVATION

1. Structure of the TCR/CD3 Complex

As mentioned briefly above, the TCR consists of a covalently linked heterodimer of an acidic glycoprotein α chain and a basic or uncharged glycoprotein β chain (reviewed by Bentley and Mariuzza, 1996). At the cell surface, the TCR is noncovalently associated with the CD3 proteins (γ, δ, and ε chains) as well as a ζζ homodimer or ζη heterodimer (Figure I-1). A typical TCR/CD3 complex has the stoichiometry TCRαβ:CD3γδε$_2$:ζ$_2$. Interestingly, the TCR complex requires all of its members for expression - during T cell development, the CD3 γ, δ, and ε chains and the ζ and η genes are expressed prior to the TCR α and β genes. Once the TCR α and β genes are rearranged and expressed, the α and β proteins associate with the CD3 proteins in the ER. TCR assembly proceeds via association of the α subunit with CD3δε proteins and the β subunit with CD3γε proteins to form αδε and βγε trimers (Kearse *et al.*, 1995). These trimers then associate to form αδε–βγε complexes, at which point the α–β disulfide bond is formed. In this process, assembly of the αδε trimer is rate-limiting. Finally, the ζ chains associate with the αβγδε$_2$ complexes and the completed TCR/CD3 structure is transported through the Golgi where it is further modified. Any incomplete complexes are not transported to

the cell membrane. Although the α, β, γ, δ, and ε chains are all synthesized in excess of the levels of cell surface expression, the rate-limiting step in expression of the TCR/CD3 complex on the cell surface is the synthesis and association of the ζ chains.

The cytoplasmic domains of the α and β chains are quite small and are not believed to transduce signals following antigen recognition. Instead, signal transduction is mediated by the CD3 proteins and the ζ chains. This idea is supported by the fact that anti-CD3 antibodies can stimulate a functional response from T cells which is identical to the antigen-induced response. Additionally, chimeric receptors containing the cytoplasmic domains of the CD3 proteins can induce a response which is identical to that induced by TCR cross-linking when transfected into T cell tumour lines. However, none of the CD3 or ζ proteins contain any intrinsic signaling capacity. Rather, the cytoplasmic domains of the γ, δ, ε, and ζ chains contain at least one copy of a conserved antigen recognition activation motif (ARAM) having the consensus sequence (^{D}/E)XXYXXL(X)$_{6-8}$YXXL (reviewed by Weiss and Littman, 1994; Howe and Weiss, 1995). This region is sufficient to couple these receptors to protein tyrosine kinases (PTKs) in the cell's cytoplasm which mediate signal transduction following antigen recognition.

2. T Cell Accessory Molecules

Although T cells recognize antigen through their TCR complex, other cell surface molecules are also involved in T cell activation, the so-called accessory molecules (summarized in Table I-1). These molecules include the T cell coreceptors CD4 and CD8, which also recognize the MHC molecules, as well as other cell surface proteins which recognize ligands on the APC surface. These accessory molecules not only strengthen the interaction between T cell and APC, but may also play a role in signal transduction (reviewed by Janeway and Bottomly, 1994). For example, CD4 and CD8 are associated with the PTK Lck in their cytoplasmic domains and Lck is rapidly activated following T cell antigen recognition. Interestingly, this association is not mediated through an ARAM motif, as are the other associations of PTKs with the CD3 proteins, but rather through a noncovalent cysteine-mediated interaction (Turner *et al.*, 1990).

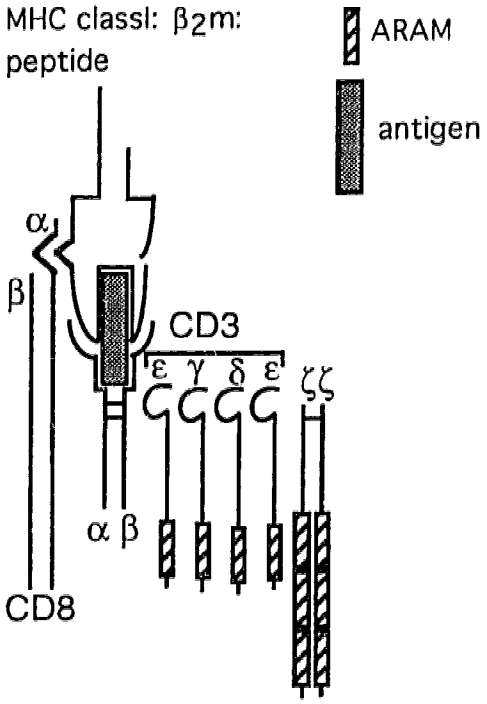


FIGURE I-1: Structure of the TCR/CD3 Complex.
The α and β subunits of the TCR recognize antigen in the context of the MHC class I protein (for $CD8^+$ T cells), which is found in association with β2-micoglobulin (β2m). The TCR is found in association with the CD3 proteins (γ, δ, ε, and ζ chains), which contain the ARAM motifs involved in signal transduction. CD8 is also involved in antigen recognition and recognizes invariant regions of the MHC protein.

3. Signaling During Activation

The response of a T cell to antigen recognition depends on the T cell type and its stage of maturity. Naive T cells are quiescent (in the G_0 phase of the cell cycle) and appear as small cells with a thin rim of cytoplasm containing a few mitochondria and ribosomes. Upon antigen recognition, the T cell re-enters the cell cycle and begins to proliferate (clonal expansion) and grow so that the activated T cell is larger than the unstimulated cell. Subsequent to or during the proliferation stage the T cell also proceeds from the cognitive phase (antigen recognition) to the effector stage. For Th cells this means the T cell begins to secrete cytokines. For CTLs, the differentiation results in the acquisition of the cytolytic machinery and the activated

CTL contains electron dense cytoplasmic granules which contain this machinery. Further interaction with the antigen results in the exocytosis of these granule contents (see below). However, despite these final differences in differentiation, the initial sequence of events following TCR cross-linking is the same regardless of cell type (reviewed by Cantrell, 1996; Zenner *et al.*, 1995; Weiss and Littman, 1994).

The earliest biochemical change detectable following TCR cross-linking is tyrosine phosphorylation of cellular proteins. These phosphorylations are carried out by the PTKs associated with the ARAM motifs of the γ, δ, ε, and ζ chains as well as Lck associated with CD4/CD8. Two families of PTKs have been implicated in TCR/CD3 signaling - those related to Src and members of the Syk/ZAP-70 family (Cooke *et al.*, 1991; Howe and Weiss, 1995; Rudd, 1990; Weiss and Littman, 1994). These classes of PTKs differ in ways which are likely to be function-related. The Src kinases (Lck and Fyn) contain a unique amino terminal domain containing a myristylated glycine, thought to be involved in membrane localization. Syk/ZAP-70 kinases are not myristylated and therefore likely to be cytoplasmically localized. The association between these kinases and the ARAM motifs is mediated by conserved domains within the PTKs called Src homology 2 (SH2) domains which recognize phosphorylated tyrosine residues (Gauen *et al.*, 1992). In the unactivated T cell the ζ chain is constitutively phosphorylated and a fraction of the cellular ZAP-70 is associated with it through its SH2 domain (van Oers *et al.*, 1994). Following TCR/CD3 activation, a Src kinase (Lck or Fyn) phosphorylates the tyrosine residues of the ARAM sequences in the γ, δ, ε, and ζ chains as well as ZAP-70 associated with the ζ chain. Additional ZAP-70 is then recruited to the membrane where it is docked by interacting with the phosphotyrosine residues in the ARAM sequences through its SH2 domain (Chan *et al.*, 1991; 1992; Irving *et al.*, 1993). ZAP-70 is activated, again by tyrosine phosphorylation, and goes on to transduce the signal further downstream.

Multiple proteins become phosphorylated following TCR cross-linking and while some are yet to be identified, it is known that the CD3 proteins and the ζ chains are themselves phosphorylated (Straus and Weiss, 1993) as are the PTKs Lck, Fyn and Syk/ZAP-70. Other substrates include the cell surface proteins CD5 and CD6, phosphatidylinositol 3-kinase, and the γ1 isoform of phosphatidylinositol-phospholipase C (PI-PLCγ1) (Secrist *et al.*, 1991). Significantly, tyrosine phosphorylation precedes activation of PI-PLCγ1 activity and inhibitors of tyrosine phosphorylation prevent both TCR-induced phosphorylation of PI-PLCγ1 and induction of phospholipase activity (Graber *et al.*, 1992; June *et al.*, 1990a, 1990b) placing the PTKs upstream of PI-PLCγ1.

The phosphorylation of PI-PLCγ1 leads to its activation, and it proceeds to hydrolyze the membrane phospholipid phosphatidylinositol 4,5-bisphosphate (PIP_2) to produce the second messengers inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG). IP_3 mediates the release of calcium from intracellular stores (the ER) resulting in a sustained increase in intracellular calcium levels. One of the roles of calcium is to act in conjunction with DAG to activate protein kinase C (PKC), a serine/threonine kinase which goes on to phosphorylate other proteins. The sustained increase in calcium also favours the formation of calcium-calmodulin complexes which activate several enzymes including the phosphatase calcineurin, a key regulatory molecule in T cell activation (Clipstone and Crabtree, 1992). Calcineurin dephosphorylates the cytoplasmic phosphoprotein NF-AT which then translocates to the nucleus where it activates transcription. The importance of calcium release and PKC activation to T cell activation can be demonstrated *in vitro* - the PKC activator phorbol myristate acetate (PMA) and the calcium ionophore ionomycin act synergistically to duplicate some of the differentiation and mitotic events seen during T cell activation.

In addition to the calcium-dependent activation of kinases, a calcium-independent pathway exists. Activation of the Src kinases leads to phosphorylation of the cytoplasmic protein Vav (Bustelo *et al.*, 1992). The tyrosine phosphorylated form of Vav has increased guanine nucleotide exchange activity for Ras *in vitro* (Gulbins *et al.*, 1993) thereby promoting the formation of Ras-GTP complexes. The Ras-GTP complex interacts with and activates Raf-1 (Siegel *et al.*, 1990), a serine/threonine kinase which regulates the activity of a kinase cascade including the Mek and MAP kinases (Crews and Erikson, 1993) resulting in activation of the Fos and Jun transcription factors (Jain *et al.*, 1993a, 1993b). In T cells, MAP kinase activation has also been associated with PKC activity (Nel *et al.*, 1990; Siegel *et al.*, 1990).

The final stage in signaling following T cell activation is the induction of transcription of function-related genes. In Th cells, these genes encode cytokines. In CTLs, transcription of the mRNA for the IL-2 receptor is induced. Following subsequent exposure to both IL-2 and antigen, the genes encoding the cytolytic proteins, including perforin and the granzymes (see below), are transcriptionally induced. These cytolytic proteins are stored in the cytoplasmic granules of the activated CTL. The sequence of events during T cell activation is summarized in Figure I-2.

TABLE I-1: T CELL ACCESSORY MOLECULES[1]

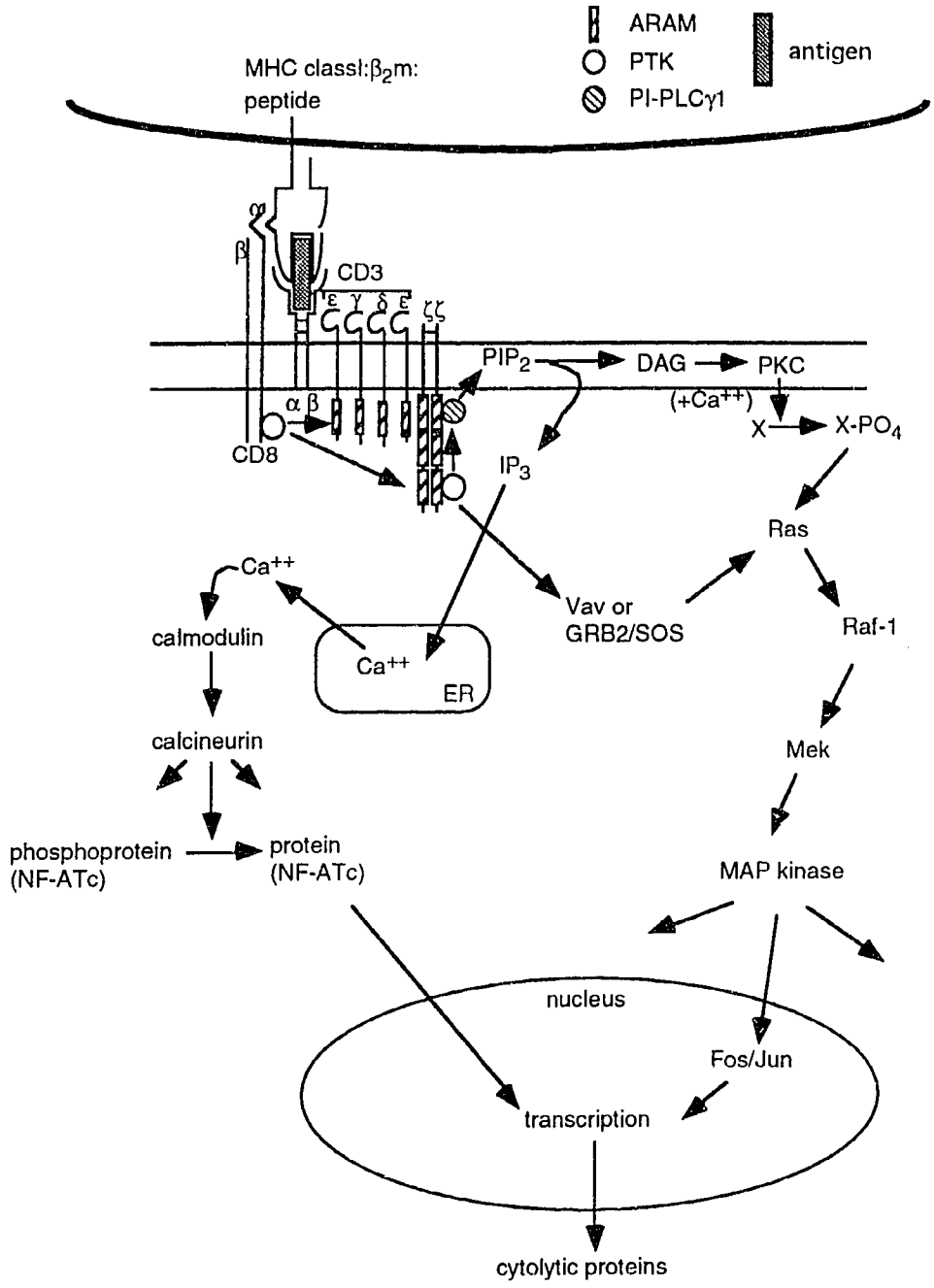
T cell protein	distribution	ligand on target	function in T cells	
			adhesion	signaling
CD4	TCRαβ+ class II MHC restricted T cells, macrophages	class II MHC	+	+
CD8	TCRαβ+ class I MHC restricted T cells	class I MHC	+	+
CD11aCD18 (LFA-1[2])	all bone marrow-derived cells	ICAM-1[3] ICAM-2	+	+
CD49CD29	leukocytes, other cells	matrix molecules, VCAM-1[4]	+	+
CD28	all CD4+ T cells; 50% CD8+ T cells	B7	?	+
CD2	>90% mature human T cells; >70% human thymocytes	LFA-3	+	+
CD45R	all immature and mature leukocytes	?	+	+
CD5	all T cells and thymocytes	?	+	+
Ly6	immature and mature T and B cells, various other cells	?	?	+
CD43	leukocytes, except circulating B cells	?	?	+
CD44	thymocytes, T cells, granulocytes, macrophages, erythrocytes, fibroblasts	collagen, fibronectin, hyaluronate	+	?

[1] Adapted from Abbas *et al.*, 1994
[2] LFA - lymphocyte function-associated
[3] ICAM - intercellular adhesion molecule
[4] VCAM - vascular cell adhesion molecule

FIGURE I-2: Signals Generated During T Cell Activation.

The $CD8^+$ T cell recognizes foreign antigen presented in the context of the MHC Class I molecule (which is associated with β2-microglobulin [β2m]) through its TCR/CD3 complex. CD8 recognizes invariant portions of the MHC molecule. Together, activation of these proteins results in activation of protein tyrosine kinases (PTKs), which are associated with the ARAM motifs found in the cytoplasmic domains of the CD3 proteins or associated with the α chain of CD8. The PTKs phosphorylate themselves and surrounding proteins, including PLCγ1. PLCγ1 hydrolyzes PIP_2 to produce the second messengers IP_3 and DAG. IP_3 induces the release of intracellular calcium from the ER. DAG and calcium together activate protein kinase C (PKC) which phosphorylates cellular proteins and leads to the activation of the Ras/Raf/MAP kinase cascade, culminating in induction of transcription. This cascade can also be activated by Vav, which is phosphorylated during T cell activation. Calcium also leads to the activation of calcineurin, a phosphatase, which can dephosphorylate the transcription factor NF-AT. The end result of these activations is the induction of transcription of genes required for the T cell to carry out its effector functions.

Adapted from Abbas *et al.*, 1994


ARAM
PTK
PI-PLCγ1
antigen
MHC classI:β2m:
peptide
α
β
CD3
ε γ δ ε ζζ
PIP2
DAG
PKC
(+Ca++)
X
X-PO4
α β
CD8
IP3
Ras
Ca++
calmodulin
Ca++
ER
Vav or
GRB2/SOS
Raf-1
calcineurin
Mek
phosphoprotein
(NF-ATc)
protein
(NF-ATc)
MAP kinase
nucleus
Fos/Jun
transcription
cytolytic proteins

II MECHANISMS OF CYTOTOXICITY

Activation of $CD8^+$ T cells following antigen recognition results in the acquisition of cytolytic machinery by the CTL. Two major mechanisms can account for the ability of CTLs to lyse target cells: granule-mediated and Fas-mediated cytotoxicity (reviewed in Kägi *et al.*, 1996; Atkinson and Bleackley, 1995; Berke, 1995; Smyth, 1995). Granule-mediated cytotoxicity involves the proteins found in a CTL's lytic granules, while Fas-mediated cytotoxicity involves the binding of a cell surface receptor on the target cell by a complementary cell surface protein on the CTL. The use of knock-out mice have confirmed that these two pathways can account for the majority of CTL-mediated cytotoxicity (Kägi *et al.*, 1994a, 1994b; Lowin *et al.*, 1994).

A. GRANULE EXOCYTOSIS MODEL OF KILLING

Although the mechanisms used to recognize target cells clearly differ between CTLs and NK cells, abundant evidence indicates that the "lethal hit" inflicted by these cells on their targets involves the contents of their electron dense granules. The granule exocytosis model of cytotoxicity states that, following conjugate formation with a target cell bearing a foreign antigen, the lytic granule is vectorally secreted in a calcium-dependent manner into the intercellular space between target cell and effector cell. The granule contents then cause target cell death by inflicting a "lethal hit". Often the lethal hit involves the formation of membrane lesions on the target cell which are visible by electron microscopy (Dourmashkin *et al.*, 1980; Dennert and Podack, 1983). The granule exocytosis model of killing is summarized in Figure I-3.

1. Nature of the Lytic Granule and Degranulation

The lytic granule is a "secretory lysosome" having characteristics of both regulated secretory granules and lysosomes (reviewed by Griffiths, 1995). In most cells, lysosomal and secretory granule components are separated in the *trans*-Golgi and packaged into distinct organelles (Kelly, 1985; Burgoyne and Morgan, 1993). In contrast, in the CTL lysosomal and secretory proteins are sorted and packaged into the same organelle (Burkhardt *et al.*, 1989, 1990; Peters *et al.*, 1991). This is perfectly demonstrated by a summary of proteins found in the lytic granule, presented in Table I-2. While the granule contains function-related proteins such as the granzymes and perforin, it also contains lysosomal enzymes such as β-glucoronidase. Some proteins, such as granzymes A and B and the lysosomal proteins, are targeted to the lytic granule through the mannose-6-phosphate receptor (Griffiths and Isaaz,

1993) which was originally defined as a lysosomal trafficking molecule. However, at least one other mechanism mediates this trafficking since perforin is correctly targeted in CTLs from patients with I-cell disease, in which the mannose-6-phosphate modification cannot be made (Griffiths, 1995).

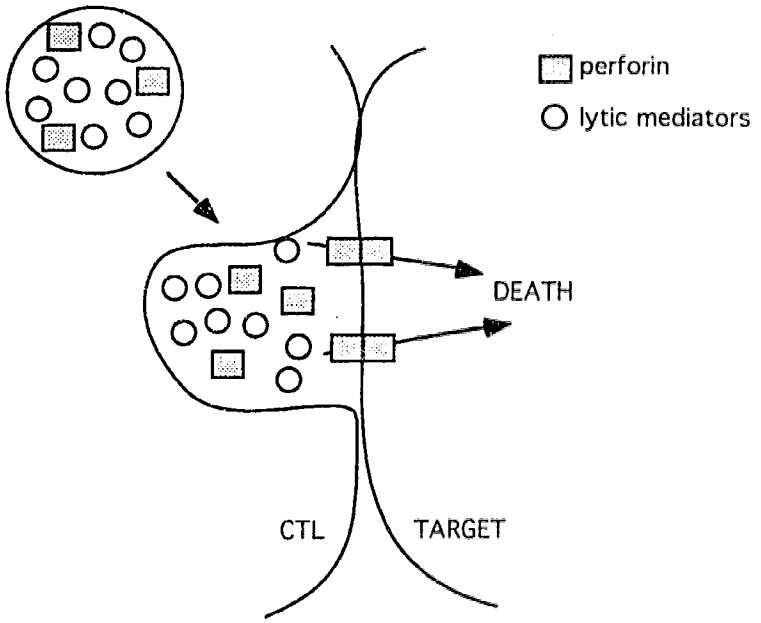


FIGURE I-3: The Granule Exocytosis Model of Cytotoxicity
Following TCR recognition of the target cell, lytic granules within the TCR are mobilized towards the target cell. Exocytosis results in the release of the granule contents, including the pore-forming protein perforin and other lytic proteins (including the granzymes) into the intercellular space. Perforin forms a pore in the target cell membrane, thereby allowing other lytic mediators to enter the target cell where they induce death by a poorly-defined mechanism.

Like lysosomes, the lytic granule is an acidic vesicle with an internal pH of 5.5 (Henkart *et al.*, 1987; Masson *et al.*, 1990). This acidity is maintained by the presence of a proton pump in granule membranes, and is an essential part of the granule - inhibition of its activity with concanamycin A results in granule breakdown

and loss of cytotoxicity of the CTL (Kataoka *et al.*, 1994). The acidic pH may also play a role in protecting the CTL from lysis due to its own granule contents (see below).

Following target cell recognition by the CTL the granules, microtubule-organizing centers and the Golgi apparatus of the CTL all reorient towards the point of contact with the target cell (Yannelli *et al.*, 1986; Geiger *et al.*, 1982; Kupfer and Dennert, 1984; Kupfer *et al.*, 1985). This reorientation helps to ensure that the exocytosed granule contents are directed toward the target cell, and that bystander killing (that is, killing of cells which have not been specifically recognized by the CTL) is minimized. The granules move along the CTL's microtubules in a kinesin-dependent manner (Burkhardt *et al.*, 1993). Kinesin activity in turn can be regulated by phosphorylation of kinesin-associated proteins (McIlvain *et al.*, 1994), thus the phosphorylation events initiated by TCR/CD3 activation are directly linked to the exocytosis of granule contents, consistent with a requirement for phosphorylation for degranulation (Anel *et al.*, 1994a).

The cytoskeletal rearrangements involved in granule exocytosis may be mediated by a family of small GTPases. Two GTPases, Rac and Rho, have been shown to regulate mast cell secretion (Price *et al.*, 1994) and have been identified in CTLs (Lang *et al.*, 1992), along with the hematopoietic cell-specific GTPase Rac2 which is upregulated following T cell activation (Reibel *et al*, 1991). Rac and Rho mediate the cytoskeletal rearrangements which precede degranulation (Norman *et al.*, 1994), suggesting that CTL degranulation may be regulated by the same GTPases involved in mast cell secretion.

CTLs are known to be serial killers, that is, each CTL is capable of detaching from a target, recognizing a new target cell, and then lysing that cell. Recently, Isaaz *et al.* (1995) have demonstrated that following TCR triggering of degranulation, new lytic proteins are synthesized in the CTL. While some of these proteins are correctly sorted to the lytic granule, almost one third of the lytic proteins are constitutively secreted. Granule killing remains directed toward the target cell, however, this constitutive secretory pathway is nondirectional and results in bystander lysis. Recent evidence suggests that this constitutive secretion may represent a means by which the CTL can regulate an immune response, by modifying cytokine secretion (see below).

TABLE I - 2: SUMMARY OF GRANULE CONTENTS

Protein	Function	Reference
perforin (cytolysin)	pore-former	Groscurth *et al.*, 1987 Podack *et al.*, 1985 Young *et al.*, 1986a
granzymes	proteolysis	see Table I-3
dipeptidyl peptidase I (cathepsin C)	granzyme activation	Smyth *et al.*, 1995 McGuire *et al.*, 1993
chondroitin sulfate	complexes with perforin and the granzymes	Tschopp and Masson, 1987 Masson *et al.*, 1990 Peters *et al.*, 1991
calreticulin	binds perforin Ca^{++} binding	Dupuis *et al.*, 1993 Burns *et al.*, 1992
TIA-1	RNA binding	Anderson *et al.*, 1990 Tian *et al.*, 1991
leukalexin	TNF-like cytokine	Liu *et al.*, 1987
leukophysin	granule mobility	Abdelhaleem *et al.*, 1991, 1996
mannose-6-phosphate receptor	protein targeting	Burkhardt *et al.*, 1990 Peters *et al.*, 1991 Griffiths and Isaaz, 1993
H^+-ATPase	acidification	Kataoka *et al.*, 1994
cathepsin D	lysosomal enzyme	Tschopp and Nabholz, 1990
arylsulfatase	lysosomal enzyme	Tschopp and Nabholz, 1990
β-glucoronidase	lysosomal enzyme	Tschopp and Nabholz, 1990
β-hexosamidase	lysosomal enzyme	Tschopp and Nabholz, 1990
lamp-1	lysosomal protein	Peters *et al.*, 1991
lamp-2	lysosomal protein	Peters *et al.*, 1991
CD63	lysosomal protein	Peters *et al.*, 1991

2. Granule Proteins

A summary of known granule proteins is shown in Table I-2. The roles that many of these proteins play within the granule and/or during CTL-mediated cytotoxicity is unknown. Some of these proteins are lysosomal proteins and likely play no role in CTL-mediated cytotoxicity, however, others clearly have functional roles in target cell killing.

Perforin

Perforin (cytolysin, pore-forming protein, C9-related protein) is the granule protein responsible for the calcium-dependent lytic activity of the CTL. Perforin was originally isolated from the lytic granules of NK and CTL cells, and was subsequently shown to be capable of inducing target cell lysis in the presence of calcium (Masson and Tschopp, 1985; Liu *et al.*, 1986; Podack *et al.*, 1985; Zalman *et al.*, 1987). In the lytic granules, perforin is in monomeric form and is found in association with proteoglycans (in a pH-dependent manner) and calreticulin (see below). Granule exocytosis releases perforin into the extracellular space where it is exposed to calcium and neutral pH. This neutral pH causes perforin to be released from the proteoglycans (Persechini *et al.*, 1989) and the perforin monomers bind the target cell, possibly by recognizing phosphorylcholine molecules on the cell surface (Tschopp *et al.*, 1989), and insert into the target cell lipid bilayer in a calcium-dependent manner (Yue *et al.*, 1987; Blumenthal *et al.*, 1984; Tschopp *et al.*, 1989; Young *et al.*, 1987; Ishiura *et al.*, 1990). The perforin monomers then aggregate in the target cell membrane to form pores which can be visualized by electron microscopy (EM) (Dourmashkin *et al.*, 1980; Dennert and Podack, 1983). Recently, Young has reported that while only 3-4 perforin monomers are required to form a functional channel, it takes 10-20 monomers to form an EM-visible channel. It is likely that these smaller pores which retain activity are actually more physiologically relevant than the larger ones which could perturb membrane permeability and result in cell death by osmosis (Liu *et al.*, 1995).

Perforin itself is able to induce lysis in a number of cell types, a finding which led to the suggestion that perforin alone accounts for CTL-induced cytolysis. However, perforin cannot induce target cell DNA fragmentation (Duke *et al.*, 1989), an event which precedes membrane damage during CTL attack (Duke *et al.*, 1983; Cohen *et al.*, 1985; Cohen, 1991; Golstein *et al.*, 1991), suggesting that perforin alone cannot mediate all of the events involved in CTL-mediated cytotoxicity and that other proteins, probably contained in the lytic granules, are involved (Munger *et al.*, 1988).

It is now believed that the prime role of perforin is not in cytolysis but rather to allow other cytotoxic mediators to enter the target cell and deliver the lethal hit (Liu *et al.*, 1995).

Two models have been proposed whereby perforin can mediate the entry of CTL lytic proteins into the target cell. In the first, perforin pores may disrupt intracellular homeostasis and initiate a repair process whereby the target cell attempts to repair the membrane damage caused by perforin insertion by endocytosing the affected area. During the repair process, lytic granule contents which were released with the perforin may be taken up by endocytosis. This model has been supported by the finding that reagents which block endocytosis affect CTL-mediated cytotoxicity (Shi *et al.*, 1992a).

In the second model, perforin pores simply act as channels through which the lytic mediators pass freely into the target cell. This model awaits confirmation. In either of these models, perforin plays a critical, yet indirect, role in granule-mediated killing. Of course, *in vivo* both mechanisms could occur simultaneously to ensure lytic protein uptake by the target cell.

Granzymes

The granzymes are a family of CTL-specific serine proteases (reviewed by Smyth and Trapani, 1995) which colocalize with perforin to the cytolytic granules (Redmond *et al.*, 1987; Ojcius *et al.*, 1991). Table I-3 shows a summary of the known murine granzymes and their properties. These proteases are synthesized as inactive precursors with an activation dipeptide at the amino terminus and require removal of this dipeptide for enzyme activation (Caputo *et al.*, 1993), a process believed to be involved in protecting the CTL from its own lytic proteins (see below).

Although evidence is only just beginning to accumulate regarding the biological role of the granzymes in CTL-mediated killing it has been known for some time that protease inhibitors can protect cells from cell-mediated lysis (Helgason *et al.*, 1995; Kaiser and Hoskin, 1992; Hudig *et al.*, 1991; Brogan and Targan, 1986). However, it was not known whether the granzymes or intracellular protease(s) were a target for these inhibitors. Loading of cells with chymotrypsin, proteinase K or trypsin has been found to cause cell lysis accompanied in most cases by DNA fragmentation and nuclear damage (Williams and Henkart, 1994) suggesting that proteases are involved in the induction of target cell death and that the granzymes likely exert their effect inside the cell. Multiple mechanisms have been proposed by

which the granzymes may induce target cell death, including the activation of endogenous endonucleases (Smyth *et al.*, 1994).

The first two granzymes to be identified were granzyme A and granzyme B. These proteases have received the most attention thus far, probably because human homologues for these proteins have been isolated while human homologues of the other murine granzymes have not been found. Additionally, in murine CTL stimulated *ex-vivo* only granzymes A and B, and maybe minor amounts of C, are expressed (Garcia-Sanz *et al.*, 1990; Ebnet *et al.*, 1991) suggesting that these granzymes are key mediators of CTL-mediated cytotoxicity. Quantitative polymerase chain reaction (PCR) of granzyme transcript levels in CTLs activated by mitogen, allogeneic cells or anti-CD3 revealed that only granzyme B transcripts correlated with cytotoxicity for all modes of stimulation (Prendergast *et al.*, 1992) suggesting that granzyme B may be a direct effector in the lytic process. Unfortunately, granzyme A levels were not examined in this study and it is not known whether granzyme A expression also correlates with cytotoxicity in response to these different activators.

While granzyme A has substrate specificity resembling trypsin (cleavage after Arg or Lys), granzyme B has a substrate specificity which is unique among eukaryotic serine proteases. Molecular modeling of the murine homologue revealed that the side chain of the murine granzyme B residue Arg^{208} partially fills the specificity pocket of the protease, predicting a requirement for acidic residues (Asp or Glu) at the P_1 site (Murphy *et al.*, 1988). Subsite mapping and inhibitor studies confirmed that granzyme B cleaves following aspartic acid residues (Odake *et al.*, 1991; Poe *et al.*, 1991). Finally, replacement of Arg^{208} with a glycine residue converted the substrate specificity of granzyme B from cleavage after Asp residues to cleavage following hydrophobic residues, demonstrating that Arg^{208} is indeed responsible for determining substrate specificity (Caputo *et al.*, 1994).

Interestingly, granzyme A exists as a homodimer, whereas granzyme B is monomeric. When combined with their differing substrate specificities, it seems likely that granzyme A and granzyme B have separate cellular substrates, a fact supported by the findings of Irmler *et al.* (1995) who found that granzyme A cleaves and activates pro-Interleukin-1β (pro-IL-1β) while granzyme B does not. Granzyme A has also been shown to cleave various intracellular and extracellular proteins, including nucleolin (Pasternack *et al.*, 1991), collagen type IV (Simon *et al.*, 1991), and the thrombin receptor (Suidan *et al.*, 1994) although these studies were performed using *in vitro* systems.

Both granzymes are thought to play a role in the induction of target cell DNA fragmentation. Two groups (Shi *et al.*, 1992b; Hayes *et al.*, 1989) have shown that granzyme A can induce target cell DNA fragmentation in a perforin-dependent manner. Similar studies using granzyme B purified from rat NK cells (called fragmentin-2 but having homology to granzyme B) showed a similar result with this protease (Shi *et al.*, 1992a, 1992b). Other groups have confirmed these results using a complementary technique. The noncytotoxic rat mast cell tumour line RBL, which can be triggered to degranulate by the immunoglobulin E (IgE)-specific Fc receptor, was transfected with perforin or the granzymes either alone or in combination and then transfectants were tested for their ability to lyse IgE-coated target cells. Cells expressing granzyme A alone were found to be noncytolytic and cells expressing perforin alone were cytolytic but failed to induce target cell DNA fragmentation. However, the combination of granzyme A and perforin could induce both target cell lysis and DNA fragmentation (Shiver and Henkart, 1991; Shiver *et al.*, 1992). Similar studies using granzyme B yielded similar results. Again, coexpression of granzyme B with perforin could enhance cytolytic and nucleolytic activity of these cells compared to cells expressing perforin alone (Nakajima *et al.*, 1995a). Interestingly, perforin/granzyme A- or perforin/granzyme B-expressing RBL were not as effective against tumour cell targets as RBL expressing all three cytolytic proteins, suggesting that there is a synergism between the two granzymes (Nakajima *et al.*, 1995a).

Besides DNA fragmentation, granzyme A may also play a role in other aspects of target cell killing. Talento *et al.* (1992) found that expression of an antisense granzyme A construct in a cloned CTL line interfered not only with target cell DNA fragmentation but also with release of ^{51}Cr-labeled proteins (as a measure of cytolytic activity), suggesting granzyme A plays either a direct or indirect role in the induction of target cell membrane damage. These results were confirmed by Nakajima and Henkart (1994) who showed that pre-loading of target cells with aprotinin (a granzyme A inhibitor) suppressed both cytolysis and DNA fragmentation in target cells exposed to RBL expressing both granzyme A and perforin. In contrast to this, Ebnet *et al.* (1995) have shown that both the CTL and NK cells derived from granzyme A-deficient mice are indistinguishable from wild type cells in causing target cell membrane damage, death (through a process called apoptosis), and DNA fragmentation, seeming to suggest that granzyme A is not essential for cell-mediated cytotoxicity. Therefore the role of granzyme A during CTL-mediated killing is still unresolved.

Similar studies have been more successful at determining the role of granzyme B. Besides the above-mentioned work, other experiments have accumulated data to suggest that granzyme B is involved in the induction of target cell DNA fragmentation, and may play a secondary role in regulating membrane damage. Bochan *et al.* (1995) have demonstrated that stable transfection of an NK cell which contains no granzyme A (Su *et al.*, 1994) with an antisense granzyme B construct inhibits the lytic ability of these cells (measured as ^{51}Cr release) by >95%. However, their results may be a consequence of looking at NK cell-mediated killing. Using CTL, NK and lymphokine-activated killers (LAKs) isolated from mice homologous for a null mutation in the granzyme B gene, other workers have shown that granzyme B plays a critical and nonredundant role in the rapid induction of target cell DNA fragmentation and apoptosis (Heusel *et al.*, 1994; Shresta *et al.*, 1995) and in NK cells (but not in CTL or LAK cells) granzyme B may also play a role in inducing membrane damage (Shresta *et al.*, 1995). The reduced DNA fragmentation in target cells treated with these effectors is due to reduced kinetics since longer incubation times resulted in target cell DNA fragmentation. Therefore, other granzymes, possibly granzyme A, may be able to induce DNA fragmentation but granzyme B is involved in its rapid induction. Consistent with a role for granzyme B in inducing DNA damage, two groups (Trapani *et al.*, 1996; Pinkoski *et al.*, 1996) have recently shown that granzyme B localizes to the nucleus of a target cell.

Although the evidence is confusing and contradictory at times, there definitely seems to be a role for both granzymes A and B in the induction of target cell death. The requirement for multiple proteases during CTL-mediated killing, each with different substrate specificities, is not surprising in light of the fact that CTLs are primarily involved in the removal of viral-infected and tumourigenic cells - both of these mutant cell types being potentially lethal. Therefore, the CTL has developed multiple mechanisms to ensure the death of these cells.

Besides their roles in the elimination of tumourigenic and virus-infected cells, granzymes may also mediate immunomodulatory functions by interacting with cell surface receptors. Sower *et al.* (1996) have recently shown that catalytically active, but not inactive, granzyme A can stimulate IL-6, IL-8 and tumour necrosis factor α (TNFα) production by human peripheral blood monocytes and purified monocytes. Interestingly, although these cells possess a cell surface receptor for the serine protease thrombin, and granzyme A has previously been shown to be capable of cleaving and activating the thrombin receptor (Suidan *et al.*, 1994), this activity of granzyme A against monocytes was found to be separate from that mediated by

TABLE I - 3: SUMMARY OF GRANZYMES[1]

Protease	Synonym	Specificity (residue at P_1)	References
Granzyme A	HF	Arg/Lys	Gershenfeld and Weissman, 1986 Pasternack and Eisen, 1985 Masson *et al.*, 1986a, 1986b
	SE1		Young *et al.*, 1986b
	CTLA-3		Brunet *et al.*, 1986
	TSP-1		Simon *et al.*, 1986
Granzyme B	CCP1	Asp/Glu	Lobe *et al.*, 1986a, 1986b, 1988 Masson and Tschopp, 1987
	SE2		Young *et al.*, 1986b
	CTLA-1		Brunet *et al.*, 1986
Granzyme C	CCP2	Asn/Ser	Lobe *et al.*, 1986a, 1986b, 1988 Jenne *et al.*, 1988a
Granzyme D	CCP5	Phe/Leu	Bleackley *et al.*, 1988a, 1988b Jenne *et al.*, 1988b Prendergast *et al.*, 1991
Granzyme E	CCP3	Phe/Leu	Bleackley *et al.*, 1988a, 1988b Jenne *et al.*, 1988b Prendergast *et al.*, 1991
	MCSP-2		Kwon *et al.*, 1988
Granzyme F	CCP4	Phe/Leu	Bleackley *et al.*, 1988a, 1988b Jenne *et al.*, 1988b, 1991 Prendergast *et al.*, 1991
	MCSP-3		Kwon *et al.*, 1988
Granzyme G	CCP6	Phe/Leu	Masson and Tschopp, 1987 Jenne *et al.*, 1989

[1]Only mouse granzymes are shown here. In addition, fragmentin-1 and fragmentin-2 have been isolated from rat NK cells and have homology to granzyme A and granzyme B respectively (Shi *et al.*, 1992a, 1992b). Human homologues of granzymes A and B have been identified (Gershenfeld *et al.*, 1988; Caputo *et al.*, 1990, Schmid and Weissman, 1987; Caputo *et al.*, 1988; Trapani *et al.*, 1988) as well as granzyme H (HuCCPX)(Meier *et al.*, 1990; Haddad *et al.*, 1991) and granzyme 3 (Hameed *et al.*, 1988).

thrombin. This implies the existence of a separate cell surface receptor for granzyme A and further suggests that once activated, CTLs may modulate the immune response by constitutive secretion of their granule contents (as demonstrated by Isaaz *et al.*, 1995; Sower *et al.*, 1996).

Granzyme B has also been shown to have an extracellular substrate, although not a beneficial one in this instance. Froelich *et al.* (1993) have found that granzyme B secreted from CTLs in a rheumatoid joint may contribute to cartilage loss during rheumatoid arthritis by degrading the aggrecan proteoglycan matrix synthesized by chondrocytes. Thus, in addition to their roles in the deletion of target cells during an immune response, the granzymes may be responsible for other events within the body, both beneficial and pathologic.

Other Granule Proteins

Besides perforin and the granzymes, additional granule proteins have been identified, although in many cases the roles of these proteins are unknown.

Dipeptidyl peptidase I (DPPI, cathepsin C), a cysteine protease with specificity for cleaving dipeptides from the amino terminus of proteins, has been found in cytolytic granules. DPPI has recently been shown to activate both granzyme A (Kummer *et al.*, 1996) and granzyme B (McGuire *et al.*, 1993; Smyth *et al.*, 1995) from their zymogen forms, suggesting a role for DPPI in granzyme activation once the zymogens have reached the granules, thereby ensuring protection of the CTL from its own lytic proteins.

A second mechanism for protecting CTLs is due to the presence of proteoglycans in the lytic granule (Schmidt *et al.*, 1985; Stevens *et al.*, 1987, 1989; Serafin *et al.*, 1986). The granule, as a secretory vesicle, has an acidic pH of 5.5 (Henkart *et al.*, 1987; Masson and Tschopp, 1990). At this pH both perforin (Tschopp and Masson, 1987) and the granzymes (Peters *et al.*, 1991) are bound to chondroitin sulfate and are maintained in an inactive state ensuring CTL protection from the action of these proteins. Following granule exocytosis, these complexes are exposed to neutral pH which releases the chondroitin sulfate from the lytic proteins, allowing them to act on the target cell.

It has long been known that perforin polymerizes in the presence of calcium and it seems that the lytic granules may possess a mechanism, besides association with proteoglycans, which prevents perforin polymerization within the cell. Lytic granules have been reported to contain the calcium-binding protein calreticulin (Dupuis *et al.*, 1993), a protein whose expression is induced following T cell

activation (Burns *et al.*, 1992). When first isolated, this protein was believed to be localized only to the ER since it possesses the carboxy terminal ER retention signal KDEL (single letter amino acid code [Michalak *et al.*, 1992]). Although it is not known how calreticulin escapes the ER, one possibility is that calreticulin "escorts" perforin out of the ER and to the granules (Dupuis *et al.*, 1993; Burns *et al.*, 1994). While bound to perforin, the KDEL retention signal of calreticulin may be masked, allowing it to exit the ER. It has been shown that although granzymes A and B are targeted to the granules by the mannose-6-phosphate receptor (Griffiths and Isaaz, 1993) another mechanism must account for perforin targeting (Griffiths, 1995), a mechanism which may involve calreticulin. Calreticulin may also serve to sequester calcium away from perforin and maintain it in a monomeric state. Alternatively, if calreticulin enters the target cell it could contribute to the calcium flux seen in target cells following CTL attack (Allbritton *et al.*, 1988a).

The use of monoclonal antibodies directed against lytic granule contents allowed Anderson *et al.* (1990) to identify TIA-1, a 15-kDa RNA-binding protein whose expression is restricted to NK cells and CTLs, and is upregulated following CTL activation (Anderson *et al.*, 1990; Cesano *et al.*, 1993). Interestingly, TIA-1 has been found to induce DNA fragmentation in digitonin-permeabilized cells (Tian *et al.*, 1991), suggesting that this protein may play a role in inducing target cell DNA fragmentation during CTL attack.

Leukalexin is a TNF-like molecule with an undetermined role in CTL-mediated cytotoxicity (Liu *et al.*, 1987). One possibility is that it binds an as yet unidentified target cell surface receptor and may be able to transduce a death signal in much the same way as TNF and the ligand of Fas (see below).

Leukophysin was identified as a 28-kDa membrane glycoprotein found in the granules of leukocytes (Abdelhaleem *et al.*, 1991). Using monoclonal antibodies to leukophysin to isolate a full length cDNA clone, Abdelhaleem *et al.* (1996) found that the nucleotide sequence encoding this protein is highly homologous to the 3' end of RNA helicase A. Interestingly, the cDNA contains a repetitive motif similar to synaptophysin I, a protein involved in synaptic vesicle exocytosis, suggesting that leukophysin may be involved in granule exocytosis. Intriguingly, this protein was found associated with granzyme A-negative granules and vesicles in $CD8^+$ CTLs, thereby defining a subset of granules which are distinct from the classical granzyme-containing granules. The role of this subset remains unidentified, but they may function to transport either granzymes (besides granzyme A and B) or other granule proteins to the cell surface.

Other granule proteins have been less well-studied and their roles, if any, in CTL-mediated cytotoxicity are unknown.

B. FAS-MEDIATED CYTOTOXICITY

The granule exocytosis model to explain CTL-mediated cytotoxicity has a strict requirement for calcium - the degranulation process is calcium-dependent, and the binding, insertion and polymerization of perforin in target cell membranes requires the presence of calcium. However, some target cells can be lysed in the absence of calcium or detectable levels of perforin or the granzymes, a fact which casts doubt on the granule exocytosis model of CTL killing (Helgason *et al.*, 1992; Allbritton *et al.*, 1988b; Berke and Rosen, 1988; Ostergaard *et al.*, 1987; Trenn *et al.*, 1987).

Further studies into this apparent discrepancy using cells in which the perforin-mediated pathway had been "knocked out" revealed some insights (Kägi *et al.*, 1994a, 1994b; Kojima *et al.*, 1994; Lowin *et al.*, 1994; Walsh *et al.*, 1994a, 1994b). These studies demonstrated considerable lytic activity in CTLs lacking perforin, and therefore the granule-mediated pathway of killing - evidence supporting the existence of a perforin-independent cytolytic pathway. This pathway is now known to be mediated by cell surface proteins.

1. Characteristics of Fas and FasL

In 1989, two groups reported the isolation of mouse-derived antibodies which could induce apoptotic death in human cells (Yonehara *et al.*, 1989; Trauth *et al.*, 1989). The cell surface markers recognized by these antibodies were designated either Fas or APO-1 (now also known as CD95). Expression cloning resulted in the isolation of the Fas cDNA (Oehm *et al.*, 1992; Watanabe-Fukunaga *et al.*, 1992a; Itoh *et al.*, 1991) and identified this protein as a type I transmembrane protein belonging to the tumour necrosis factor/nerve growth factor (TNF/NGF) superfamily of receptors. It has been proposed that all members of this family function as trimers (Banner *et al.*, 1993; Peitsch and Tschopp, 1995; reviewed by Smith *et al.*, 1994) a result supported by the finding that receptor oligomerization is required to transduce the death signal (Dhein *et al.*, 1992).

Transfection of the cDNA encoding the Fas antigen confirmed that this protein could transduce an apoptotic signal (Oehm *et al.*, 1992; Rouvier *et al.*, 1993). Mutation analysis of the cytoplasmic domain of Fas showed that a region of this domain was both necessary and sufficient for transducing the death signal (Itoh and

Nagata, 1993). This domain was subsequently dubbed the "death domain" and is conserved in the 55-kDa TNF receptor TNFRI (Tartaglia *et al.*, 1993), which also transduces a death signal following receptor ligation, as well as in the *Drosophila* protein REAPER, which plays a central role in programmed cell death during development (White *et al.*, 1994; Golstein *et al.*, 1995). The ability of the death domain to aggregate accounts for its ability to transduce a death signal. Boldin *et al.* (1995a) have shown that these death domains spontaneously aggregate when transfected into HeLa cells and transduce an apoptotic signal, suggesting that a mechanism must exist which maintains Fas in an unaggregated (inactive) form. Peter *et al.* (1995) have recently shown that sensitivity to Fas killing is modulated by surface sialylation, suggesting that the negative charges on Fas due to this sialylation could maintain the molecule in a monomeric state. Additionally, cytoplasmic proteins may associate with Fas and be displaced by signal transducers following activation (see below).

The structure of Fas and its homology to the TNF/NGF receptors suggested that Fas may be a receptor for a novel cytokine. The cDNA encoding the ligand for Fas (FasL) was subsequently cloned (Suda *et al.*, 1993) and found to code for a type II transmembrane protein belonging to the TNF family of cytokines. Expression of FasL in COS cells renders these cells cytotoxic towards target cells bearing Fas, confirming that Fas is a death-inducing receptor and FasL is its ligand (Suda *et al.*, 1993).

Fas was originally found at low levels on many cell types within the body, but high levels are found in mouse thymus, liver, heart, lung, kidney, ovary, testis and eye (Watanabe-Fukunaga *et al.*, 1992a). In addition, functional, soluble forms of Fas have been identified (Cascino *et al.*, 1995; Hughes and Crispe, 1995). Although resting T cells express low levels of Fas, T cell activation results in upregulation of surface Fas levels on these cells (Trauth *et al.*, 1989), a fact that may hint at the function of Fas (see below). FasL has been detected in testis, small intestine, kidney, lung, spleen, thymus, and eye (Griffith *et al.*, 1995; Suda *et al.*, 1993) as well as on activated T cells (Stalder *et al.*, 1994; Nagata and Golstein, 1995; Suda *et al.*, 1995) and in soluble form (Tanaka *et al.*, 1995). This presence of FasL on cytolytic T cells suggested that FasL could be a cytolytic mediator.

It is now believed that T cell activation results in FasL expression and that FasL then engages Fas on the target cell surface, resulting in activation of the target cell's death program (Figure I-4). Interestingly, both $CD4^+$ and $CD8^+$ cells may express FasL following activation, so this mechanism may account for the occurrence

of $CD4^+$ killer cells. (Stalder *et al.*, 1994). Since no other mechanisms have been found to account for CTL-mediated cytotoxicity, granule-mediated and Fas-mediated killing are believe to constitute the two major mechanisms used, although interactions between TNF and the TNF "death receptor" may also play a role. In addition, a novel member of the TNF family has recently been identified, called Apo-2 ligand (Apo2L)/TNF-related apoptosis-inducing ligand (TRAIL), which can induce apoptosis in both lymphoid and non-lymphoid targets. Since neither soluble Fas nor soluble TNFRI suppresses the death-inducing activity of Apo2L, this novel cytokine is believed to act through a third, yet unidentified receptor. This Apo2-Apo2L system may also contribute to the cytotoxicity of CTLs (Pitti *et al.*, 1996; Wiley *et al.*, 1995).

2. Potential Signal Transducers

In the past year a number of groups have used the yeast two-hybrid system to identify proteins associated with the cytoplasmic domain of Fas, which may play a role in signal transduction. In this regard, Fas-associated death domain protein (FADD, also known as MORT1 [Chinnaiyan *et al.*, 1995; Boldin *et al.*, 1995b]) and RIP (Stanger *et al.*, 1995) have been identified and contain regions similar to the Fas death domain (reviewed by Cleveland and Ihle, 1995). Indeed, overexpression of FADD/MORT1 or RIP has been shown to induce apoptosis. FADD/MORT1 and RIP are also involved in transducing the death signal from TNFRI by associating with another death domain-containing protein, TRADD (Chinnaiyan *et al.*, 1996a; Hsu *et al.*, 1996a; Varfolomeev *et al.*, 1996; Hsu *et al.*, 1995), suggesting that there may be "cross-talk" between the TNF and Fas death receptors (Varfolomeev *et al.*, 1996). These results may explain the findings of Glass *et al.* (1996) who showed that FasL expression is necessary but not sufficient for Fas-mediated apoptosis. That is, transduction of a death signal may require both Fas and TNFRI to be ligated, and involve the concerted effects of TRADD, FADD/MORT1 and RIP activation. Additional support for this hypothesis comes from the work of Zhou *et al.* (1996) who demonstrated that autoimmune and lymphadenopathy are accelerated in mice with a Fas deficiency and no TNFRI, compared to mice deficient in Fas only, suggesting that these two cell surface receptors play similar roles. Catalytic activity of FADD/MORT1, if any, is unknown, however, in studies demonstrating that RIP is recruited to the TNFRI signaling complex in a TNF-dependent manner, RIP was shown to possess serine/threonine kinase activity (Hsu *et al.*, 1996b).

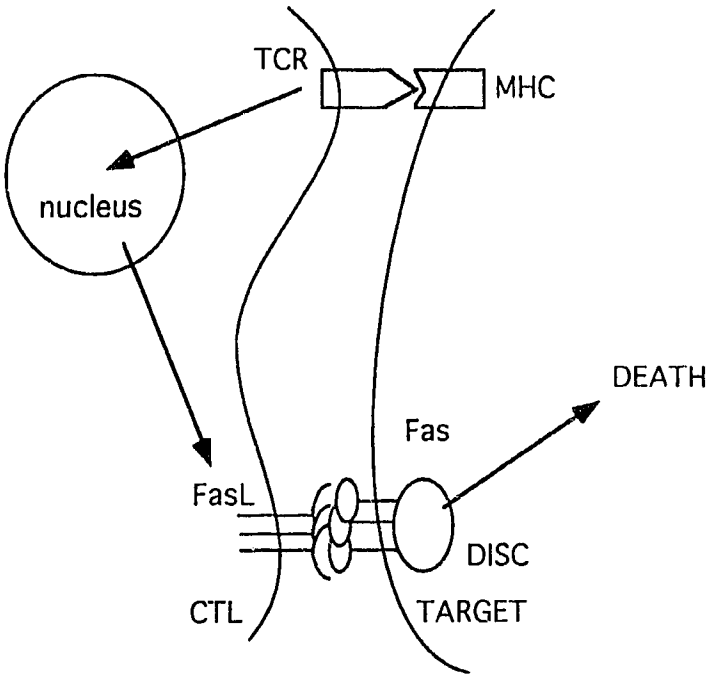


FIGURE I-4: Fas-Mediated Cytotoxicity
The TCR recognizes foreign peptide presented by the target cell's MHC proteins. Cross-linking of the TCR results in the generation of signals within the CTL which eventually lead to upregulation of Fas ligand (FasL) levels on the CTL surface. FasL binds to Fas on the target cell, resulting in trimerization of the receptor. A number of proteins within the target cell associate with the death domain of Fas to form the death-inducing signaling complex (DISC), which initiates a signal resulting in target cell death.

It now seems that FADD/MORT1 actually acts as an adaptor molecule rather than possessing catalytic activity itself. Two groups have recently identified a protein which interacts with FADD/MORT1 and contains a region with homology to a family of cysteine proteases related to Interleukin-1β Converting Enzyme (ICE). These proteases appear to be key mediators of apoptosis (see below). This novel protein, named FLICE/MACH, is activated upon Fas ligation by an unknown mechanism. It is thought that this allows FLICE/MACH to initiate a series of events culminating in apoptotic cell death. Since this protein associates with Fas, this is believed to

represent the "top" of the cascade of events activated by Fas ligation (Boldin *et al.*, 1996; Muzio *et al.*, 1996; reviewed by Fraser and Evan, 1996).

A third protein identified using this approach was FAP-1 (Fas-associated phosphatase [Sato *et al.*, 1995]). Although no association with Fas under physiological conditions was shown, FAP-1 seems to associate with the carboxy terminus of Fas (Sato *et al.*, 1995), a region previously identified as containing a negative regulatory domain (Itoh and Nagata, 1993). In addition, gene transfer of FAP-1 to Jurkat cells yielded a cell line which was more resistant to Fas-mediated apoptosis (Sato *et al.*, 1995) suggesting that Fas could transduce the death signal through the use of kinases and the signal could be modulated by phosphatases. Interestingly, our lab has recently determined that there is an association between Fas and the Src PTK Fyn (Atkinson *et al.*, 1996), again suggesting the involvement of kinases in transducing the signal. The role of kinases in Fas-mediated apoptosis is somewhat controversial however, and will be discussed in detail below.

Finally, FAF-1 (Fas-associated protein factor 1) has also been identified through two-hybrid studies (Chu *et al.*, 1995). Although FAF-1 contains no significant homology to any known proteins, it has an interesting ability to interact only with wild type Fas and not with Fas containing a point mutation in the cytoplasmic domain. The further finding that FAF-1 associates with Fas in mammalian cells suggests that FAF-1 may play a role in the transduction of the death signal following Fas ligation.

Using co-immunoprecipitation as a means of identifying associated proteins, Kischkel *et al.* (1995) identified four proteins associated with Fas. These proteins were designated CAP 1 through 4, for cytotoxicity-dependent APO-1-associated protein. The CAPs were found only to co-immunoprecipitate with the cross-linked (active) form of Fas, and not the monomeric form, suggesting a role for these proteins in signal transduction. CAP1 and CAP2 were identified as phosphorylated versions of FADD/MORT1 while CAP3 and CAP4 have since been identified as alternate versions of the FADD/MORT1-associated cysteine protease FLICE/MACH (Muzio *et al.*, 1996). This complex of signaling molecules associated with Fas has been designated the death-inducing signaling complex (DISC [Kischkel *et al.*, 1995]).

3. Downstream Signaling

The reports on downstream signaling events which transduce the apoptotic signal from Fas are somewhat confusing. While one group has shown that calcium flux is an important factor (Oshimi and Miyazaki, 1995), others have not found this to

be the case (Vignaux *et al.*, 1995). Similar studies on the role of tyrosine phosphorylation have produced similarly conflicting reports - Eischen *et al.* (1994) have reported that tyrosine phosphorylation is an early and requisite event for Fas signaling, a result which has been contradicted (Schraven and Peter, 1995). The discovery of both a kinase and a phosphatase associated with Fas (see above) would seem to suggest that phosphorylation is a downstream signaling event. In addition, yet another group has shown that a number of proteins are tyrosine phosphorylated following Fas ligation (Cifone *et al.*, 1993). Finally, Su *et al.* (1995) have recently demonstrated that sensitivity to Fas-mediated apoptosis correlates with expression of hematopoietic cell protein tyrosine phosphatase (HCP) in eleven human and murine Fas-bearing lymphoma cell lines, again suggesting that tyrosine phosphorylation and dephosphorylation may play a role in modulating the death signal.

Other signaling pathways during Fas-mediated apoptosis are more defined. Fas ligation results in the activation of at least four different phospholipases: acidic sphingomyelinase (aSMase) (Cifone *et al.*, 1993, 1995); neutral sphingomyelinase (nSMase), phosphatidylcholine-specific phospholipase C (PC-PLC) and phospholipase A_2 (PLA_2) (Cifone *et al.*, 1995). Activation of aSMase, nSMase, and PC-PLC results in the release of the second messenger ceramide which can activate ERK-2 which, in turn, activates PLA_2. These activities belong to at least two independent pathways: activation of nSMase and PLA_2 are insufficient to induce apoptosis, however, activation of PC-PLC results in activation of aSMase and ceramide production, leading to the induction of apoptosis (Cifone *et al.*, 1995).

Involvement of ceramide as a second messenger during apoptosis has been confirmed in a number of ways - cell-permeable synthetic ceramides can induce apoptosis (Dressler *et al.*, 1992; reviewed by Hannun and Obeid, 1995; Pushkareva *et al.*, 1995) and, in an *in vitro* system, ceramide can induce apoptotic activity in cytoplasmic extracts isolated from normal (i.e., nonapoptotic) cells (Martin *et al.*, 1995a).

The GTP-binding protein $p21^{ras}$ is also activated during Fas-mediated killing (Gulbins *et al.*, 1995) and inhibition of this activity by a neutralizing antibody or a dominant negative Ras mutant can interfere with Fas-induced apoptosis. Ceramides, which are released during Fas killing, are capable of activating Ras, suggesting that Fas ligation results in the release of ceramides which go on to activate Ras and a kinase cascade similar to that involved in TCR/CD3 signaling.

Interestingly, Taupin *et al.* (1995) have found that an RNA-binding protein called TIAR translocates from the nucleus to the cytoplasm during Fas-mediated

cytotoxicity. This is intriguing since it suggests a mechanism conserved with the granule-mediated pathway to cell death. Granules have been shown to contain the 15-kDa isofomr of the RNA binding protein TIA-1 (Anderson *et al.*, 1990; Cesano *et al.*, 1993) which is structurally related to the carboxy terminus of TIAR and can induce DNA fragmentation in permeabilized cells (Tian *et al.*, 1991). Interestingly, Tian *et al.* (1995) recently reported the characterization of a serine/threonine kinase which is rapidly dephosphorylated and activated following Fas ligation. Activation of this FAST (Fas-activated serine/threonine) kinase leads to the phosphorylation of the 40- and 53-kDa isoforms of TIA-1, an event which precedes the onset of DNA fragmentation. Not only does this provide a common feature with granule-mediated cytotoxicity (that is, the possible involvement of RNA binding proteins in DNA fragmentation), but it also represents another example of the involvement of both phosphatases and kinases in Fas-mediated cytotoxicity.

Finally, Um *et al.* (1996) have shown that reactive oxygen intermediates are key mediators of Fas-induced monocyte apoptosis, demonstrated by the fact that antioxidants such as glutathione and *N*-acetylcysteine can abolish the apoptotic action of anti-Fas antibody on activated human peripheral blood monocytes. Other studies have also suggested this involvement of reactive oxygen species in apoptosis suggesting that they may constitute universal mediators (reviewed by Slater *et al.*, 1995; Korsmeyer *et al.*, 1995).

4. Role of Fas-FasL Interactions

One question which arises is the role of the Fas pathway in cell-mediated cytotoxicity. Is this merely a second mode of cell death or does it have a more significant role in immune responses? The use of mice with natural mutations in the Fas death pathway seem to be providing the answer. Mice containing the *lpr* (lymphoproliferation, a mutation in the Fas antigen [Watanabe-Fukunaga *et al.*, 1992b]) or *gld* (generalized lymphoproliferative disorder, a mutation in FasL [Lynch *et al.*, 1994; Ramsdell *et al.*, 1994; Takahashi *et al.*, 1994]) mutation exhibit similar phenotypes of lymphadenopathy and splenomegaly (reviewed in Nagata and Suda, 1995). In these mutant mice, elimination of mature activated T cells is defective, suggesting that Fas and FasL are involved in down-regulating the immune response. Interestingly, mutations in Fas have recently been associated with a human autoimmune lymphoproliferative syndrome (Fisher *et al.*, 1995; Rieux-Laucat *et al.*, 1995).

Mature unactivated T cells express low levels of Fas but are not sensitive to FasL (Owen-Schaub *et al.*, 1992). During activation, Fas and FasL levels are upregulated and the cell becomes FasL-sensitive. Therefore, in the process of activation, T cells gain the ability both to kill and be killed through the Fas pathway. There is now accumulating evidence to suggest that Fas-FasL interactions are responsible for activation-induced T cell death. A number of groups have shown that activation-induced death of T cell hybridomas, preactivated T cells and T cell lines occurs through a Fas-dependent pathway (Ju *et al.*, 1995; Brunner *et al.*, 1995; Alderson *et al.*, 1995; Dhein *et al.*, 1995). Interestingly, in single-cell cultures these cells undergo Fas-mediated apoptosis, indicating a *cis* interaction can induce death, as well as a *trans* interaction. It is now thought that, in an area of high density of T cells, such as an infection, the activated T cells are likely to be colliding with each other as well as potential target cells. This would result in Fas-FasL interactions resulting in death of the T cell. Thus, while the immune response is in progress it is also being inactivated (reviewed by Lenardo, 1996; Lynch *et al.*, 1995). Interestingly, recent work has demonstrated that NK cells stimulated through the Fc receptor (Eischen *et al.*, 1996) or by phorbol myristate acetate/ionomycin (Montel *et al.*, 1995) have increased expression of FasL compared to unstimulated cells, suggesting that NK cells may also kill through the Fas-FasL pathway. Similar to CTLs, during activation the NK cell Fas receptors also become competent to deliver the death signal suggesting that cell-cell interactions may also be involved in downregulating the levels of activated NK cells (Eischen *et al.*, 1996). This proposal has come under close scrutiny recently, however, with the demonstration that *in vivo* activated CTLs are not susceptible to FasL-induced cell death (Ehl *et al.*, 1996; Tucek-Szabo *et al.*, 1996), implying the existence of a Fas-independent pathway of elimination. Additionally, Mollereau *et al.* (1996) have recently shown that CD2- (but not CD3-) induced apoptosis proceeds through a Fas-independent pathway which requires protein tyrosine phosphorylation, suggesting that this Fas-independent death pathway may contribute to the elimination of T cells expanding during an immune response. Finally, Zheng *et al.* (1995) have shown that autoregulatory apoptosis of mature T cells can also occur through the TNF-TNFRI system, suggesting that two distinct mechanisms - Fas-FasL and TNF-TNFRI - can account for T cell deletion following an immune response.

In addition to a role in cytotoxicity and activation-induced T cell apoptosis, recent evidence has suggested a role for Fas in apoptosis of nonlymphoid cell types. Fas and FasL are known to be co-expressed in tissues such as the small intestine,

prostate and uterus in which apoptosis is implicated in control of cell turnover and tissue homeostasis (Kyprianou and Isaacs, 1988; Rotello *et al.*, 1992), suggesting a role for Fas-FasL interactions during physiological cell turnover (French *et al.*, 1996).

Additionally, expression of FasL in nonlymphoid tissues may represent a way of maintaining immune privilege. FasL expression in the testis and eye confers immune privileged status on these tissues, probably by allowing FasL-bearing cells within these tissues to eliminate any Fas positive T cells which enter the tissue (Griffith *et al.*, 1995; Bellgrau *et al.*, 1995), thereby preventing any lymphocyte-mediated damage. Recent work has demonstrated expression of FasL at other immune-privileged sites such as the brain, adrenal gland, uterus, ovary, and prostate (French *et al.*, 1996) implicating a role for FasL in maintaining immune privilege.

5. Specificity in Fas-Mediated Cytotoxicity

If Fas-FasL interactions are involved in CTL-mediated cytotoxicity, and since FasL is a membrane bound protein, how does the T cell ensure that only the recognized target cell is lysed? One possibility is that FasL expression is directed towards the point of interaction with the target cell.

Another intriguing hypothesis is that FasL may reside in the lytic granule membranes rather than on the cell surface (Griffiths, 1995). Following T cell activation, FasL would then be directionally expressed as a result of the fusion between the granule membrane and the plasma membrane during degranulation. Support for this model is the fact that although Fas-mediated killing is calcium-independent, expression of FasL on the CTL surface has been found to require calcium (Vignaux and Golstein, 1994; Anel *et al.*, 1994b; Kojima *et al.*, 1994). This calcium could be required for the calcium-dependent granule exocytosis that would bring FasL to the cell surface. Once activated, the T cell is known to constitutively secrete a portion of its granule contents (Isaaz *et al.*, 1995) in a nondirectional manner, meaning that FasL expression would eventually spread to the entire T cell surface, and the T cell would become capable of initiating the death signal in another cell, or initiating its own suicide program by *cis* ligation of Fas. In this manner, only after T cells had lysed a number of target cells would they be likely to be eliminated, ensuring that the immune response is not terminated prior to eradication of the initial antigen.

C. OTHER CONSIDERATIONS

1. CTL Protection from Lysis

One of the questions arising in the study of CTL-mediated cytotoxicity is how the T cell is able to induce target cell lysis but remain intact itself. Studies have shown that CTLs and NK cells are much more resistant to lytic granule contents than other cells (Kranz and Eisen, 1987; Nagler-Anderson *et al.*, 1988; Golstein, 1974) and that this resistance correlates with cytotoxicity (Liu *et al.*, 1989). Certain mechanisms during T cell activation are in place to protect the CTL from its granule contents, such as the fact that granzymes are not activated from the zymogen form until they reach the granules, and the binding of proteoglycans to perforin and the granzymes to maintain them in an inactive state. However, it has been proposed that the CTL possesses certain membrane features which protects it from its lytic proteins once they are exocytosed (Jiang *et al.*, 1990; Müller and Tschopp, 1994; Blakely *et al.*, 1987; McFarland *et al.*, 1995; Martin *et al.*, 1988; Zalman *et al.*, 1988). Another possibility is that degranulation results in the exposure of a protective layer of proteoglycan on the CTL surface, which was on the inside surface of the lytic granule and is exposed by exocytosis, and protects the CTL from its lytic proteins.

Perforin is also inhibited by lipoproteins found in the serum, a mechanism believed to limit bystander lysis once perforin has been released from CTLs, and which may protect the CTL from its lytic proteins (Tschopp *et al.*, 1986).

2. The Role of Target Cell DNA Fragmentation

Although both granzyme A, and particularly granzyme B, have been shown to induce target cell DNA fragmentation (Shi *et al.*, 1992a, 1992b), and DNA fragmentation is seen in Fas-mediated cytotoxicity (Oshimi and Miyazaki, 1995; Enari *et al.*, 1995a; Owen-Schaub *et al.*, 1992), recent reports have shown that the target cell nucleus is not required for either mode of killing following CTL attack (Nakajima *et al.*, 1995b; Ucker *et al.*, 1992; Schulze-Osthoff *et al.*, 1994). Indeed, enucleated cytoplasts have been found to be as sensitive to CTL attack as the parental cell line (Nakajima *et al.*, 1995b). However, given the nature of the target cells recognized by CTLs, the role of CTL-induced DNA fragmentation may not be to promote apoptosis, but rather to degrade any viral or mutated DNA that may be present in the cell.

III. APOPTOSIS

When a CTL lyses a target cell, the target dies by undergoing a series of events termed apoptosis. Apoptosis was originally described as a morphological phenomenon occurring in two distinct phases (Kerr *et al.*, 1972). In phase I, the cytoplasm of the cell condenses but the morphology of the mitochondria and the ribosomes is maintained. The chromatin condenses and forms crescent-shaped aggregates lining the nuclear membrane and the nucleolus fragments. During this phase the cell's DNA is fragmented into oligonucleosomal-sized pieces, a process mediated by a Ca^{++}/Mg^{++}-dependent endonuclease. The nucleus eventually breaks up into several fragments. The plasma membrane also invaginates and the cell separates into membrane-bound fragments containing morphologically normal mitochondria and other organelles. These "apoptotic bodies" are rapidly phagocytosed during phase II either by surrounding cells or by macrophages (Schwartzman and Cidlowski, 1993; Savill *et al.*, 1993).

It has only been recently that some of the biochemical events involved in the induction of this form of cell death have been elucidated. Only mediators with some relevance to CTL-mediated cytotoxicity will be discussed, the reader is referred elsewhere for more in-depth reviews (Kroemer *et al.*, 1995; Schwartzman and Cidlowski, 1993).

A. THE ICE/CED-3 PROTEASES

1. ICE and a Possible Role in Apoptosis

In recent years, interest has focused on a family of cysteine proteases related to Interleukin-1β converting enzyme (ICE) as putative apoptotic mediators. ICE was originally identified as the proteolytic activity responsible for cleaving the inactive 33-kDa pro-IL-1β precursor to produce the active 17.5-kDa cytokine (Thornberry *et al.*, 1992; Cerretti *et al.*, 1992; Molineaux *et al.*, 1993; Miller *et al.*, 1993; Nett *et al.*, 1992). ICE itself is generated as an inactive precursor of 45-kDa. Proteolytic activation, involving cleavage after Asp residues, results in the production of the active form of the enzyme - a heterodimer consisting of p20 and p10 subunits (Thornberry *et al.*, 1992).

Early studies on IL-1β activation revealed that ICE has substrate specificity requiring Asp at P_1 (Sleath *et al.*, 1990), a finding confirmed by the discovery that IL-1β is activated by cleavage at two sites, each after an Asp residue (Howard *et al.*, 1991). Since this coincides with the cleavages occurring during ICE activation, it has been proposed that ICE may be autocatalytic (Thornberry *et al.*, 1992; Wang *et al.*,

1994a; Howard *et al.*, 1995). The crystal structure of active ICE seems to suggest a mechanism by which autoactivation can proceed. In the active form of ICE, two p10/p20 heterodimers form a homodimer which contains two active sites. If the homodimer is formed from two p45 precursors such that the p10 subunit of one precursor interacts with the p20 subunit of the opposing precursor, the cleavage sites required for activation are positioned correctly such that one precursor, once activated, can cleave and activate the other p45 (Walker *et al.*, 1994; Wilson *et al.*, 1994). Subsequent studies in baculovirus-infected *Sf*9 cells have confirmed that many family members (see below) are autocatalytic (Fernandes-Alnemri *et al.*, 1995a, 1995b).

Interest in ICE as an apoptotic mediator began when it was discovered that the protein product of the *Caenorhabditis elegans* gene *ced-3*, which had previously been shown to be essential for the induction of apoptosis during nematode development (Ellis and Horvitz, 1986; Yuan and Horvitz, 1990; Hedgecock *et al.*, 1983), shared 29% sequence identity with ICE (Yuan *et al.*, 1993). Overexpression of either ICE or Ced-3 in Rat-1 fibroblasts induced apoptosis, a property dependent upon the catalytic activity of ICE (Miura *et al.*, 1993). In addition, apoptosis of dorsal root ganglion neurons following growth factor withdrawal could be prevented by expression of the poxvirus-encoded serine protease inhibitor CrmA (cytokine response modifier A [Gagliardini *et al.*, 1994]), a serpin identified as an ICE inhibitor (Komiyama *et al.*, 1994; Ray *et al.*, 1992), further suggesting that ICE is involved in the induction of apoptosis.

Finally, use of the ICE inhibitors acetyl-Tyr-Val-Ala-Asp-aldehyde (Ac-YVAD-CHO) and acetyl-YVAD-chloromethylketone (AC-YVAD-cmk) arrested apoptosis of motoneurons *in vitro* as a result of neurotrophic factor withdrawal (Milligan *et al.*, 1995). Additionally, cell death of both motoneurons and interdigital cells was prevented *in vivo* using Ac-YVAD-cmk, further suggesting involvement of ICE (or a related protease, see below) during developmental cell death of both motoneurons and other cells.

Studies of mice lacking ICE have demonstrated that ICE does not play a unique role in apoptosis induced by dexamethasone or UV-radiation (Li *et al.*, 1995; Kuida *et al.*, 1995) but may play a role in Fas-mediated apoptosis (Kuida *et al.*, 1995). These results suggest that other members of the ICE/Ced-3 family (see below) play a more crucial role in the induction of apoptosis than ICE itself.

2. Other ICE/Ced-3 Proteases

Subsequent studies resulted in the isolation of a number of ICE/Ced-3 proteases. Each of these proteases is generated as an inactive precursor requiring cleavage after Asp residues for activation, and has substrate specificity requiring an Asp at P_1. Phylogenetic analysis has revealed that these proteases fall into three subfamilies (Duan *et al.*, 1996) - the ICE family (ICE, TX/Ich-2/ICE_{rel}II, ICE_{rel}III); the NEDD2 family (Ich-1 and Nedd-2); and the CPP32 family (consisting of Ced-3, CPP32/Apopain/Yama, Mch3α/CMH-1/ICE-LAP3, FLICE/MACH, and Mch2α). These families are summarized in Table I-4.

Some of these proteases occur in both active and alternately spliced, inactive forms, specifically ICE (Alnemri *et al.*, 1995); Ich1 (Wang *et al.*, 1994), Mch2 (Fernandes-Alnemri *et al.*, 1995a), Mch3 (Fernandes-Alnemri *et al.*, 1995b) and MACH (Boldin *et al.*, 1996). It has been postulated that these inactive isoforms may act as regulators of ICE/Ced-3 activity by sequestering active p10/p20 heterodimers in an inactive homodimer, since these inactive forms are still able to interact with an active isoform (Alnemri *et al.*, 1995) Therefore, a competition between cell death-protective and cell death-inducing subunits exists, and the result of this competition determines whether a cell lives or dies (Shaham and Horvitz, 1996). Indeed, Gu *et al.* (1995a) have recently shown that the ICE/Ced-3 precursors can form different quaternary structures *in vivo* and that there is hetero-oligomerization between family members, suggesting that the induction of apoptosis by mammalian proteases is a very complex process and depends upon the interactions of many proteins. Additionally, active heterodimers may contain subunits derived from different family members - it has recently been shown that the large subunit from Mch3α can interact with the small subunit from CPP32, and *vice versa,* to form an active complex *in vivo*, (Fernandes-Alnemri *et al.*, 1995b). In this regard, the ICE/Ced-3 family is similar to the Bcl-2 family of apoptotic regulators (see below) in that some members induce apoptosis while others protect against it and there is hetero-oligomerization between family members, suggesting that a cell's propensity to undergo apoptosis depends on the levels of each of the ICE/Ced-3 proteins within the cell.

As mentioned above, studies of ICE-deficient mice suggested that ICE does not play a unique role in the induction of apoptosis, and that other ICE/Ced-3 proteases play a more critical role. However, studies involving overexpression or "knockout" of a single family member should be treated with caution since different family members with the same target substrates are present within the same cell implying that no protease plays a unique role. Evidence is also accumulating to

TABLE I-4: THE ICE/CED-3 PROTEASES

protease	species	effect on apoptosis	substrates	References
The ICE Family:				
ICE	murine human	induction	pro-IL-1β PARP[1,2] CPP32 actin	Nett *et al.*, 1992 Thornberry *et al.*, 1992 Cerretti *et al.*, 1992 Gu *et al.*, 1995b Tewari *et al.*, 1995a Mashima *et al.*, 1995 Kayalar *et al.*, 1996
ICEβ	human	induction	?	Alnemri *et al.*, 1995
ICEγ	human	induction	?	Alnemri *et al.*, 1995
ICEδ	human	protection	NA[3]	Alnemri *et al.*, 1995
ICEε	human	protection	NA	Alnemri *et al.*, 1995
TX/Ich-2/ $ICE_{rel}II$	human	induction	PARP	Faucheu *et al.*, 1995 Kamens *et al.*, 1995 Munday *et al.*, 1995 Gu *et al.*, 1995b
$ICE_{rel}III$	human	induction	?	Munday *et al.*, 1995
The NEDD2 Family:				
Nedd-2	murine	induction	PARP	Kumar *et al.*, 1992, 1994 Gu *et al.*, 1995b
Ich-1L	human	induction	?	Wang *et al.*, 1994b
Ich-1S	human	protection	NA	Wang *et al.*, 1994b

[1]PARP - poly(ADP-ribose) polymerase

[2] at 50-100 times the concentration required for IL-1β activation

[3]NA - the alternately spliced, protective forms are inactive as proteases and therefore have no substrates

CONTINUED OVERLEAF

TABLE I-4: THE ICE/CED-3 PROTEASES (CONTINUED)

Protease	Species	effect on apoptosis	substrates	References
The CPP32 Family:				
Ced-3	*C.elegans*	induction	?	Yuan *et al.*, 1995
CPP32	human hamster	induction	PARP[1] pro-Mch3α U1-70kDa[2] DNA-PK[3] SREBPs[4] D4-GDI[5]	Fernandes-Alnemri *et al.*, 1994, 1995b Tewari *et al.*, 1995a Nicholson *et al.*, 1995 Lazebnik *et al.*, 1994 Casciola-Rosen *et al.*, 1996 Song *et al.*, 1996 Wang *et al.*, 1995a, 1996 Na *et al.*, 1996
Mch2α	human	induction	lamin A	Fernandes-Alnemri *et al.*, 1995a Orth *et al.*, 1996
Mch2β	human	protection	NA[6]	Fernandes-Alnemri *et al.*, 1995a
Mch3α/ CMH-1/ ICE-LAP3	human	induction	PARP SREBPs	Fernandes-Alnemri *et al.*, 1995b Lippke *et al.*, 1996 Duan *et al.*, 1996 Pai *et al.*, 1996
Mch3β	human	protection	NA	Fernandes-Alnemri *et al.*, 1995b
FLICE/ MACH	human	induction	PARP	Boldin *et al.*, 1996 Muzio *et al.*, 1996

[1]PARP - poly(ADP-ribose) polymerase
[2]U1-70kDa - 70-kDa protein component of the U1 small nuclear ribonucleoprotein
[3]DNA-PK - DNA-dependent protein kinase (catalytic subunit)
[4]SREBP - sterol regulatory element binding protein
[5]D4-GDI - hematopoietic cell GDP dissociation inhibitor of Rho family GTPases
[6]NA - the alternately spliced, protective forms are inactive as proteases and therefore have no substrates

suggest a proteolytic cascade during apoptosis, as some of the ICE/Ced-3 proteases are able to activate other family members. For example, ICE may be autocatalytic (Thornberry *et al.*, 1992; Quan *et al.*, 1996) and can activate CPP32 (Tewari *et al.*, 1995a) while CPP32 can activate Mch3α but not *vice versa* (Fernandes-Alnemri *et al.*, 1995b) suggesting sequential activation of ICE, CPP32 and Mch3α during apoptosis. To this end, Enari *et al.* (1996) have recently demonstrated sequential activation of ICE-like and CPP32-like proteases in Fas-mediated apoptosis. This may constitute a central death pathway with the cell being able to enter the pathway at different points, depending on the apoptotic stimulus.

Besides the previously identified substrates of ICE/Ced-3 proteases - pro-IL-1β, sterol regulatory element binding proteins (SREBPs), poly(ADP-ribose) polymerase (PARP), the 70-kDa protein component of the small nuclear ribonucleoprotein (U1-70kDa), DNA-dependent protein kinase (DNA-PK), D4-GDI, actin and lamin A - a number of other proteins which are proteolytically cleaved during apoptosis may also serve as substrates including the protein kinase C δ isoform (Emoto *et al.*, 1995), the cytoskeletal proteins Gas2 and fodrin (Brancolini *et al.*, 1995; Martin *et al.*, 1995b), other nuclear lamins (Lazebnik *et al.*, 1995; Weaver *et al.*, 1996) and possibly the larger isoforms of the PITSLRE family of cdc2-related kinases (Lahti *et al.*, 1995). In addition, the nuclear-mitotic apparatus (NuMA) is cleaved during apoptosis at levels comparable to the degree of oligonucleosomal DNA laddering (Hsu and Yeh, 1996; Weaver *et al.*, 1996). These results suggest that the nuclear degradation seen during apoptosis may be a result of site-specific proteolysis of key structural proteins.

3. ICE/Ced-3 Proteases and Autoimmunity

Interestingly, three of the proteins identified as ICE/Ced-3 substrates (U1-70kDa, DNA-PK, PARP) have been identified as autoantigens involved in autoimmune disease. These autoantigens, along with others targeted in the human autoimmune disease systemic lupus erythrematosus, are found clustered in two discrete populations of surface structures on apoptotic cells: apoptotic bodies arising from the condensed, fragmented nucleus, and surface blebs arising from the fragmented rough ER (Casciola-Rosen *et al.*, 1994a). This clustered targeting of antigens is thought to reflect the susceptibility of these molecules to a common biochemical modification occurring in apoptotic cells. Since these proteins are now known to be proteolytically processed during apoptosis to distinct fragments by the ICE/Ced-3 proteases (Casciola-Rosen *et al.*, 1994b, 1995, 1996; Lazebnik *et al.*,

1994; Kaufmann *et al.*, 1993), this implies a link between these proteases and autoimmunity.

During the selection of the TCR repertoire in the thymus T cells are exposed to self peptides in the context of self MHC presented by thymic APCs. Any T cell which recognizes and responds to these complexes is deleted such that the final TCR repertoire should recognize only foreign antigen-self MHC complexes. In this manner, self-tolerance is induced. However, tolerance is only induced to self peptides displayed at sufficiently high levels. Some T cells are able to escape tolerance induction, even though they recognize self antigen, because their cognate peptides are not efficiently presented in the thymus, tolerance being induced only to efficiently presented (immunodominant) determinants. Although such T cells are potentially autoreactive, under normal circumstances these cells do not participate in immune responses because the body's APCs do not efficiently present their cryptic (subdominant) determinants (Gammon and Sercarz, 1989; Sercarz *et al.*, 1993; Lipham *et al.*, 1991; Cibotti *et al.*, 1992). However, if their cryptic determinant is subsequently revealed, these autoreactive cells may become pathogenic and initiate an immune response. As the immune response develops, the tolerance to immunodominant determinants may also be broken (Mamula *et al.*, 1992; Mamula, 1993) resulting in the initiation of autoimmune disease.

It has been suggested that the specific proteolysis occurring during apoptosis, which results in the appearance of novel peptide fragments, may also result in unveiling of a cryptic determinant. Interestingly, Casciola-Rosen *et al.* (1995) have found that autoimmune antisera from several different individuals specifically recognizes proteolytic fragments generated during apoptosis. These workers propose a model for autoimmunity whereby apoptosis results in the cleavage of U1-70kDa, PARP, DNA-PK and other autoantigens within a cell by ICE/Ced-3 proteases. When the apoptotic bodies of the cell are phagocytosed, peptides derived from proteins found in these bodies are presented in the context of MHC Class II molecules on the surface of the phagocytic cell. If a protein fragment generated during proteolysis by the ICE/Ced-3 proteases contains a cryptic determinant which is efficiently presented, this may trigger an autoimmune response mediated by T cells which escaped tolerance induction. If tolerance to the self protein is thus broken, this autoreactive T cell response may subsequently diversify to other areas of the molecule which were previously tolerated and autoimmune disease ensues.

In this manner, apoptosis may result in induction of an autoimmune response to proteins which were previously tolerated, by the activity of the ICE/Ced-3

proteases and generation of specific protein fragments. One important consideration in this model, however, is the mechanisms of apoptosis by thymocytes. When thymocytes are eliminated during selection, they die by apoptosis (Surh and Sprent, 1994). It is therefore necessary to determine whether the specific proteolytic events occurring in "general" apoptosis also occur in apoptosis during thymocyte selection. If the thymocytes do not utilize similar proteolytic events, this could explain the persistence of potentially autoreactive T cells. That is, that they were not exposed to the necessary peptide antigens (generated by apoptosis of neighbouring cells) during selection. It remains to be seen whether the ICE/Ced-3 proteases play a significant role in apoptosis during selection of the TCR repertoire.

4. ICE/Ced-3 Proteases and CTL-Mediated Cytotoxicity

ICE/Ced-3 proteases have also been implicated as playing a role in target cell death following CTL attack. The finding that ICE is activated by cleavage after Asp residues, which coincides with the substrate specificity of granzyme B, led to the hypothesis that the ICE precursor could act as a granzyme B substrate (Vaux *et al.*, 1994). In this proposal, CTL-mediated killing would involve a cascade of proteolytic events, in much the same manner as the blood clotting pathway. Although we have found no evidence that the ICE precursor acts as a granzyme B substrate (Chapter III, Darmon *et al.*, 1994) this did not preclude another ICE/Ced-3 protease from being a granzyme B target. Indeed we (and others) have recently shown that the precursor of CPP32 acts as an intracellular substrate for granzyme B (Chapter IV, Darmon *et al.*, 1995; Quan *et al.*, 1996; Martin *et al.*, 1996). In addition, other workers have demonstrated that granzyme B can activate the related proteases CMH-1/Mch3α/ICE-LAP3 (Gu *et al.*, 1996; Chinnaiyan *et al.*, 1996b) and FLICE/MACH (Muzio *et al.*, 1996). Since CPP32 has been shown to cleave a number of nuclear proteins such as PARP (Tewari *et al.*, 1995a; Nicholson *et al.*, 1995), SREBPs (Wang *et al.*, 1996, 1995a), U1-70kDa (Casciola-Rosen *et al.*, 1996) and DNA-PK (Casciola-Rosen *et al.*, 1996; Song *et al.*, 1996), it is believed that CPP32 is involved primarily in the induction of the nuclear events of apoptosis, and that granzyme B induces target cell DNA fragmentation by cleaving and activating CPP32 (and other CPP32-related proteases). We have recently obtained evidence to support this finding (Chapter V; Darmon *et al.*, 1996). It is not known how granzyme B induces membrane blebbing, if at all, but presumably this occurs by cleavage of another substrate, possibly another ICE/Ced-3 protease.

Accumulating evidence suggests that ICE/Ced-3 proteases are also involved in Fas-mediated cytotoxicity. Both CrmA, and another virus-encoded antiapoptotic protein which inhibits ICE/Ced-3 proteases, p35 (Clem *et al.*, 1991; Bump *et al.*, 1995; Xue and Horvitz, 1995; Clem and Miller, 1994) can inhibit Fas-mediated apoptosis when expressed in cells exposed to anti-Fas antibody (Beidler *et al.*, 1995; Tewari and Dixit, 1995) and in particular could inhibit cleavage of U1-70kDa (Tewari *et al.*, 1995b) or PARP (Beidler *et al.*, 1995). Although both U1-70kDa and PARP are substrates of CPP32 (and related proteases), CrmA reportedly cannot inhibit CPP32 (Nicholson *et al.*, 1995; Fernandes-Alnemri *et al.*, 1995b), although this is somewhat controversial (Tewari *et al.*, 1995a), suggesting that CrmA acts at a point other than CPP32. Using CTLs derived from human peripheral blood as effectors, Tewari *et al.* (1995c) showed that CrmA could inhibit CTL-mediated cytotoxicity and that, despite the fact that CrmA had previously been shown to inhibit granzyme B (Quan *et al.*, 1995), the inhibitory effect of CrmA expression was on the calcium-independent (Fas-mediated) pathway of cytolysis. Although this is convincing evidence of the involvement of an ICE/Ced-3 protease, the specific protease involved cannot be identified since each of these proteins (CrmA and p35) can inhibit multiple family members.

Other groups have reported the inhibitory effect of a tetrapeptide aldehyde containing the sequence YVAD (single letter amino acid code) on Fas-mediated apoptosis (Enari *et al.*, 1995a; Los *et al.*, 1995). This YVAD sequence was originally identified as an ICE inhibitor although it may also inhibit other family members, again leaving the exact nature of the protease involved unclear. Using an *in vitro* system, several groups have shown that lysates from anti-Fas treated cells can induce apoptotic changes in the nuclei of normal cells, and that this can be inhibited by the YVAD aldehyde (Enari *et al.*, 1995b; Chow *et al.*, 1995; Schlegel *et al.*, 1995), again suggesting the involvement of an ICE/Ced-3 protease.

Although this evidence seems to suggest the involvement of a protease closely related to ICE in Fas-mediated killing, Schlegel *et al.* (1996) have found no evidence of IL-1β processing activity in lysates from cells treated with anti-Fas antibody, a result which we have confirmed (Chapter VI; Darmon and Bleackley, 1996) indicating that this ICE-like activity is not due to ICE itself. In fact, Schlegel *et al.* determined that CPP32 is a key mediator in Fas-induced apoptosis, however, we believe that this is a consequence of their model system - in our hands CPP32 is not activated in all cells in response to Fas ligation and is therefore not essential (Chapter VI; Darmon and Bleackley, 1996).

Interestingly, apoptosis mediated by the *Drosophila* protein REAPER, which contains a death domain similar to that found in Fas, has been shown to require an ICE-like protease for the induction of both apoptosis and ceramide release, which seems to place an ICE-like protease very early in the signaling pathway initiated by Fas ligation (Pronk *et al.*, 1996). Significantly, the identification of FLICE/MACH as an ICE/Ced-3 protease which interacts with FADD/MORT1 seems to link Fas ligation with the activation of ICE/Ced-3 proteases (Boldin *et al.*, 1996; Muzio *et al.*, 1996). Presumably, activation of FLICE/MACH following Fas ligation results in the activation of downstream proteases such as those related to CPP32, although this is yet to be demonstrated. The recent demonstration of sequential activation of ICE-like and CPP32-like proteases during Fas-mediated apoptosis (Enari *et al.*, 1996) may support this role for FLICE/MACH in the induction of cell death.

B. BCL-2-RELATED PROTEINS

1. The Bcl-2 family

Another family of proteins implicated in the control of apoptosis correspond to the members of the Bcl-2 family and the proteins with which they interact. Bcl-2 was first identified as the gene involved in the development of human follicular lymphoma (Tsujimoto *et al.*, 1984). The chromosomal 14:18 translocation associated with these cancers brings the *bcl-2* gene under control of the immunoglobulin heavy chain enhancer, resulting in constitutively high expression of Bcl-2 in B cells containing the translocation (Graninger *et al.*, 1987). Since the translocation usually occurs within the 3' untranslated region of *bcl-2* the resulting protein is not altered. Because of its association with cancer, Bcl-2 was believed to represent an oncogene, however, studies of transfections revealed that Bcl-2 actually functions by inhibiting apoptosis rather than by promoting cell growth (reviewed by Hawkins and Vaux, 1994). Since its first discovery Bcl-2 has been found to be capable of protecting cells against a wide variety of insults, including growth factor withdrawal, hypoxia, heat shock and drugs.

Further evidence suggesting a role for Bcl-2 in controlling apoptosis came from studies of the nematode *C. elegans*. The *C. elegans* cell death gene *ced-9*, which protects cells against cell death (and is therefore antagonistic to Ced-3), encodes a member of the Bcl-2 family of proteins (Hengartner *et al.*, 1992; Hengartner and Horvitz, 1994). Ced-9 and Bcl-2 are functionally related and Bcl-2 can substitute for Ced-9 *in vivo* (Vaux *et al.*, 1992a), further suggesting a role for Bcl-2 in inhibiting apoptosis.

Other proteins related to Bcl-2 have since been identified and are summarized in Table I-5 (reviewed by Farrow and Brown, 1996; White, 1996). These proteins form homo- and heterodimers *in vivo* with other members of the family, much like the ICE/Ced-3 proteins. Bax was the first related protein to be identified and although it was originally believed that Bax may work in concert with Bcl-2 to prevent cell death, it is now known that Bax actually opposes Bcl-2 function and induces cell death. The current model for Bcl-2 and Bax control of cell death proposes that they act as a "rheostat" - when Bcl-2 is in excess, Bcl-2 homodimers predominate and the cell is protected against apoptosis. When Bax is in excess, Bax homodimers predominate and the cell is susceptible to apoptosis in response to an external signal. Since the other family members can also dimerize a cell's propensity to die in response to an apoptotic signal may depend upon the total number of apoptotic inducers versus the total number of apoptotic protectors. Similar interactions may occur within the ICE/Ced-3 family, as mentioned above, since some members are protectors and some are inducers. Therefore, for the same reasons outlined for the ICE/Ced-3 proteases, studies of overexpression or "knockout" of only one family member must be approached with caution.

2. Role of Bcl-2 Proteins in CTL-Mediated Cytotoxicity

The mechanism of action of the Bcl-2 family proteins is currently unknown. These proteins possess no known catalytic activity making elucidation of their roles more difficult. As a result, cellular localization was used in an attempt to generate hints as to their function. Localization of Bcl-2 to mitochondrial, rough ER and nuclear membranes (Hockenberry *et al.*, 1990; Krajewski *et al.*, 1993; Lithgow *et al.*, 1994) led to the suggestion that Bcl-2 and related proteins may be involved in regulating the levels of reactive oxygen species (ROS) in cells (reviewed by Slater *et al.*, 1995; Korsmeyer *et al.*, 1995; Hockenberry *et al.*, 1993). In this model of apoptosis, ROS are generated early during the apoptotic process, possibly as a result of mitochondrial oxidative metabolism or through the arachidonic acid pathway of signaling, and are critical to the completion of cell death. Mitochondrial respiration is an unlikely source of ROS however, since apoptosis still occurs in cells lacking mitochondrial DNA (Jacobson *et al.*, 1993) suggesting that the arachidonic acid pathway, initiated by phospholipase A_2 (PLA_2), is the primary source of ROS during apoptosis. In normal cells, Bcl-2 proteins may control the levels of ROS and maintain the cell in its usual oxidation state. During apoptosis the levels of ROS generated may surpass the "buffering" capacity of the Bcl-2 proteins and may have a

number of effects in the apoptotic cell - direct oxidative damage to DNA and oxidation of intracellular proteins (which may modify their function or ability to be recognized by other proteins). Interestingly, this may also represent a means of "tagging" proteins for degradation by cellular proteases, particularly the ICE/Ced-3 proteins. Two studies have recently shown that Bcl-2 (Chinnaiyan *et al.*, 1996c; Boulakia *et al.*, 1996) and BclxL (Chinnaiyan *et al.*, 1996c), which repress a common cell death pathway (Chao *et al.*, 1995), act upstream of the ICE/Ced-3 proteases CPP32 and Mch3α/CMH-1/ICE-LAP3. In the ROS-mediated model of apoptosis, Bcl-2 and Bcl-xL block the production of excess ROS which thereby halts oxidative modification of cellular proteins. Since cellular proteins would not be tagged for degradation, the ICE/Ced-3 proteases would not recognize their target proteins and apoptosis would not proceed. However, under hypoxic conditions, under which production of ROS is expected to be minimal, Bcl-2 can still protect against apoptosis suggesting that ROS are not required for apoptosis and that Bcl-2 functions independently of ROS (Jacobson and Raff, 1995; Shimizu *et al.*, 1995). Therefore, the exact roles of ROS and the Bcl-2 proteins in apoptosis remain to be determined.

The implication of Bcl-2 in regulating ROS-induced apoptosis is intriguing in light of the fact that PLA_2 has been shown to be activated during Fas-mediated apoptosis (Cifone *et al.*, 1995). In addition, thiol reductants and antioxidants have been shown to have a protective effect against both TNF- and Fas-mediated killing, further implicating ROS as playing a role in cell death in response to Fas ligation (Um *et al.*, 1996; Talley *et al.*, 1995; Albrecht *et al.*, 1994; Chang *et al.*, 1992; Mayer and Noble, 1994; Schulze-Osthoff *et al.*, 1992). It would therefore be expected that Bcl-2 proteins would be able to "buffer" the levels of ROS generated by PLA_2 in response to Fas ligation, and therefore protect against Fas-induced apoptosis. However, this result has not been consistently produced - some groups have reported that Bcl-2 can protect against Fas-induced death (Schröter *et al.*, 1995; Torigoe *et al.*, 1994; Itoh *et al.*, 1993) while others have found no effect (Chiu *et al.*, 1995; Memon *et al.*, 1995; Vaux *et al.*, 1992b). Jaattela *et al.* (1995) have suggested that Bcl-2 and Bcl-xL inhibit activation of PLA_2 following Fas ligation, suggesting that the Bcl-2 proteins act upstream of arachidonic acid metabolism. Strasser *et al.* (1995) have recently shown that Bcl-2 and Fas regulate distinct pathways to cell death so the exact role of Bcl-2 proteins in this mode of killing remains to be determined.

TABLE I-5: THE BCL-2 FAMILY PROTEINS

Protein	Effect on Apoptosis	Reference
Bcl-2	protection	Vaux *et al.*, 1988 Hockenberry *et al.*, 1990
Ced-9	protection	Hengartner *et al.*, 1992 Hengartner and Horvitz, 1994
Bax	induction	Oltvai *et al.*, 1993
BclxL	protection	Boise *et al.*, 1993
BclxS	induction	Boise *et al.*, 1993
Bak	induction	Farrow et al., 1995 Chittenden *et al.*, 1995 Kiefer *et al.*, 1995
BAG-1[1]	protection	Takayama *et al.*, 1995
BAD	induction	Yang *et al.*, 1995
Mcl-1	protection	Kozopas *et al.*, 1993 Reynolds *et al.*, 1994
A1	protection[2]	Lin *et al.*, 1993
Nbk/Bik1[1]	induction	Boyd *et al.*, 1995
African Swine Fever Virus HMW5-HL	protection[2]	Neilan *et al.*, 1993
Epstein Barr Virus BHRF1	protection	Cleary *et al.*, 1986 Henderson *et al.*, 1993
Adenovirus E1B 19K	protection	White *et al.*, 1992 Chiou *et al.*, 1994 Farrow *et al.*, 1995

[1] these proteins share no homology to Bcl-2 but act as binding partners
[2] based on sequence homology to Bcl-2

In contrast, there is no evidence to suggest the involvement of PLA_2 in granule-mediated killing so Bcl-2 family members would not be expected to protect against granule-mediated cytotoxicity. Indeed, some workers have found this to be the case (Chiu *et al.*, 1995) although again this has not been consistently produced, with other workers reporting that Bcl-2 protects target cells during granule-mediated killing (Schröter *et al.*, 1995). One group has reported that Bcl-2 protects against neither Fas nor granule-mediated killing (Vaux *et al.*, 1992b) so the exact role of Bcl-2 proteins in CTL-mediated cytotoxicity remains unknown. Interestingly, Takayama *et al.* (1995) have shown that co-expression of BAG-1 and Bcl-2 protects cells against several stimuli, including CTL attack, whereas Bcl-2 alone does not, suggesting that expression of both BAG-1 and Bcl-2 may be required to see an effect.

C. A CELL CYCLE LINK

Interestingly, some of the events of apoptosis, such as chromatin condensation and nuclear membrane dissolution, are mirrored in mitosis, leading to a possible cell cycle link between apoptosis and cellular proliferation. Indeed, Nishioka and Welsh (1994) have found that quiescent cells (in the G_0 stage of the cell cycle) are more resistant to CTL-induced DNA fragmentation than G_1 cells, suggesting that susceptibility to apoptosis may be linked to cell cycle stage.

Progression through the cell cycle is regulated by kinases, and kinase activity is dependent upon association with cyclin molecules. Thus, these kinases are termed cyclin-dependent kinases or cdks. The G_2/M transition during the cell cycle is dependent upon the serine/threonine kinase Cdc2 (also known as cdk1) (Nurse, 1990). During the S and G_2 stages, Cdc2 is found in association with cyclin B, and is maintained in a catalytically inactive state by phosphorylation of Tyr^{15}, located within the ATP-binding region of Cdc2. This phosphorylation is carried out by the kinase Wee1 or Mik1 (Parker and Piwnica-Worms, 1992; McGowan and Russell, 1993; Heald *et al.*, 1993; Lundgren *et al.*, 1991). Wee1 in turn is regulated by the kinase Nim1/Cdr1 (Wu and Russell, 1993; Parker *et al.*, 1993; Coleman *et al.*, 1993). At the onset of mitosis, Cdc2 is activated by dephosphorylation, a function carried out by Cdc25 (Gould *et al.*, 1990). In addition to Cdc25, Wee1, Mik1, and Nim1/Cdr1, Cdc2 activity is also regulated by Cdk2 which plays an essential role in regulating G_1/S transition (Guadagno and Newport, 1996).

Inappropriate activation of Cdc2 or Cdc25 (which activates Cdc2) causes cells to undergo mitotic catastrophe (Heald *et al.*, 1993; Krek and Nigg, 1991), a process which morphologically resembles apoptosis. Interestingly, premature activation of

Cdc2 has been observed in some forms of apoptosis, for example, Cdc2 is activated during taxol-induced apoptosis (Donaldson *et al.*, 1994); Cdc2 and Cdk2 kinase activity, associated with cyclin A, increases following TNFα or drug-induced death of HeLa cells (Meikrantz *et al.*, 1994); and activity of the Cdc2/cyclin B complex increases during activation-induced T cell death, a process requiring cyclin B expression (Fotedar *et al.*, 1995). Interestingly, Schröter *et al.* (1996) have recently suggested that activation of Cdc2 may be linked to DNase I activity, implying a connection between cell cycle proteins and DNA fragmentation.

Cdc2 has also been implicated in CTL-mediated cytotoxicity. In 1994, Shi *et al.* found that Cdc2 activation, through dephosphorylation, occurred during apoptosis of YAC-1 cells treated with perforin and granzyme B, and that this activation was necessary since blocking Cdc2 activity could prevent granzyme B-induced death. Further studies revealed that Wee1, the kinase which negatively regulates Cdc2 activity, rescues target cells from granzyme B/perforin-induced apoptosis (Chen *et al.*, 1995), thereby strengthening the link between CTL-mediated cytotoxicity and cell cycle regulation. It is not known how granzyme B can result in Cdc2 activation, since Cdc2 is not cleaved during granzyme B-induced death.

Cdc2 is not involved in all forms of apoptosis however. As mentioned above, Meikrantz *et al.* (1994) have found that levels of Cdk2/cyclin A kinase activity increase during TNFα or drug-induced apoptosis in HeLa cells. Apoptosis in this system can be inhibited by expression of dominant negative mutants of Cdc2, Cdk2, and Cdk3, but not by dominant negative mutants of Cdk5. It was also found that Cdc2, Cdk2, and Cdk3 formed complexes with cyclin A *in vivo*, while Cdk5 did not, suggesting a role for cyclin A-associated kinases in apoptosis (Meikrantz and Schlegel, 1996). In addition, a kinase distantly related to Cdc2 called p58PITSLRE induces apoptosis when overexpressed (Lahti *et al.*, 1995). Intriguingly, larger isoforms of the PITSLRE family are proteolytically cleaved to a novel, catalytically active 50-kDa form during Fas-mediated killing. Although some results suggest that a serine protease is involved, it is quite possible that this cleavage is mediated by an ICE/Ced-3 protease.

In other systems, apoptosis is able to proceed in the absence of Cdc2/cyclin B activity. Neurons, which are fully differentiated and postmitotic, are still able to undergo apoptosis even though Cdc2 is not expressed, although cyclin D1 is induced during cell death (Freeman *et al.*, 1994) and is an essential mediator (Kranenburg *et al.*, 1996). Thymocytes treated with etoposide or dexamethasone do not contain increased Cdc2 activity (Norbury *et al.*, 1994), a result which is consistent with the

findings of Fotedar *et al.* (1995), who showed that dexamethasone-induced thymocyte apoptosis was not inhibited by the expression of antisense cyclin B. Thus, it seems that the involvement of Cdc2 and/or other cell cycle proteins is determined by the stimulus which induces apoptosis, and, within the same cell, the pathway culminating in apoptosis may or may not involve Cdc2/cyclin B.

IV. AIMS OF THE THESIS

Although 10 years had elapsed since the first discovery of the granzymes, the exact mechanism of their action was still unresolved at the beginning of this work. Both granzyme A and granzyme B had been shown to play a role in inducing target cell DNA fragmentation, but the exact events involved were unclear, and it had been widely assumed that this was achieved through the activation of an endonuclease, yet to be identified.

Therefore, it was of prime interest to identify intracellular substrates for the granzymes. The first half of the work presented here outlines studies resulting in the identification of an intracellular substrate for granzyme B, which turns out to be an ICE/Ced-3 protease. The second part of this work examines the role of this substrate, and other ICE/Ced-3 proteases, in both granule- and Fas-mediated cytotoxicity.

CHAPTER II - MATERIALS AND METHODS

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CELL CULTURE

Effector Cells

CTL21.9 is a cloned cytotoxic T cell line using the granular exocytosis mechanism of killing (Garner *et al.*, 1994) that requires antigenic stimulation and is IL-2-dependent. These cells were stimulated on a 7-day cycle with irradiated splenocytes from Balb/c (H-2^d) mice as stimulators for 3 days at a stimulator:responder ratio of 20:1. Following the three day incubation, dead cells were removed by sedimentation through a Ficoll/metrizoate gradient. Cells were maintained in RHFM (RPMI 640 media [Gibco] supplemented with 5% (v/v) fetal calf serum (Gibco) and 100 μM β-mercaptoethanol) and containing 60 units/ml recombinant IL-2.

C57BL/6J (H-2^b) mice homozygous for a null mutation in the granzyme B gene, and wild type control mice, were a generous gift of Dr. Tim Ley (Washington University Medical School, St. Louis, MO) and have been previously described (Heusel *et al.*, 1994). Cytotoxic cells lacking granzyme B (GB KO) or wild type control cells (GB WT) were generated as follows: Splenocytes from these mice were activated in primary mixed lymphocyte cultures in the presence of IL-2 using irradiated splenocytes from Balb/c mice as stimulators for three days. Dead cells were removed by sedimentation through a Ficoll/metrizoate gradient. The initial stimulation used a stimulator:responder ratio of 1:1; all subsequent stimulations used a ratio of 20:1. The resulting cells were maintained in RHFM plus IL-2, and stimulated on a 7-day cycle as outlined for CTL21.9 cells (above).

All of the above cells were activated with anti-CD3 antibody (2C11, 1:500 dilution) for 24 hours prior to use as cytotoxic effectors.

PMM-1 is a cytolytic hybridoma previously derived from primary peritoneal exudate lymphocytes (Kaufmann *et al.*, 1981) and is grown by continuous culture in RHFM in 5% CO_2 at 37°C. Activation for use as cytotoxic cells was achieved by stimulation for 3 hours with phorbol-12-myristate-13-acetate (PMA, 10 ng/ml) and ionomycin (3 μg/ml).

"Target" Cells

The L1210 (H-2^d) variant transfected with mouse Fas cDNA (L1210-Fas) was kindly provided by Dr. Pierre Golstein (Centre d'Immunologie INSERM-CNRS de Marseille-Luminy, Marseille, France; Rouvier *et al.*, 1993) and is maintained by

continuous culture in RHFM. EL4 (H-2^b) and YAC-1 (H-2^a) mouse lymphomas, P815 (H-2^d) mouse mastocytoma cells, and the human T cell leukemia line Jurkat were maintained by continuous culture in RHFM.

Other Cells

COS-M5 cells were grown at 37°C, 5% CO_2 in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal calf serum, penicillin and streptomycin.

The cytotoxic T cell line MTL 2.8.2 was previously generated from CBA/Balb/c mice (Bleackley *et al.*, 1982) and is an IL-2-dependent cell line that can proliferate in the absence of antigen. These cells were cultured in RHFM containing 30 units/ml recombinant human IL-2.

TRANSFECTION OF COS CELLS

Granzyme B Expression Vector

An *Eco*RI fragment containing the cDNA encoding enzymatically active murine granzyme B (CCP1a; Caputo *et al.*, 1993) was subcloned into the mammalian expression vector pAX142 which contains the EF1α promoter, enhancer and introns (a generous gift of Dr. Robert Kay), a derivative of the vector pAX114 (Kay and Humphries, 1991). This vector was used in preference to other expression systems since we have previously found that murine granzyme B contains a cryptic splice site which results in truncated transcripts if vectors containing the SV40 small t-antigen intron are used (Fujinaga and Bleackley, unpublished data). The cDNA encoding the zymogen form of murine granzyme B (CCP1) and an active site Ser-Ala mutant (S183A; Caputo *et al.*, 1994) were also subcloned into this expression plasmid.

Preparation of DNA for Transfections

DNA for COS cell transfections was prepared by CsCl density gradient centrifugation as follows: overnight cultures of bacteria were grown in LB media plus antibiotics. Bacteria were collected by centrifugation at 6000 rpm for 10 minutes, and resuspended in 10 ml GTE (0.9% glucose; 25 mM Tris-Cl, pH 8.0; 10 mM EDTA). Lysozyme was then added to the bacteria (10 ml of a 10 mg/ml solution in GTE) and the cell suspension was incubated at room temperature for 10 minutes. Bacteria were lysed by the addition of 40 ml of 0.2 M NaOH, 1% SDS followed by incubation on ice for 10 minutes, and then cellular debris was precipitated by the addition of 30 ml 3 M potassium acetate (pH ~5.5). Following incubation on ice for 10 minutes, cellular debris was pelleted by centrifugation at 8000g for 10 minutes.

The supernatant was decanted and DNA was precipitated by the addition of 90 ml of isopropanol, and pelleted (following incubation at room temperature for 10 minutes) by centrifugation at 15,000g for 30 minutes. The pellet was washed with 70% ethanol, and dried in a desiccator. The DNA pellet was resuspended in 4 ml TE, to which was added 4.4 g CsCl, 0.4 ml 10 mg/ml ethidium bromide. Any precipitate formed was removed by centrifugation at 15,000g for 10 minutes. The supernatant at this point was transferred to a polyallomer ultracentrifuge tube (Seton Scientific) and spun in a VTi65 rotor at 58,000 rpm at 20°C overnight (16 hours minimum).

The following day the plasmid band was removed from the ultracentrifuge tube using a syringe and needle, and then extracted three times with an equal volume of NaCl-saturated isopropanol in order to remove the ethidium bromide. The DNA was then precipitated by addition of two volumes of TE, NaCl to a final concentration of 0.1 M, and finally ethanol (two volumes). The sample was then incubated at -20°C for 30 minutes. DNA was isolated by centrifugation at 15,000g for 30 minutes, the pellet was washed with 70% ethanol, and then resuspended in TE. Quantitation was by ethidium bromide fluorescence using calf thymus DNA as a standard.

Transfection of COS Cells

Transient transfections of COS cells were performed by the DEAE-dextran method as follows: cells were seeded at a density of 10^6 cells per 90-mm plate and allowed to grow overnight. Transfection was by addition of 5 ml Dulbecco's modified Eagle's medium containing 10 mM Hepes, pH 7.15; 200 µg/ml DEAE-dextran; 0.8 µg/ml plasmid DNA; and 500 µM chloroquine for 6 hours at 37°C, 5% CO_2. Transfection medium was removed following this incubation and cells were shocked for one minute by addition of 3 ml of 10% dimethyl sulfoxide (DMSO) in phosphate-buffered saline (PBS) containing 0.5 mM $CaCl_2$ and 1.0 mM $MgCl_2$. Cells were rinsed with PBS and then culture continued by addition of 10 ml of fully-supplemented Dulbecco's modified Eagle's medium at 37°C, 5% CO_2 until 48 hours following addition of the DNA.

Generation of COS Cell Lysates

Suspensions of COS cells were prepared 48 hours following addition of the DNA by treatment with trypsin followed by suspension in 10 ml fully-supplemented cell culture medium. Cells were washed with 10 ml PBS, and lysed by suspension in COS cell lysis buffer containing 1% Triton X-100; 50 mM Tris, pH 8.0; and 1 M NaCl (200 µl/2x10^6 cells), followed by incubation on ice for 30 minutes. Nuclei were

removed by centrifugation at 10,000g, for 10 minutes at 4°C. Supernatants were stored at -70°C until use. Protein concentrations were determined by the bicinchoninic acid (BCA) assay kit (Pierce) using bovine serum albumin as a standard.

ISOLATION OF PRO-mICE cDNA

Designing Primers for PCR of pro-mICE

Oligonucleotide primers for PCR amplification of the murine ICE cDNA were based on the published sequence of mICE (Nett *et al.*, 1992, Genbank accession number L03799) and had the following sequences:

pICE1: 5'-GGAA**GGATCCGC**ATGGCTGACAAGATCCTG-3' (forward primer containing *Bam*HI site (bold) and initiation codon (underlined)); and

pICE2: 5'-GGAA**CTGCAG**TCATCTAAGGAAGTATTGG-3' (reverse primer containing *Pst*I site (bold)).

Primers were synthesized on a model 392 DNA/RNA Synthesizer (Applied Biosystems Inc.).

cDNA Synthesis

RNA was isolated from the cytotoxic T cell line MTL 2.8.2 using guanidinium thiocyanate (Chirgwin *et al.*, 1979). RNA samples were denatured by incubating 5 µg of total RNA with 250 ng oligo dT primer (Promega) in a total volume of 14 µl at 96°C for 3 min. Samples were chilled on ice and reagents added in the following order (final concentrations given): 50 mM Tris, pH 8.3; 75 mM KCl; 3 mM $MgCl_2$; 10 mM dithiothreitol; 50 units RNasin (Promega) and 1 mM dNTPs in a final volume of 25 µl. The reaction mix was preincubated at 37°C prior to addition of 100 units Moloney murine leukemia virus reverse transcriptase (Bethesda Research Laboratories). Reactions were incubated at 37°C for 30 min followed by 99°C for 5 min. 5 µl of the reverse transcriptase reaction was used directly in the PCR reaction.

PCR of pro-mICE cDNA

Each 50 µl PCR reaction contained 10 mM Tris, pH 8.3; 50 mM KCl; 0.01% gelatin; 1.5 mM $MgCl_2$; 200 µM dNTPs; 2 units Taq polymerase (Boehringer Mannheim); 400 nM of each primer pICE1/pICE2 and 5 µl template cDNA. Samples were denatured at 94°C for 3 min then subjected to 35 amplification cycles in a Hybaid thermocycler (Bio/Can Scientific). Each PCR cycle consisted of a 1 min denaturation step (94°C), a 1 min annealing step (48°C) and a 1 min extension step

(72°C). Finally, products were extended for 5 min at 72°C. Resulting products were analyzed by agarose gel electrophoresis.

Subcloning of pro-mICE cDNA

The pro-mICE PCR product generated above was subcloned into the pGEM-3Z plasmid (Promega) as a 1200 bp *Bam*HI/*Pst*I fragment following purification from a 1% low melting point agarose gel. One clone (mICE-3) was selected for further analysis and the sequence was verified using terminator cycle sequencing (based on the method of Sanger *et al.*, 1977) on an Applied Biosystems Model 373A DNA Sequencer.

IN VITRO TRANSCRIPTION AND TRANSLATION

Plasmid Vectors

The cDNA encoding pro-CPP32 (Genbank accession number U13738) was a generous gift of Dr. Don Nicholson (Merck Frosst Centre for Therapeutic Research, Montreal, PQ) and contains a silent mutation (G^{234} to A). This cDNA was cloned as an *Xba*I fragment in the *Xba*I site of the vector pBluescript II SK+ (Stratagene) in the T7 orientation.

The cDNA encoding PARP (Genbank accession number M32721) was a generous gift of Dr. Mark Smulson (Georgetown University School of Medicine, Washington, DC) and is cloned as an *Xho*I fragment in the T7 orientation in the vector pBluescript II SK+ (Stratagene).

The cDNA encoding pro-IL-1β (Genbank accession number X02532) was a gift of Dr. Mike Tocci (Merck Research Laboratories, Rahway, NJ) and is cloned as an *Eco*RI/*Pst*I fragment in the vector pGEM-3Z (Promega).

Plasmid DNA for *in vitro* transcription was prepared using a commercially available plasmid preparation kit (Qiagen).

***In Vitro* Transcription and Translation**

The construct mICE-3 was linearized by digestion with *Pst*I. The vector containing pro-CPP32 was linearized by digestion with *Not*I. The pGEM-3Z-pro-IL-1β DNA was linearized by digestion with *Pst*I. Following linearization, DNA was isolated by phenol:chloroform extractions followed by ethanol precipitation. Pelleted and dried DNA was resuspended in sterile water. RNA transcripts were produced by using T7 RNA polymerase (Bethesda Research Laboratories) and a Promega *in vitro* transcription kit. Template DNA was destroyed after the transcription reaction by

treatment with *E. coli* DNaseI (Promega). The size and purity of the RNA transcript was confirmed by electrophoresis on a denaturing agarose gel. *In vitro* translation was performed in a total volume of 50 µl using a rabbit reticulocyte lysate kit from Promega. Each reaction included 40 µCi of TRAN^{35}S-LABEL (ICN Biomedicals Inc.) or 40 µCi of [^{35}S]Met (Dupont-NEN). Resulting translation products were analyzed by SDS-PAGE followed by autoradiography. Synthesis of the resulting proteins was shown to be RNA-dependent by performing the *in vitro* translation in the absence of RNA.

In other instances the CPP32 or PARP plasmids were used to drive the synthesis of [^{35}S]Met-labeled pro-CPP32 or [^{35}S]Met-labeled PARP by coupled transcription/translation using a TNT kit from Promega. These translation mixes were purified by gel permeation fast protein liquid chromatography (FPLC) to remove unincorporated [^{35}S]Met and constituents of the reticulocyte lysate.

GENERATION OF MTL 2.8.2 LYSATES

MTL 2.8.2 cells were washed with PBS and removed from monolayers by treatment with PBS containing 20 mM EDTA, followed by suspension in 10 ml cell culture medium. Cells were washed with 10 ml PBS, and lysed by suspension in COS cell lysis buffer (200 µl/2x10^6 cells), followed by incubation on ice for 30 minutes. Nuclei were removed by centrifugation at 10,000g, for 10 minutes at 4°C. Supernatants were stored at -70°C until use. Protein concentrations were determined by the BCA assay kit (Pierce) using bovine serum albumin as a standard.

FPLC SEPARATION OF GRANZYMES

FPLC

FPLC separation was through a modification of the method of Hanna *et al.* (1993). Cell lysate from 3.45x10^7 MTL 2.8.2 cells was generated as above, and 15 µg of total cell protein was checked for enzymatic activity (see below) prior to separation by FPLC. NaCl was added to the cell lysate to a final concentration of 390 mM. FPLC over a Mono-S HR 5/5 column (Pharmacia) was as follows: the column was first equilibrated in buffer A (50 mM MES, pH 6.1; 25 mM NaCl; 2 mM $CaCl_2$). Lysate was filtered and then applied to the column and the flow through was collected. Elution was by a 30 ml linear gradient from buffer A to buffer B (50 mM MES, pH 6.1; 1 M NaCl; 2 mM $CaCl_2$). Fractions of 1 ml were collected throughout the elution period and a 10 ml wash with buffer B immediately following elution.

Throughout the elution a plot of OD_{280} (protein content) was generated. Based on this plot, 10 µl of fractions 13 to 39 were assayed for enzymatic activity (see below).

Concentration of FPLC Fractions

500 µl of FPLC fractions 20 (granzyme B) and 24 (granzyme A) from the granzyme separation were concentrated to a volume of 25 µl using Millipore Ultrafree-MC 10,000 NMWL filter units by centrifugation at 6400 rpm for ~45 minutes. The fractions were then made up to a total volume of 100 µl in COS cell lysis buffer, and then assayed for enzymatic activity as outlined below.

ENZYMATIC ASSAYS

Granzyme Activity

COS Cell Lysates

For Asp-ase activity due to granzyme B, COS cell lysates containing 50 µg of total protein were made to 50 µl with COS cell lysis buffer and added to 450 µl of assay buffer containing 0.1M Hepes, pH 7.0; 0.3 M NaCl; 1 mM EDTA; 0.1 mM *tert*-butyloxycarbonyl-Ala-Ala-Asp-thiobenzyl ester (Enzyme Systems Products) and 0.11 mM dithiobis(2-nitrobenzoic acid) (DTNB) and incubated at 23°C for 20 min before reading the optical density at 405 nm. Colour development due to nonspecific indicator reduction was determined by performing identical assays without substrate. Negative controls were prepared by the addition of 10 µl of COS cell lysis buffer to the same reactions including substrate and indicator. Positive controls were prepared by performing the same assay with 15 µg MTL 2.8.2 total protein.

To determine the effect of the *in vitro* translated proteins on enzymatic activity, 1 µl of translation mix per reaction was combined with the lysate and then the assay proceeded as usual.

FPLC Fractions

FPLC fractions 13 to 39 were assayed for Asp-ase (granzyme B), BLT-esterase (granzyme A) and Chym-ase (other granzymes) activity as follows: 10 µl of each fraction was combined with the following (final concentrations given) in a 96-well plate to a final volume of 202 µl: 0.1 M Hepes, pH 7.0; 0.3 M NaCl; 1 mM EDTA and 0.11 mM DTNB. For Asp-ase assays, 0.1 mM *tert*-butyloxycarbonyl-Ala-Ala-Asp-thiobenzyl ester was added. For BLT esterase activity, 0.1 mM Nα-carbobenzoxy-L-Lys-thiobenzyl ester (Sigma) was added. For chymotrypsin-like activity, 0.1 mM N-succinyl-Ala-Ala-Pro-Phe-thiobenzyl ester (Sigma) was added.

Reactions were incubated at 23°C for 20 minutes prior to reading the optical density at 410 nm. Colour development due to nonspecific indicator reduction was determined by performing the identical assays without substrate. Negative controls were prepared by the addition of 10 µl of FPLC buffer B to the same reactions including substrate and indicator. Positive controls were prepared by performing the same assay with 15 µg MTL 2.8.2 total protein.

Following concentration of the FPLC fractions, similar assays were performed to ensure that enzymatic activity had been maintained.

ICE Activity

Aliquots of COS cell lysates containing 75 µg of protein were diluted to 50 µl with COS cell lysis buffer and thence made to a total volume of 0.5 ml with assay buffer (20 mM Hepes, pH 7.5; 10% glycerol; 2 mM DTT) containing 0.2 mM benzyloxycarbonyl-Tyr-Val-Ala-Asp-7-amino-4-trifluoromethyl coumarin (Enzyme Systems Products). At indicated intervals 2.5 ml assay buffer was added to each reaction and fluorescence was detected by a Perkin Elmer LS 50 Luminescence Spectrometer, with excitation at 400 nm and emission at 505 nm. Spontaneous substrate hydrolysis was detected by substituting COS cell lysis buffer for cell lysates.

TREATMENT OF CELLS WITH ANTI-FAS ANTIBODY

Treatment of Cells With Anti-Fas

L1210-Fas or YAC-1 cells were suspended in AIM-V serum-free media (Life Technologies) and treated with 1 µg/ml hamster anti-mouse Fas IgG (PharMingen) on ice for 30 minutes. Cells were then pelleted and resuspended in AIM-V containing 20 µg/ml goat anti-hamster IgG antibody, (Jackson ImmunoResearch Laboratories) in order to cross-link ligated receptors. Incubation proceeded at 37°C for the time points indicated. Jurkat cells were incubated with 100 ng/ml mouse anti-human Fas IgM (Upstate Biotechnology Inc.) in AIM-V serum free medium.

Generation of Cell Lysates

Cells were pelleted and washed with PBS, and then lysed in NP-40 lysis buffer (10 mM Tris, pH 7.4; 10 mM NaCl; 3 mM $MgCl_2$; 0.5% (v/v) NP-40). Debris was pelleted by centrifugation at 10,000g, 4°C, for 10 minutes, the supernatant was transferred to a new tube and assayed for protein content using a BCA Protein Assay kit from Pierce using bovine serum albumin as a standard. All lysates were stored at -70°C prior to use.

CLEAVAGE ASSAYS

COS Cell Lysates and pro-mICE

500 µg of total COS cell protein (from cells transfected with pAX142-CCP1a or from mock-transfected cells) was combined with COS cell lysis buffer to a total volume of 110 µl. Control reactions contained 45 ng of human recombinant ICE (a generous gift of Nancy Thornberry, Merck Research Labs, Rahway, NJ) in a total volume of 110 µl. To each of these were added 30 µl of assay buffer containing 0.1 M Hepes, pH 7.0; 0.3 M NaCl; 1 mM EDTA and 10 µl of *in vitro* translated [^{35}S]pro-mICE. Samples were incubated at 37°C and at specific time points (0, 2, 4, 8, and 16h) a 30 µl aliquot was removed and analyzed by 12% SDS-PAGE and visualized by autoradiography.

FPLC Fractions and pro-CPP32

5 µl of concentrated FPLC fraction containing granzyme A (fraction 24) or granzyme B (fraction 20) or 5 µl of MTL 2.8.2 lysate (~10 µg total protein) was combined with 5 µl of [^{35}S]pro-CPP32 and incubated at 37°C for 4 hours. Samples were resolved by 12% SDS-PAGE and visualized by autoradiography.

COS Cell Lysates and pro-CPP32

COS cell lysate (50 µg total protein) was made to a total volume of 15 µl with COS cell lysis buffer to which was added 5 µl of 5X BAADT assay buffer (0.5 M Hepes, pH 7.5; 1.5 M NaCl; 5 mM EDTA; 0.25% Triton X-100). This mixture was then combined with 5 µl of *in vitro* translated [^{35}S]pro-CPP32 at 37°C for 4 hours. Samples were resolved by 12% SDS-PAGE and visualized by autoradiography.

COS Cell Lysates and PARP

COS cell lysate (50 µg total protein) was combined with [^{35}S]PARP (3 µl) and 4 µl 5X BAADT buffer at 37°C for 60 minutes. Samples were resolved by 14% SDS-PAGE and cleavage products were visualized by fluorography.

For the inhibitor study, COS cell lysates expressing enzymatically active recombinant granzyme B (50 µg total protein) were preincubated at 37°C for 20 minutes with the following: aprotinin (Calbiochem; 2µg/ml), phosphoramidon (Calbiochem; 8.5 µM), chymostatin (Calbiochem; 100 µM), leupeptin (Boehringer Mannheim; 100 µM), E-64 (Calbiochem; 10 µM), iodoacetamide (Sigma; 5 mM), *N*-ethylmaleimide (Sigma; 5 mM), or Ac-DEVD-CHO (a generous gift of Dr. Don

Nicholson; 100 nM) prior to addition of 3 µl of [^{35}S]PARP and further incubation at 37°C for 60 minutes. Samples were analyzed as above.

Anti-Fas-Treated Cell Lysates and pro-IL-1β

Lysates of cells treated with anti-Fas antibody (50 µg total protein in a final volume of 27 µl) were incubated with 3 µl of *in vitro* translated [^{35}S]pro-IL-1β in a total volume of 30 µl at 37°C overnight. Samples were analyzed by 12% SDS-PAGE followed by autoradiography. In some cases, 45 ng of purified human recombinant ICE was added to the reaction to check for the presence of an ICE inhibitor.

***IN VIVO* CLEAVAGE OF CPP32**

COS Cell Lysates

COS cell lysates expressing recombinant active granzyme B, inactive zymogen or the active site mutant were generated and 50 µg total cell protein was then incubated at 37°C for 1 hour. Samples were then resolved by 12% SDS-PAGE followed by electroblotting (see below)

CTL21.9 and Target Cells

CTL21.9 effector cells (stimulated with anti-CD3 for 24 hours) were combined with target cells (EL4 or YAC-1) at an effector-to-target ratio of 1:1. At time zero and following incubation at 37°C for 4 hours, CTLs were removed from the target cells using Dynabeads (Dynal) as follows: all cells were pelleted and resuspended at $2x10^7$ cells/ml in coating medium (Hanks buffered saline solution plus 10% (v/v) fetal calf serum, 20 mM Hepes) to which rat anti-mouse CD8α (Serotec) was then added (1:200 dilution). Cells were incubated for 30 minutes at 4°C with mixing. After washing the cells twice with coating medium and resuspending at $2x10^7$ cells/ml, Dynabeads were then added to the suspension (4 beads per CTL21.9 cell). Incubation proceeded at 4°C for 1 hour with mixing. CTL21.9 cells were then removed by magnetic separation. Target cells were pelleted, washed with PBS, and then lysed in Nicholson lysis buffer (10 mM Hepes/KOH, pH 7.4; 2 mM EDTA; 0.1% (v/v) CHAPS; 5 mM DTT) at 10^8 cells/ml. Cellular debris was pelleted by centrifugation at 10,000g for 10 minutes at 4°C and then the supernatant was stored at -70°C prior to further use. Protein concentration was determined using a Biorad Protein Assay kit (based on the Bradford dye-binding procedure) using bovine serum albumin as a standard. Target cell lysate (50 µg total protein) was then separated by 12% SDS-PAGE followed by electroblotting (see below).

GB KO/WT Cells and Target Cells

GB KO and GB WT effector cells (stimulated with anti-CD3 for 24 hours) were combined with EL4 or YAC-1 target cells (10^6 targets per sample) at an effector-to-target ratio of 1:1 and incubated at 37°C for 0, 4 or 24 hours. All cells were then pelleted, washed with PBS, and lysed in 40 µl Nicholson lysis buffer. Cellular debris was removed by centrifugation and then the supernatant was stored at -70°C until further use. The entire sample was subsequently separated by 12% SDS-PAGE followed by electroblotting (see below).

PMM-1 and Target Cells

PMM-1 effectors (stimulated with PMA and ionomycin for 3 hours) were combined with EL4 or YAC-1 cells (10^6 targets per sample) at an effector-to-target ratio of 5:1 and incubated at 37°C for 4 hours. All cells were then pelleted, washed with PBS, and then lysed in 40 µl Nicholson cell lysis buffer. Cellular debris was removed by centrifugation, and then the supernatant was stored at -70°C. The entire sample was subsequently separated by 12% SDS-PAGE followed by electroblotting (see below).

Western Blotting

Electroblotting to PVDF was performed using a semi-dry transfer system from Tyler Research Instruments. Blots were blocked for 1 hour at room temperature in 5% skim milk in tris-buffered saline (TBS). Primary antibody (rabbit anti-CPP32, 1:2000 dilution, generously provided by Dr. Don Nicholson) was added in 5% skim milk and allowed to bind at 4°C overnight. The blots were then washed three times for 15 minutes each in TBS at room temperature. The secondary antibody (donkey anti-rabbit, 1:5000 dilution, from Amersham) was then added in 5% skim milk for 1 hour at room temperature. Blots were again washed as above and then developed using the ECL detection kit from Amersham.

CHROMIUM RELEASE (CYTOLYSIS) ASSAYS

Target cells were labeled with ^{51}Cr (Dupont-NEN, 100 µCi/10^6 cells) for 60 to 90 minutes at 37°C. Cells were then pelleted and the supernatant removed. Labeled cells were resuspended in 10 ml cell culture media, followed by incubation at 37°C for an additional 30 minutes. Cells were then washed twice with PBS and resuspended in culture media at 10^5 cells/ml.

100 µl of labeled target cells were combined with 100 µl of activated effector cells (at the appropriate concentration) in a 96-well V-bottom plate. Some assays contained concanavalin A (ConA) at a final concentration of 2 µg/ml, peptide inhibitors (diluted from stock solutions of 20 mM in DMSO) at the final concentrations indicated, or EGTA and $MgCl_2$ at final concentrations of 2 mM and 4 mM respectively. Spontaneous release was determined by incubating targets in the absence of effectors. Incubation proceeded at 37°C for the time shown. Total counts were obtained by counting 50 µl of the cell suspension. All samples were set up in triplicate.

Following incubation, cells were pelleted by centrifugation and 100 µl of supernatant was transferred to borosilicate glass tubes for counting. Samples were counted in a LKB Wallac 1270 Rackgamma II gamma counter.

CTL cytolytic activity was calculated using the following formula:

% lysis = 100 x (sample-spontaneous release)/(total-spontaneous release).

TRITIUM RELEASE (DNA FRAGMENTATION) ASSAYS

Target cells were labeled with [^{3}H]thymidine (Dupont-NEN) for 24 hours prior to the assay. On the day of the assay, cells were pelleted and resuspended in cell culture media for 60 minutes at 37°C to allow clearance of unincorporated tritium. Following this incubation, cells were washed twice with PBS and then resuspended in cell culture medium at 10^6 cells/ml.

100 µl of labeled target cells were combined with 100 µl of activated effector cells (at an appropriate concentration) in microcentrifuge tubes. Total and spontaneous counts were obtained by incubating labeled targets in the absence of effectors. Incubation proceeded at 37°C for the time shown. Some assays contained ConA at a final concentration of 2 µg/ml or peptide inhibitors (diluted from stock solutions of 20 mM in DMSO) at the final concentrations indicated. All samples were set up in triplicate.

Following the incubation period, total counts were obtained by addition of 200 µl of 2% SDS in 0.2 M NaOH to the cells followed by vortexing for 30 seconds. The entire 400 µl was transferred to scintillation vials for counting, but only half the total number of counts was used in the calculations.

The remaining samples were harvested by addition of 200 µl of 1% Triton X-100 in TE followed by vortexing for 30 seconds. Nuclei were pelleted by centrifugation at 10,000g for 10 minutes at 4°C, then 200 µl of supernatant was transferred to scintillation vials for counting.

The scintillant ACS (5 ml, Amersham) was added to each vial and samples were counted in a Beckman LS 7800 scintillation counter.
CTL activity was calculated using the following formula:
% lysis = 100 x (sample-spontaneous release)/(total/2-spontaneous release).

INHIBITION OF CPP32 ACTIVITY BY PEPTIDE INHIBITORS

Purified human CPP32 was a generous gift of Dr. Don Nicholson of Merck Frosst, Montréal, and was provided at a concentration of 6 units/µl. This stock solution was diluted 1:50 in ICE II buffer (50 mM PIPES/KOH, pH 6.5; 2 mM EDTA; 0.1% (w/v) CHAPS; 5 mM DTT) for the inhibitor study. The diluted CPP32 (1 µl) was combined with 1 µl of inhibitor stock (diluted in DMSO) in a final volume of 20 µl and incubated at 37°C for 15 minutes to allow binding of the inhibitor to CPP32. Following the preincubation, 5 µl of *in vitro* translated, FPLC-purified [^{35}S]PARP was added to each reaction and incubation proceeded at 37°C for an additional 60 minutes. Reactions were separated by 12% SDS-PAGE and cleavage was assessed by autoradiography.

CHAPTER III - ICE IS NOT A GRANZYME B SUBSTRATE

CHAPTER III - ICE IS NOT A GRANZYME B SUBSTRATE[1]

INTRODUCTION

Cytotoxic T lymphocytes (CTLs) destroy target cells bearing foreign antigens by one of two mechanisms: Mechanism one involves membrane permeabilization and the induction of apoptotic cell death through the exocytosis of cytolytic granules within the CTL towards the target cell. Mechanism two requires the ligation of a cell surface receptor (Fas/APO-1/CD95) on the target cell by Fas ligand (FasL) on the CTL. Fas then transduces a death signal which results in apoptotic cell death of the target (reviewed by Berke, 1995; Atkinson and Bleackley, 1995; Smyth, 1995). This CTL-mediated cytotoxicity is important for the destruction of viral-infected and tumourigenic cells, and is responsible for organ transplant rejection and autoimmune disease (reviewed by Berke, 1995). Additionally, the Fas-FasL system may be responsible for the down-regulation of immune responses, as well as the maintenance of immune privilege. The currently accepted model for granule-mediated cytotoxicity (Henkart, 1985) postulates that there are three main steps involved in target cell lysis: recognition by CTL of a target cell; delivery of a "lethal hit" to the target cell by exocytosis of CTL granule contents; and detachment of the CTL from the target allowing the CTL to seek and destroy new targets. The CTL cytoplasmic granules have been found to contain a family of seven serine proteases (granzymes A to G) as well as the pore-forming protein perforin. Perforin may facilitate the entry of the proteases into the target cell (reviewed by Liu *et al.*, 1995) where they trigger apoptosis by a poorly understood mechanism.

Murine granzyme B (Cytotoxic Cell Proteinase-1/CCP1) represents a prototype of six of these serine proteases (granzymes B to G). Originally identified as a CTL-specific gene (Lobe *et al.*, 1986a, 1986b) whose expression correlated with the induction of cytotoxicity (Lobe *et al.*, 1986b), it was subsequently localized to the cytoplasmic granules of CTLs (Redmond *et al.*, 1987; Ojcius *et al.*, 1991). Quantitative PCR of granzymes B to G was used to analyze granzyme expression in response to various modes of T cell activation. The results revealed that only granzyme B expression correlates with the development of cytotoxicity in T cells in response to stimulation by mitogen, allogeneic cells or anti-CD3 (Prendergast *et al.*, 1992), suggesting that it may be a direct effector in the lytic process. Recent evidence

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[1] A version of this chapter has been published: Darmon, A.J., Ehrman, N., Caputo, A., Fujinaga, J., and Bleackley, R.C. (1994) *J. Biol. Chem.* **269**, 32042-32046

suggests a role for granzyme B in the induction of target cell DNA fragmentation, a hallmark of apoptosis. Shi *et al.* (1992a, 1992b) have shown that a serine protease (fragmentin-2) purified from rat natural killer (NK) cell granules, and having homology to granzyme B, is able to rapidly induce DNA fragmentation and apoptosis in YAC-1 target cells in the presence of perforin. In complementary studies, Nakajima *et al.* (1995a) expressed both granzyme B and perforin in a noncytotoxic rat mast cell line which can be triggered to degranulate by the IgE-specific Fc receptor. Their results show that cells expressing both granzyme B and perforin have enhanced cytolytic and nucleolytic activity compared to cells expressing perforin alone, again suggesting a role for granzyme B in the induction of target cell DNA fragmentation. Finally, using CTL, NK and lymphokine-activated killer (LAK) cells isolated from mice homozygous for a null mutation in the granzyme B gene, other workers have shown that granzyme B plays a critical role in the early induction of target cell DNA fragmentation and apoptosis in allogeneic target cells (Heusel *et al.*, 1994; Shresta *et al.*, 1995). In addition, in NK cells, but not in CTL or LAK cells, granzyme B may play a critical role in inducing membrane damage (Shresta *et al.*, 1995).

Using comparative molecular modeling of granzyme B, Murphy *et al.* (1988) predicted a requirement for aspartic or glutamic acid residues at P_1 based on the presence of an arginine side chain which partially fills the specificity pocket of granzyme B. Use of thioester substrates containing various amino acids at P_1 showed that granzyme B has Asp-ase activity (Odake *et al.*, 1991). Final confirmation of this specificity was obtained through mutagenesis analysis (Caputo *et al.*, 1994). The specificity of granzyme B is unique among eukaryotic serine proteases and there is only one other known eukaryotic protease with this substrate specificity, Interleukin-1β Converting Enzyme (ICE) (Howard *et al.*, 1991; Sleath *et al.*, 1990). At the time of these studies, no intracellular substrates for granzyme B had been identified but it was assumed that granzyme B cleaves a protein within the target cell which leads to DNA fragmentation.

ICE was identified as a cysteine protease responsible for proteolytically processing the 33-kDa pro-IL-1β to produce the 17.5-kDa biologically active IL-1β (Cerretti *et al.*, 1992; Thornberry *et al.*, 1992) by cleaving the precursor at two distinct sites: FEAD-G^{28} and YVHD-A^{117} in the human pro-cytokine (Figure III-1A). Active ICE consists of a p10/p20 heterodimer and is generated by proteolytic processing of a p45 precursor (pro-ICE; [Thornberry *et al.*, 1992]). The structure of pro-ICE is shown in Figure III-1B. Activation of ICE requires proteolysis at four different sites, each involving cleavage after Asp residues. Two of the cleavages

release an amino terminal pro-domain, while the remaining sites bracket an intervening sequence between the two subunits of the active enzyme. Since the cleavages required during ICE activation coincide with ICE substrate specificity this led to the proposal that ICE may be autocatalytic. Subsequent studies have shown that ICE is autocatalytic both *in vitro* and *in vivo*, confirming this hypothesis (Thornberry *et al.*, 1992; Wang *et al.*, 1994a; Howard *et al.*, 1995). Interestingly, the cleavages which occur during ICE activation are at Asp-X bonds, which coincides with the substrate specificity of granzyme B.

A role for ICE in programmed cell death was suggested by the results of Miura *et al.* (1993). They showed that overexpression of murine ICE (mICE) in rodent fibroblasts caused the cells to undergo apoptosis, a result dependent upon the cysteine protease activity of mICE. One model by which ICE could mediate apoptosis is by cleavage of a protein required for cell viability, leading to cellular disintegration. The possible existence of ICE substrates other than pro-IL-1β is supported by the fact that mICE mRNA is found in tissues that have not been shown to express IL-1β (Cerretti *et al.*, 1992; Nett *et al.*, 1992).

These results led to the proposal of a model for CTL-mediated cytotoxicity in which granzyme B activates ICE in the target cell by proteolytically processing the precursor molecule. The active ICE heterodimer then goes on to induce apoptosis in the target cell by the same mechanism used during development (Vaux *et al.*, 1994). This intriguing hypothesis is very appealing as it unites the mechanisms of apoptosis involved in cell-mediated cytotoxicity with that observed during programmed cell death. Alternatively, granzyme B may cleave the same cellular protein(s) as ICE, and induce apoptotic cell death in an ICE-independent manner.

The key question then is whether the ICE precursor is a substrate for granzyme B. This lab was in a unique position to address this as we have developed a heterologous expression system that allows expression of enzymatically active murine granzyme B from a modified granzyme B cDNA in the absence of other granzymes (Caputo *et al.*, 1993). This chapter reports that the ICE precursor is not a substrate for granzyme B, and proposes an alternate mechanism of CTL-mediated killing.

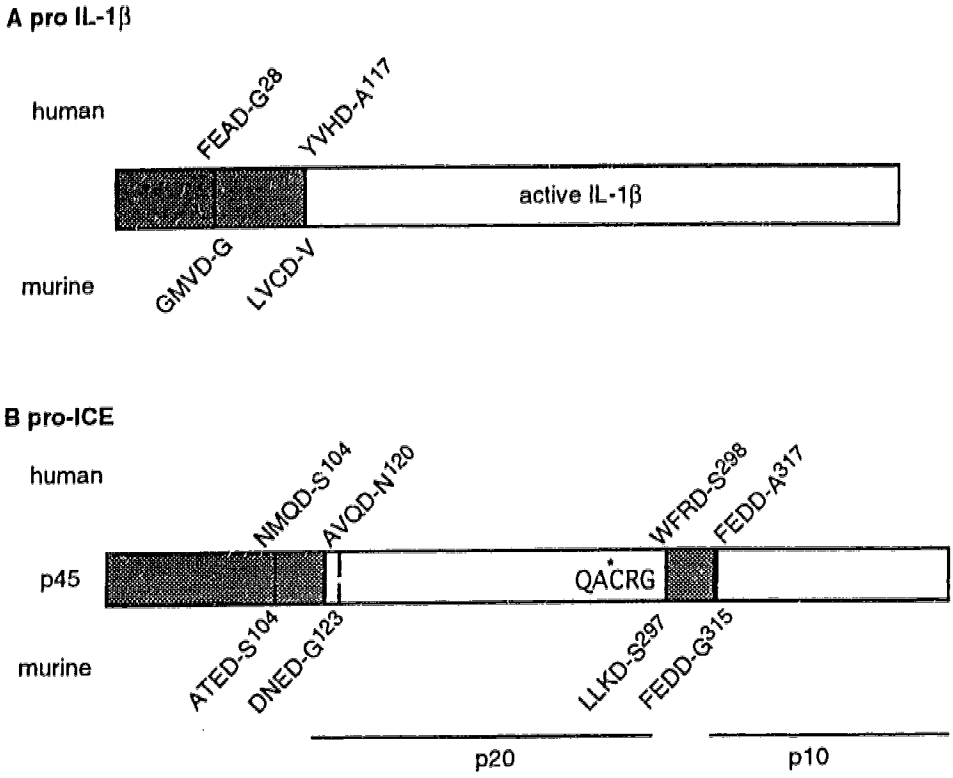


FIGURE III-1: The structure of pro-Interleukin-1β and pro-ICE.

A. Structure of human and murine pro-Interleukin-1β. Sequence of cleavage sites during proteolytic activation are shown above (human) and below (murine) the schematic. The scissile bond is indicated by a hyphen.

B. Structure of human and murine pro-ICE. Sequence at cleavage sites during proteolytic activation are shown above (human) or below (murine) the schematic. Note that murine pro-ICE lacks an Asp at a position equivalent to Asp^{119} in the human homologue, the presumed amino terminus of the active enzyme is shown by the dotted line at murine Asp^{122}. The conserved pentapeptide QACRG containing the catalytic cysteine (*) is highlighted. Scissile bonds are indicated by hyphens.

References: Howard *et al.*, 1992; Thornberry *et al.*, 1992; Cerretti *et al.*, 1992; Nett *et al.*, 1992; Molineaux *et al.*, 1993

RESULTS AND DISCUSSION

Isolation of pro-mICE cDNA

In order to determine if precursor murine Interleukin-1β Converting Enzyme (pro-mICE) is a substrate of granzyme B, the cDNA encoding pro-mICE was first isolated from MTL 2.8.2 RNA (a cytotoxic T cell line) and subcloned using a reverse transcriptase-PCR protocol (see Chapter II). The resulting cDNA was subcloned into a vector such that its transcription was under the control of the T7 RNA Polymerase promoter. This construct was sequenced to ensure there were no mutations introduced during the PCR reactions. The construct was used as a template for *in vitro* transcription using T7 RNA polymerase and the resulting RNA was *in vitro* translated in the presence of [^{35}S]Met and [^{35}S]Cys using rabbit reticulocyte lysates. Upon examination by SDS-PAGE, the primary translation product was ~45-kDa (p45, Figure III-2), representing the mICE precursor. Multiple secondary bands appeared at ~30-kDa, probably due to premature translation termination. The *in vitro* translation reaction was shown to be RNA-dependent since no bands were present on the gel when RNA was not included in the reaction (Figure III-2).

Granzyme B Does Not Cleave Pro-mICE

To determine whether granzyme B could activate mICE by processing the precursor, the radioactively-labeled pro-mICE was incubated with lysates from COS cells expressing granzyme B or with lysates from mock-transfected COS cells. This system has the advantage of facilitating the study of granzyme B in the absence of other granzymes, which may contaminate other granzyme B preparations made from CTLs. As a control, the pro-mICE translation product was incubated with human recombinant ICE. Each incubation was carried out at 37°C and aliquots were removed at various time points and boiled with SDS-PAGE sample buffer to inactivate the proteases in the aliquot. Samples were then analyzed by SDS-PAGE. The results clearly show that pro-mICE was not cleaved by granzyme B, but was cleaved to a certain extent by human recombinant ICE (Figure III-3).

The primary translation product of pro-mICE mRNA, p45, was apparently not autocatalytic since it was not able to process itself. When pro-mICE was incubated with human recombinant ICE a new band appeared at ~34-kDa (p34) after 2 hours (Figure III-3A) representing an intermediate produced by cleavage of the proenzyme. This is consistent with the results of Thornberry *et al.* (1992) who showed limited cleavage of the ICE precursor by active ICE. Importantly, this cleavage served as confirmation that our *in vitro* translated product was the mICE precursor. As with

their experiments, we did not see complete processing of the precursor to produce active heterodimer. This suggests that autocatalysis is unlikely to be a mechanism of activation for ICE. However, perhaps active ICE, once generated in a cell by an unknown mechanism, is able to activate inactive precursors. The crystal structure of ICE indicates that ICE acts as a tetramer consisting of two heterodimers. Furthermore, if an active heterodimer interacts with an inactive precursor, the cleavage sites in the precursor are juxtaposed with the catalytic site, suggesting that this mechanism of activation is entirely plausible (Walker *et al.*, 1994; Wilson *et al.*, 1995). In the system used here, perhaps the pro-mICE does not attain the correct conformation to allow this mechanism of activation to occur. This would explain the limited cleavage seen when pro-mICE is incubated with active ICE.

In contrast to the limited cleavage seen when pro-mICE was incubated with active ICE, when lysates of COS cells expressing granzyme B were used in the cleavage assay, no cleavage product was seen. Even after a 16 hour incubation, granzyme B did not cleave pro-mICE (Figure III-3B). It was estimated that the amount of granzyme B used in the attempted cleavage of pro-mICE was equivalent to that contained in 500,000 cytotoxic cells. The finding that granzyme B cannot cleave pro-mICE is not that surprising in light of molecular modeling done with granzyme B (Murphy *et al.*, 1988). In these studies it was proposed that granzyme B had substrate specificity requiring an Asp at P_1 and a second acidic residue at P_3. Only one of the ICE cleavage sites meets this requirement (FEDD-G^{315} in the murine homologue) and cleavage at only this site would not produce active enzyme, since the intervening sequence between the subunits would not be removed.

Granzyme B is Active in the Presence of Pro-mICE

These results suggest that pro-mICE is not a substrate for granzyme B. It is, however, possible that the presence of pro-mICE inhibited granzyme B in the cleavage assay or, alternatively, that there was a granzyme B inhibitor present in the reticulocyte lysate. To ensure that granzyme B was active in the presence of pro-mICE, an assay of Asp-ase activity was performed using the chromogenic substrate *tert*-butyloxycarbonyl-Ala-Ala-Asp-thiobenzyl ester (BAADT), as previously described (Caputo *et al.*, 1993). This assay was performed with the same lysates that were used in the cleavage assay, and the ratio of pro-mICE to granzyme B in these assays was the same as that used in the attempted cleavage of pro-mICE. As a positive control, lysates from the cytotoxic T cell line MTL 2.8.2, which expresses high levels of granzyme B, were used. The results, shown in Figure III-4, clearly

indicate that granzyme B was active in the presence of pro-mICE. Nonspecific colour development was found to be consistent between all samples, indicating that the reticulocyte lysate did not interfere with the assay (data not shown). When BAADT was included in the reactions, specific hydrolysis was observed in the presence of granzyme B (columns 4 and 6) but no specific hydrolysis was mediated by lysates from mock-transfected COS cells (columns 3 and 5). Importantly, the addition of reticulocyte lysate containing pro-mICE to the reaction containing granzyme B-expressing COS lysates had no effect on granzyme B activity (column 6 compared to column 4). Together, these results show that granzyme B was active in the presence of pro-mICE, and that the failure of granzyme B to cleave pro-mICE was not due to inhibition of granzyme B in the cleavage assay.

Granzyme B Can Cleave a Synthetic ICE Substrate

The results thus far support a model for granule-based cytotoxicity which does not involve activation of ICE by granzyme B. Therefore, it was desirable to test an alternative model for granule-mediated killing. That is, that granzyme B and ICE may share similar cellular substrates. The ability of lysates from COS cells expressing granzyme B to hydrolyze the synthetic ICE substrate benzyloxycarbonyl-Tyr-Val-Ala-Asp-7-amino-4-trifluoromethyl coumarin was assessed in a fluorogenic assay. The results, shown in Figure III-5, clearly show that COS lysates expressing granzyme B hydrolyzed this substrate more efficiently than lysates from mock-transfected cells. The efficiency of hydrolysis of this substrate by granzyme B in comparison to ICE could not be determined as the amount of granzyme B in the COS cell lysates was unknown. This result does, however, suggest that natural substrates of ICE may serve as substrates for granzyme B.

FIGURE III-2: *In vitro* translation of pro-mICE.

Translation using rabbit reticulocyte lysates in the absence (lane 1) or presence (lane 2) of mRNA generated by *in vitro* transcription using murine ICE precursor cDNA as a template. Each reaction contained 40 µCi of [^{35}S]Met and [^{35}S]Cys. 2 µl of the total of 50 µl was analyzed by SDS-PAGE followed by autoradiography.

p45 - murine ICE precursor (pro-mICE)

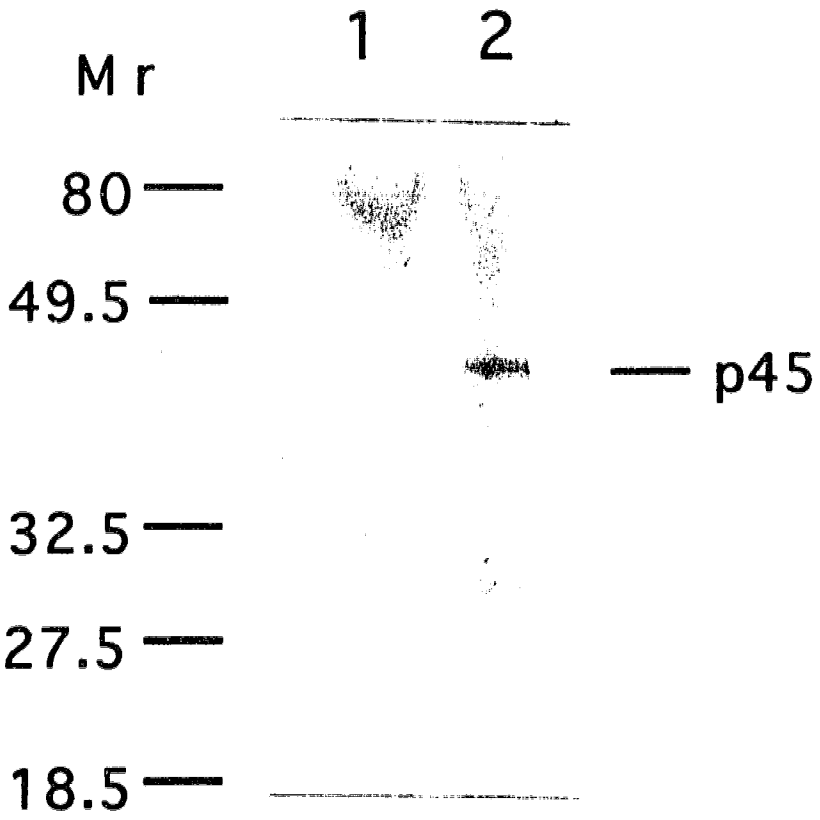
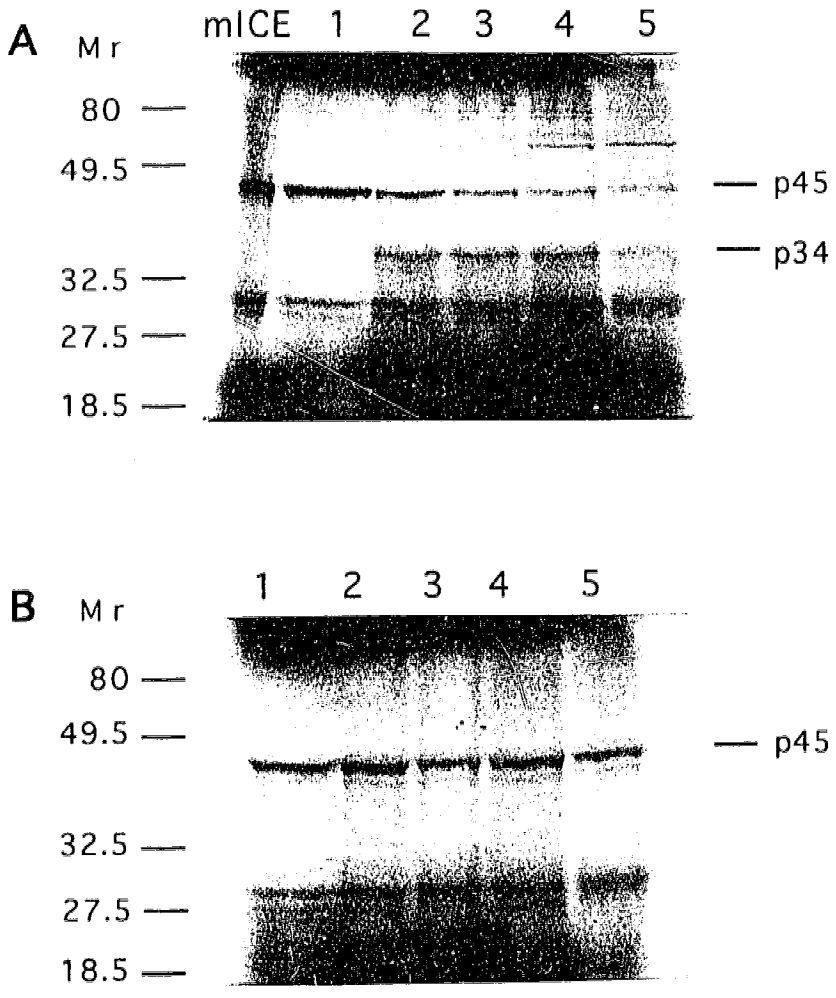

Mr
1
2
80
49.5
p45
32.5
27.5
18.5

FIGURE III-3: Cleavage of pro-mICE by ICE or Granzyme B.

10 µl of *in vitro* translated, radiolabeled mICE precursor was incubated at 37°C with 45 ng human recombinant ICE (A) or 500 µg total COS cell protein from cells transfected with a granzyme B expression vector (B). At various time points, a 30 µl aliquot was removed, boiled with SDS loading buffer then analyzed by SDS-PAGE followed by autoradiography. Lanes: mICE - translation product alone; 1 - time zero; 2 - 2 hour incubation; 3 - 4 hour incubation; 4 - 8 hour incubation; 5 - 16 hour incubation. p45 - murine ICE precursor; p34 - intermediate produced by partial cleavage of precursor.


A
Mr
mICE
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p45

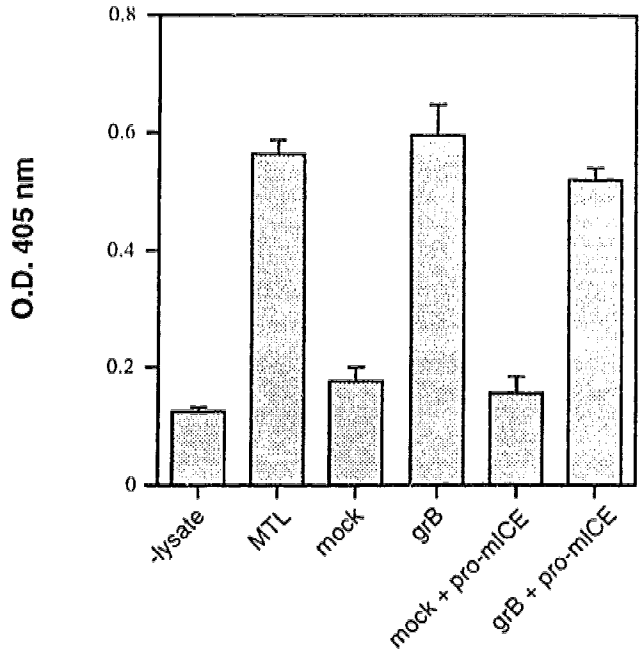

FIGURE III-4: Granzyme B is active in the presence of pro-mICE.

Assay of Asp-ase activity of COS cell lysates in the presence or absence of *in vitro* translated mICE precursor. The ratio of pro-mICE to lysate was the same as for the cleavage assay shown in Figure III-3. Mean and standard deviation of triplicate samples is shown. Assays contained the following amounts of protein: COS, 50 µg; MTL, 25 µg. MTL lysate acts as a positive control since this cell line (MTL 2.8.2, a cytotoxic T cell line) expresses high levels of granzyme B. mock - mock transfected COS cell lysate; grB - lysate of COS cells transfected with granzyme B expression vector.

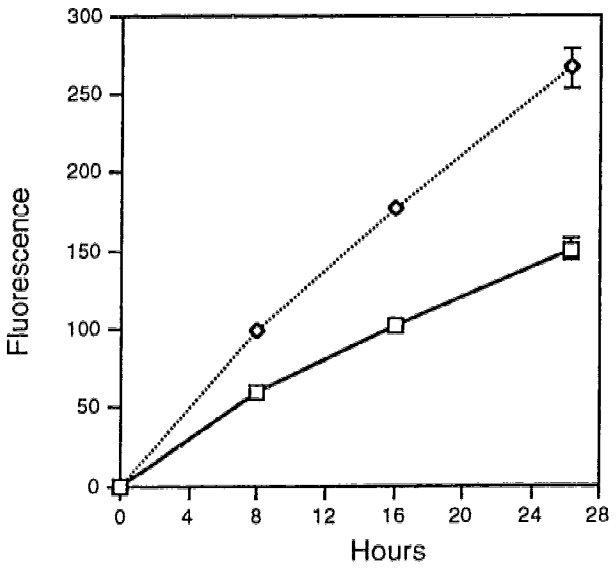

FIGURE III-5: Hydrolysis of ICE synthetic substrate by granzyme B.
Ability of COS cell lysates from cells expressing active murine granzyme B (diamonds) or from mock-transfected cells (squares) to hydrolyze the synthetic ICE substrate YVAD-AFC was determined. Mean and standard deviation of triplicate samples is shown.

The accepted model for granule-based CTL-mediated cytotoxicity proposes that CTLs recognize and bind target cells resulting in CTL activation and the release of granule contents into the intercellular space (Berke, 1995). Polymerization of perforin in the target cell membrane allows entry of the granzymes into the cell where they cleave their intracellular substrates, leading to DNA fragmentation and apoptosis. ICE has been implicated as playing a role here based on the fact that overexpression of ICE in rodent fibroblasts leads to apoptosis (Miura *et al.*, 1993). Vaux *et al.* (1994) recently proposed a model for CTL-mediated cytotoxicity which unites the two mechanisms of apoptosis involved in programmed cell death and CTL-mediated cytotoxicity. In this hypothesis, granzyme B enters the target cell and activates ICE by processing the precursor molecule to produce the active heterodimer. ICE then goes on to induce apoptosis in the target cell by the same mechanism used during programmed cell death. However, data presented here suggests that granzyme B does not function in target cells by cleaving and activating pro-mICE. Rather, the data here suggest that granzyme B and ICE may share a cellular substrate(s) which, when proteolytically processed, induces apoptotic cell death. The identity of this substrate remains to be determined.

Addendum

Subsequent to the publication of this work (Darmon *et al.*, 1994), an intracellular substrate for granzyme B was identified (Chapter IV; Darmon *et al.*, 1995). This substrate is the precursor of a protease related to, but distinct from, ICE. This protease (CPP32) is indeed a putative substrate for ICE (Tewari *et al.*, 1995a), confirming the hypothesis, presented here, that granzyme B replaces the function of ICE *in vivo* by cleaving cellular substrates of ICE (and ICE-like proteases). Further studies, presented later in this thesis (Chapter V), confirmed that ICE (and ICE-related) proteases play no role in the induction of target cell DNA fragmentation during granule-based CTL-mediated cytotoxicity, and that granzyme B functions primarily be cleaving and activating the precursors of CPP32 and CPP32-related proteases. Thus, in retrospect, this lack of cleavage of pro-mICE is not surprising, in light of the current model for granule-mediated killing. This point will be returned to later.

CHAPTER IV - CPP32 IS A GRANZYME B SUBSTRATE

CHAPTER IV - CPP32 AS A GRANZYME B SUBSTRATE[2]

INTRODUCTION

Cytotoxic T lymphocyte (CTL)-mediated cytotoxicity represents the body's major defense against virus-infected and tumourigenic cells, and contributes to transplant rejection and autoimmune disease. Two mechanisms of killing are available to the CTL: granule- and Fas-mediated. During granule-mediated killing, CTL granules are exocytosed, releasing their contents into the intercellular space between the target cell and the effector. Perforin facilitates the entry of cytotoxic cell serine proteases, the granzymes, into the target cell, where they induce apoptotic death by an enigmatic pathway (reviewed by Berke, 1995; Atkinson and Bleackley, 1995; Smyth, 1995). Granzyme B plays a critical role in the induction of DNA fragmentation and apoptosis in target cells (Shi *et al.*, 1992a, 1992b; Su *et al.*, 1994; Heusel *et al.*, 1995; Shresta *et al.*, 1995) yet its substrate is unknown. Identification of the intracellular substrate for granzyme B is therefore the key to understanding the mechanism of granule-based CTL-mediated killing. This chapter outlines the identification of such an intracellular substrate, an ICE/Ced-3 protease precursor.

Recent work on apoptosis has focused on the involvement of members of the ICE/Ced-3 family of cysteine proteases (reviewed by Henkart, 1996; Martin and Green, 1995). Each of the ICE/Ced-3 proteases is synthesized as an inactive precursor requiring cleavage at specific Asp residues to produce two subunits of ~20-kDa and ~10-kDa, which together form the active protease. Since the cleavages that occur during ICE/Ced-3 protease activation are at Asp-X bonds, they are excellent potential substrates for granzyme B (Vaux *et al.*, 1994), which has substrate specificity requiring an Asp at P_1 (Murphy *et al.*, 1988; Odake *et al.*, 1991; Caputo *et al.*, 1994). Although it has previously been shown that ICE itself is not a granzyme B substrate (Chapter III; Darmon *et al.*, 1994), evidence has suggested that other ICE/Ced-3 proteases may be more critical for the induction of apoptosis than ICE itself. In particular, studies of mice deficient in ICE have revealed that ICE does not play a unique role in apoptosis induced by dexamethasone or UV-radiation (Li *et al.*, 1995; Kuida *et al.*, 1995) but may play a role in Fas-induced apoptosis (Kuida *et al.*, 1995). These results suggest that other ICE/Ced-3 proteases may play more critical roles in the induction of apoptotic cell death than ICE itself. While most of the proteases in this family have no

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[2]A version of this chapter has been published: Darmon, A.J., Nicholson, D.W., and Bleackley, R.C. (1995) *Nature* **377**, 446-448

known substrates and have been identified by sequence homology, one ICE/Ced-3 protease with known substrates is CPP32/Apopain/Yama. This protease was originally identified by searching sequence databases (Fernandes-Alnemri *et al.*, 1994) but was subsequently isolated as the protease responsible for cleavage of the nuclear protein poly(ADP-ribose) polymerase (PARP) during the induction of apoptosis (Nicholson *et al.*, 1995). Simultaneously, Tewari *et al.* (1995a) identified CPP32 as a PARP-cleaving activity using an *in vitro* system.

PARP is a 113-kDa nuclear protein which transfers the ADP-ribose moiety from nicotinamide adenine dinucleotide (NAD) in a DNA-dependent manner to a selected number of protein acceptors involved in chromatin architecture and DNA metabolism (heteromodification) as well as to itself (automodification) (reviewed by de Murcia and Ménissier de Murcia, 1994; Lindahl *et al.*, 1995). In this capacity, PARP is involved in the maintenance of genome integrity and chromatin structure (Ding *et al.*, 1992; Smulson *et al.*, 1994; Ding and Smulson, 1994) as well as in DNA repair (Satoh and Lindahl, 1992).

The structure of PARP is shown in Figure IV-1A. This protein is multifunctional and has been divided into three domains based on partial proteolysis (Kameshita *et al.*, 1984): a 46-kDa amino terminal DNA binding domain which contains two independently folded zinc-finger-like motifs of sequence $CX_2CX_{28/30}HX_2C$ (Ménissier de Murcia *et al.*, 1989; Gradwohl *et al.*, 1990) and a bipartite nuclear localization sequence (Schreiber *et al.*, 1992); a 22-kDa central automodification domain containing the glutamic acid residues which act as acceptors for poly(ADP-ribose); and a 54-kDa carboxy terminal NAD-binding domain which contains the sequences required for PARP catalytic activity (amino acids 859-908).

Cleavage of PARP during apoptosis is believed to lead to inhibition of DNA repair processes as well as the release of negative regulation of the Ca^{++}/Mg^{++}-dependent endonuclease involved in DNA fragmentation, a hallmark of apoptosis (Tanaka *et al.*, 1984). Additionally, it may contribute to cell death via depletion of NAD and ATP in the cell, which occurs during apoptosis (Kaufmann *et al.*, 1993). Recent evidence has shown that PARP is not essential as PARP knockout mice develop normally but are more susceptible to environmental stress (Wang *et al.*, 1995b). Thus, while PARP cleavage may act as a marker for CPP32 activity, it seems likely that CPP32 induces apoptosis by cleaving several proteins. Indeed, subsequent studies have revealed that CPP32 cleaves a number of nuclear proteins, including the 70-kDa protein component of the U1 small nuclear ribonucleoprotein particle (Casciola-Rosen *et al.*, 1996), the catalytic subunit of DNA-dependent protein kinase

(Casciola-Rosen *et al.*, 1996; Song *et al.*, 1996), and sterol regulatory element binding proteins (Wang *et al.*, 1995a; 1996). It is now thought that CPP32 plays a role in the induction of the nuclear events of apoptosis by cleaving a number of nuclear proteins.

The structure of CPP32 is shown in Figure IV-1B. Like ICE, CPP32 is synthesized as an inactive precursor, although pro-CPP32 is only 32-kDa in size, compared to 45-kDa for pro-ICE. The majority of the size difference is in the amino terminal pro-domain - the pro-domain of ICE is ~11-kDa while for CPP32 the pro-domain is only 3-kDa. Additionally, while the p10 and p20 subunits are separated by an intervening sequence of ~3-kDa in pro-ICE, there is no sequence between p17 and p12 (the subunits of the active form) in pro-CPP32. Although the cleavages occurring during CPP32 activation are at Asp residues, the surrounding sequences are not conserved between CPP32 and ICE, suggesting that granzyme B could activate CPP32 and not recognize ICE.

Although ICE and CPP32 are members of the same family there are several differences between these two proteases. Firstly, CPP32 is more closely related to Ced-3 than ICE, which suggests that CPP32 may be the mammalian Ced-3 homologue. In addition, these proteases have different substrate specificity - while CPP32 is able to cleave PARP, it cannot cleave pro-IL-1β and the reverse is true for ICE. This difference in substrate specificity is illustrated by the inhibitors which specifically target these proteins: CPP32 is inhibited by the peptide aldehyde Ac-DEVD-CHO ($K_{i,CPP32}$ < 1 nM; [Nicholson *et al.*, 1995]) but not by Ac-YVAD-CHO ($K_{i,CPP32}$ = 12 μM [Nicholson *et al.*, 1995]). The reverse is true for ICE - inhibition by Ac-YVAD-CHO ($K_{i,ICE}$ = 0.76 nM, [Thornberry *et al.*, 1992]) but not by Ac-DEVD-CHO ($K_{i,ICE}$ >10 μM [Nicholson *et al.*, 1995).

The discovery of CPP32 as a protease primarily involved in the proteolysis of a nuclear enzyme was intriguing since granzyme B has also been implicated in the induction of nuclear events during granule-based CTL-mediated cytotoxicity. Shi *et al.* (1992a, 1992b) have previously shown that fragmentin-2 (a serine protease purified from rat natural killer cells and having homology to granzyme B) is able to induce rapid DNA fragmentation in YAC-1 cells in the presence of perforin. Additionally, granzyme B is necessary for the early induction of DNA fragmentation and apoptosis in allogeneic target cells lysed by CTL (Heusel *et al.*, 1994), natural killer or lymphokine-activated killer cells (Shresta *et al.*, 1994) from mice homozygous for a null mutation in the granzyme B gene. It was therefore possible that the link between granzyme B and target cell DNA fragmentation could be mediated by CPP32. The experiments outlined

in this chapter were designed to determine whether pro-CPP32 can act as a granzyme B substrate.

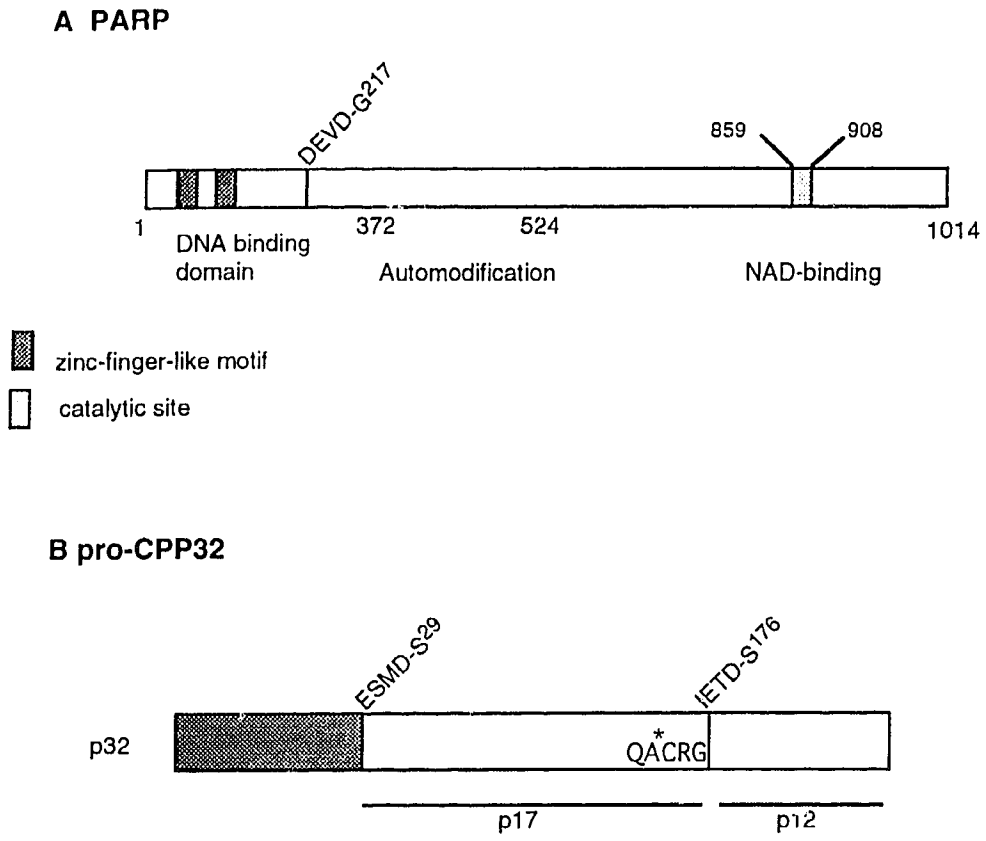


Figure IV-1: The Structure of poly(ADP-ribose) polymerase (PARP) and pro-CPP32.

A. The structure of PARP showing location of dominant features. Numbers along the bottom represent amino acid numbers and delineate the domains. DEVD represents the cleavage site of PARP during apoptosis. The scissile bond is designated by a hyphen.

B. The structure of pro-CPP32. Cleavage sites during activation are shown above the schematic. The scissile bond is indicated by the hyphen. The location of the conserved pentapeptide containing the catalytic cysteine (*) is highlighted.

References: Lazebnik *et al.*, 1994; Nicholson *et al.*, 1995

RESULTS AND DISCUSSION

FPLC Separation of Granzymes and Cleavage of pro-CPP32

In order to determine whether pro-CPP32 could act as a granzyme B substrate, granzymes A and B were first separated from each other. Beginning with lysate from the cytotoxic T cell line MTL 2.8.2, which expresses high levels of granzyme B, FPLC using a Mono-S column was carried out as outlined in Chapter II. Fractions showing the most protein content, as determined by a UV scan during chromatography, were further analyzed for enzymatic activity due to granzyme A (BLT esterase), granzyme B (Asp-ase), or other proteases related to chymotrypsin (Chym-ase). The results of these enzymatic assays are shown in Figure IV-2A. Granzyme B appears to elute as a doublet during FPLC. One possible explanation for this is alternate glycosylation. Granzyme B contains two *N*-linked glycosylation sites, and either one or both can be linked to complex carbohydrates (Griffiths and Isaaz, 1993). The relevant fractions showed little, if any, contamination by each other or any other granzymes. The fractions containing predominantly granzyme B activity (fraction 20) and granzyme A activity (fraction 24) were concentrated as outlined in Chapter II and then checked for enzymatic activity. These fractions were then tested for their ability to cleave *in vitro* translated, radiolabeled pro-CPP32. Results of this study are shown in Figure IV-2B. It was found that granzyme B was able to cleave pro-CPP32 to a band of correct size to represent active protein, while granzyme A could not. The size of the band generated by granzyme B, ~20-kDa, corresponds to cleavage between the p17 and p12 subunits of pro-CPP32 at a site with sequence IETD-S. This site conforms with the proposed substrate specificity of granzyme B based on molecular modeling requiring Asp at P_1 and a second acidic residue at P_3 (Murphy *et al.*, 1988). Interestingly, the analogous site in pro-ICE also conforms to the substrate specificity for granzyme B, although it has previously been shown that granzyme B does not cleave pro-ICE (Chapter III; Darmon *et al.*, 1994), suggesting that conformational aspects of the substrate likely play an important role in determining accessibility to proteases.

Although a band does become visible following treatment of pro-CPP32 by granzyme A, this is thought to be due to cleavage at an inappropriate site since it is not the correct size to produce active enzyme. Incubation of pro-CPP32 with whole MTL lysate resulted in disappearance of full length CPP32 precursor and a band at ~20 kDa. In other experiments this band was not always present (data not shown) due to extensive degradation in the presence of multiple granzymes. In contrast, incubation with granzyme B always gave the 20-kDa cleavage product.

Cleavage of pro-CPP32 by COS Cell Lysates

In order to establish that cleavage of pro-CPP32 was dependent on granzyme B proteolytic activity the heterologous expression system that was developed in the lab (Caputo *et al.*, 1993), which allows analysis of granzyme B in the absence of other granzymes, was used. This is the same system previously used to study ICE activation by granzyme B (Chapter III, Darmon *et al.* 1994). Enzymatically active murine granzyme B, as well as the inactive zymogen and an active site Ser-Ala mutant (S183A [Caputo *et al.*, 1994]), were expressed in COS cells and the ability of lysates of these cells to cleave *in vitro* translated, radiolabeled pro-CPP32 was assessed. It was found that COS cell lysates expressing enzymatically active granzyme B were able to cleave pro-CPP32 while those that produced inactive zymogen or the active site mutant S183A were not (Figure IV-3). The band seen in the lysates mediating cleavage was the same size as that generated when pro-CPP32 was incubated with granzyme B isolated from MTL (Figure IV-2B).

PARP-Cleaving Activity of COS Cells Expressing Granzyme B

Similar COS cell lysates were used to show that the cleavage mediated by granzyme B produced active, PARP-cleaving, endogenous CPP32. Incubation of *in vitro* translated [^{35}S]PARP with COS cell lysates expressing active granzyme B resulted in PARP cleavage to produce the 89-kDa and 24-kDa fragments found in apoptotic cells (Lazebnik *et al.*, 1994) (Figure IV-4A), suggesting that CPP32 is indeed active in these cells. In contrast, lysates containing the inactive zymogen form of granzyme B, or the active site Ser-Ala mutant (S183A) did not result in cleavage of PARP. An inhibitor study, shown in Figure IV-4B, revealed that the PARP-cleaving activity was sensitive to the cysteine protease inhibitors iodoacetamide and *N*-ethylmaleimide, but not to E-64 (another cysteine protease inhibitor), and not to any serine protease inhibitors, an inhibitor profile characteristic of the ICE/Ced-3 family of proteases (Nicholson *et al.*, 1995). Significantly, the PARP-cleaving activity was not sensitive to aprotinin, phosphoramidon or chymostatin, all inhibitors which have previously been shown to inhibit granzyme B (Poe *et al.*, 1991). The activity of these lysates was shown to be related to CPP32 since a potent, specific inhibitor of CPP32, Ac-DEVD-CHO ($K_{i,CPP32}$ < 1 nM [Nicholson *et al.*, 1995]), was able to inhibit the PARP-cleaving activity of these COS cell lysates (Figure IV-4B).

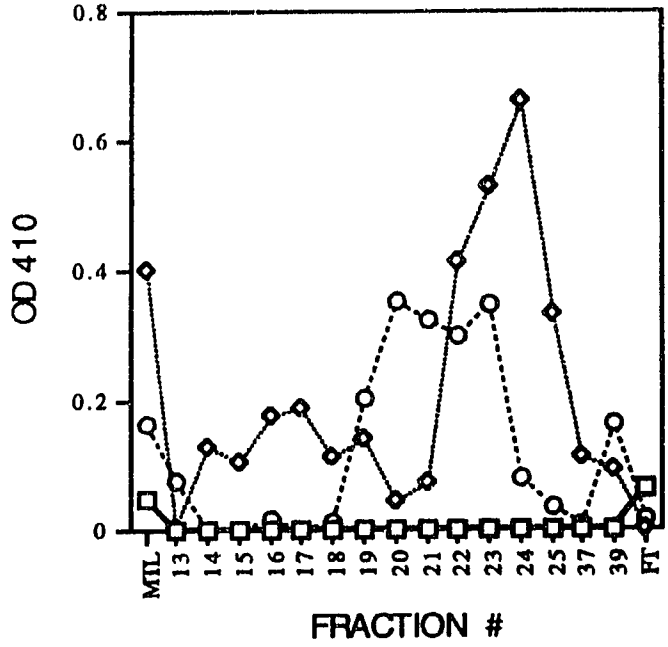
FIGURE IV-2: Enzymatic Activity and Cleavage of pro-CPP32 by FPLC Fractions from MTL 2.8.2 Cell Lysate

A. Enzymatic activity of FPLC fractions from MTL 2.8.2 cell lysate. Results of separation of granzymes from MTL 2.8.2 cell lysate by FPLC. MTL - MTL 2.8.2 lysate before purification; FT - flow through fraction from purification. diamonds - BLT esterase assay of granzyme A activity; circles - Asp-ase assay of granzyme B activity, squares - assay of chymotrypsin-like activity.

B. Cleavage of pro-CPP32 by FPLC-separated Granzyme B.

[^{35}S]pro-CPP32 was incubated with whole MTL 2.8.2 lysate (MTL), FPLC fraction 20 (granzyme B) or FPLC fraction 24 (granzyme A) for 4 hours at 37°C and then separated by SDS-PAGE. [^{35}S]pro-CPP32 alone was used as a negative control.

A


0.8
0.6
0.4
0.2
0
OD 410
MTL
13
14
15
16
17
18
19
20
21
22
23
24
25
37
39
FT
FRACTION #

B

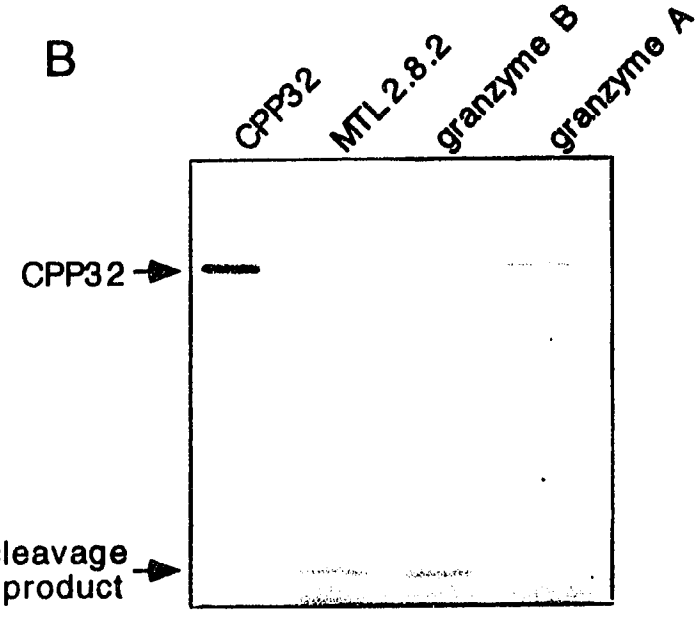

CPP32
MTL 2.8.2
granzyme B
granzyme A
CPP32
cleavage product

FIGURE IV-3: Cleavage of pro-CPP32 by granzyme B-expressing COS lysates. [^{35}S]pro-CPP32 was combined with cytosolic lysates from COS cells expressing active granzyme B, the inactive zymogen of granzyme B or an active site Ser-Ala mutant of granzyme B (S183A) for 4 hours at 37°C followed by separation by SDS-PAGE. Visualization was by autoradiography. [^{35}S]pro-CPP32 alone was used as a negative control.

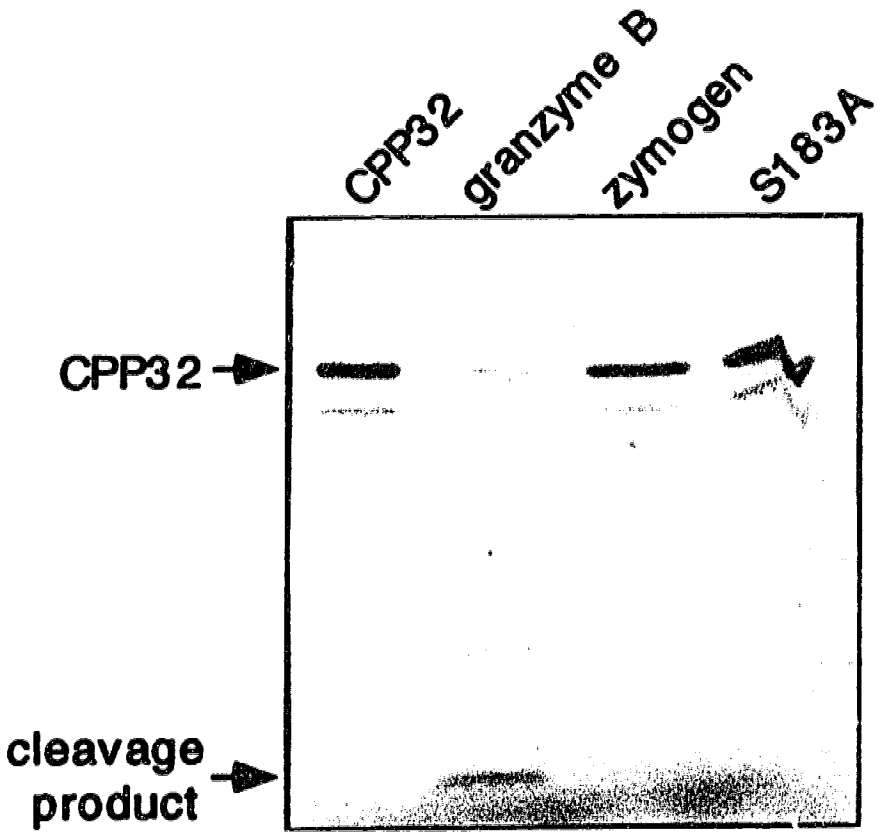
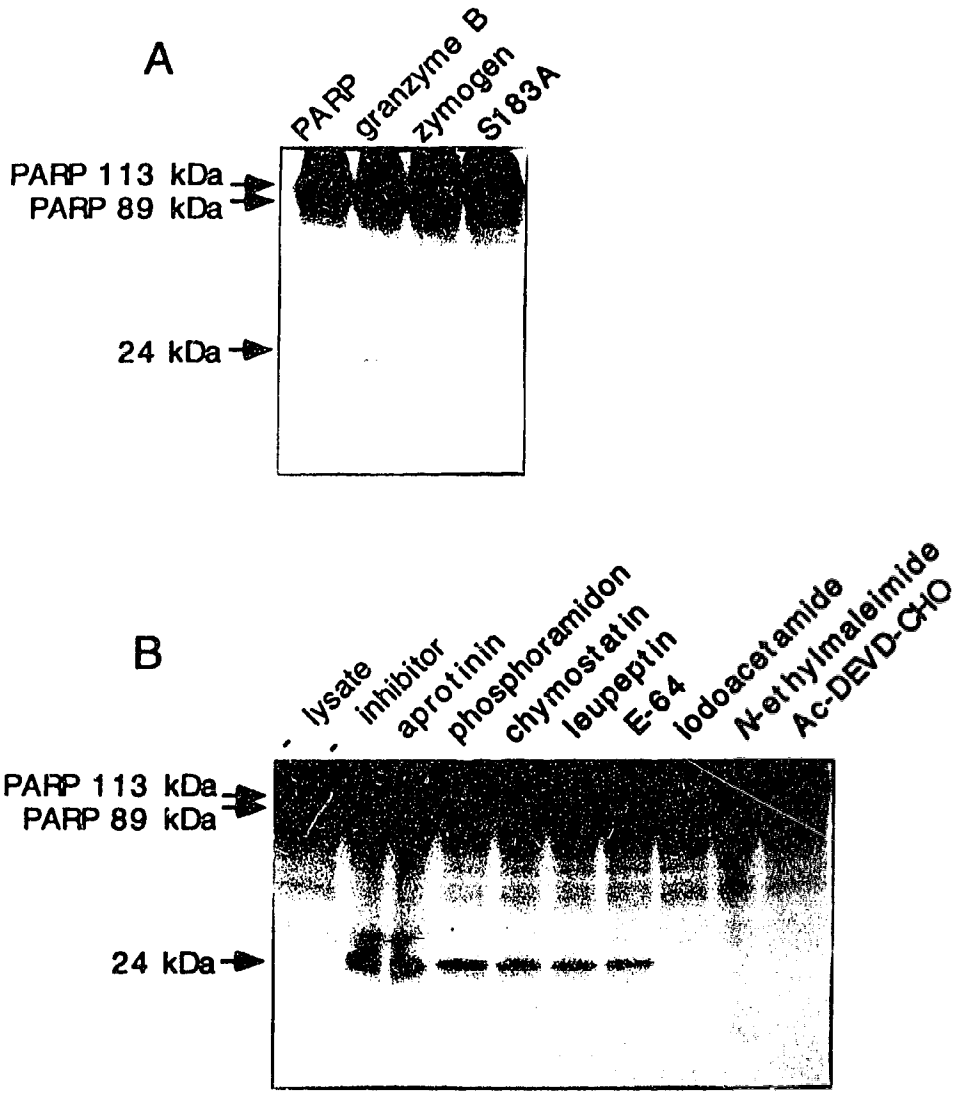

CPP32
granzyme B
zymogen
S183A
CPP32
cleavage
product

FIGURE IV-4: PARP cleaving activity of granzyme B-expressing COS cell lysates.

A - Cleavage of PARP by lysates expressing active recombinant murine granzyme B. [^{35}S]PARP was combined with cytosolic lysates from COS cells expressing active granzyme B, the inactive zymogen of granzyme B or an active site Ser-Ala mutant of granzyme B (S183A) for 4 hours at 37°C followed by SDS-PAGE separation and detection by fluorography. [^{35}S]PARP alone was used as a negative control.

B - Inhibition of PARP-cleaving activity of COS cell lysates. Cytosolic lysates from COS cells expressing active granzyme B were incubated with [^{35}S]PARP in the presence of the protease inhibitors indicated, or the synthetic tetrapeptide aldehyde inhibitor Ac-DEVD-CHO. Control samples were incubated either in the absence of COS cell lysate or in the presence of lysate but in the absence of inhibitor.


A
PARP
granzyme B zymogen S183A
PARP 113 kDa
PARP 89 kDa
24 kDa
B
lysate
inhibitor
aprotinin
phosphoramidon
chymostatin
leupeptin
E-64
Iodoacetamide
N-ethylmaleimide
Ac-DEVD-CHO
PARP 113 kDa
PARP 89 kDa
24 kDa

Cleavage of CPP32 in COS Cells

The results presented thus far suggest that pro-CPP32 is a cellular substrate for granzyme B. In addition, the presence of PARP-cleaving activity in COS cells expressing enzymatically active granzyme B (an activity which cannot be attributed to granzyme B itself, based on inhibitor studies) seems to suggest that expression of granzyme B in COS cells results in activation of their endogenous CPP32. Presence of active CPP32 in lysates from COS cells expressing granzyme B was confirmed using Western blot analysis with an antibody directed against the p17 subunit of CPP32 (Figure IV-5A). Again, active CPP32 (demonstrated by presence of the p17 subunit) was found only in lysates from COS cells expressing active granzyme B and not in lysates expressing the zymogen form of granzyme B or the active site mutant (S183A) (demonstrated by the presence of p32 only). These results suggest that expression of active granzyme B in COS cells results in activation of their endogenous CPP32, which is able to cleave PARP. Interestingly, the amount of CPP32 that was cleaved in the COS cell lysates increased following incubation at 37°C for one hour, as opposed to no pre-incubation (data not shown). CPP32 was isolated from cytosolic fractions of apoptotic cells (Nicholson *et al.*, 1995), while granzyme B contains a signal sequence (Caputo *et al.*, 1993) and is likely targeted to COS cell lysosomes by the mannose-6-phosphate receptor pathway, similar to its targeting mechanism *in vivo* (Griffiths and Isaaz, 1993). Therefore, it is likely that the observed time dependence of cleavage is due to the separate localization of granzyme B and CPP32.

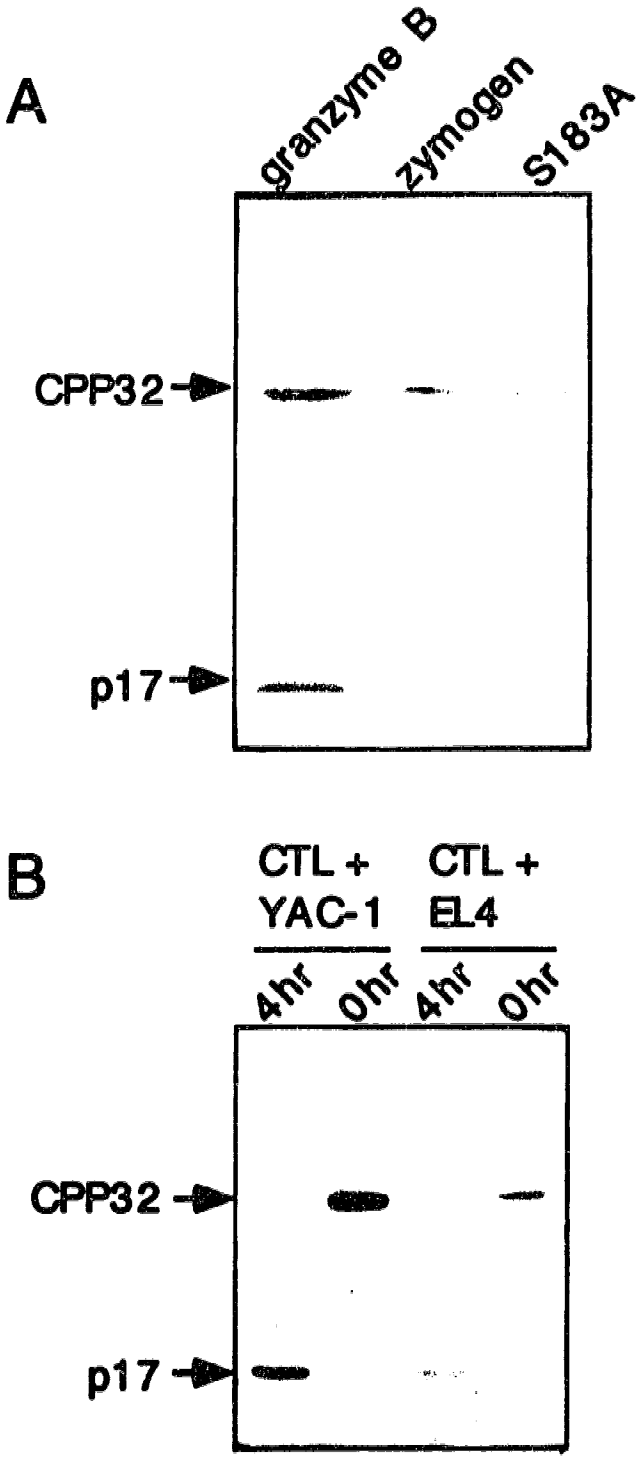
Cleavage of CPP32 in Target Cells during Granule-Mediated Killing

Finally, it was determined whether CPP32 was cleaved in target cells following attack by CTLs. In these studies, the T cell line CTL21.9, which kills through the granzyme/perforin pathway (Garner *et al.*, 1994), was used as an effector cell. Effector cells and target cells (EL4 and YAC-1) were incubated together for 4 hours and then the effectors were selectively removed using an anti-CD8 antibody followed by magnetic separation with anti-IgG-coated Dynabeads. The remaining targets were lysed and the lysates were analyzed for CPP32 status by Western blotting. The results of this experiment are shown in Figure IV-5B. In both target cell types, full length CPP32 was present in untreated target cells (p32) but was completely processed to the active form following incubation with effector cells for 4 hours (p17). These results further confirm the above findings that granzyme B cleaves and activates CPP32 during CTL-mediated cytotoxicity.

FIGURE IV-5: ***In vivo*** **cleavage of CPP32.**

A. Cleavage of CPP32 in COS cells expressing granzyme B. Lysates of COS cells expressing recombinant murine granzyme B, the inactive zymogen of granzyme B or an active site Ser-Ala mutant of granzyme B (S183A) were pre-incubated at 37°C for 1 hour prior to SDS-PAGE followed by Western blotting to detect CPP32.

B. Cleavage of CPP32 in target cells following CTL attack. CTL21.9 effector cells were incubated with YAC-1 or EL4 target cells at an effector-to-target ratio of 1:1 for 4 hours. CTLs were removed from the samples using Dynabeads and remaining target cells were lysed, lysates were resolved by SDS-PAGE and then CPP32 was detected by Western blotting.


A
granzyme B
zymogen
S183A
CPP32
p17
B
CTL + YAC-1
CTL + EL4
4hr
0hr
4hr
0hr
CPP32
p17

In conclusion, the studies presented here have resulted in the identification of an intracellular substrate for granzyme B. FPLC-separated granzyme B, and COS cell lysates expressing active granzyme B (but not inactive forms of granzyme B) could cleave *in vitro* translated pro-CPP32 to produce a band at ~20-kDa (Figure IV-2; IV-3). This band would represent the p17 subunit with an intact pro-domain, and requires cleavage at a site with sequence IETD-S. The CPP32 generated by granzyme B cleavage possesses PARP-cleaving activity, as shown by the *in vitro* cleavage assay using radiolabeled PARP as substrate (Figure IV-4). When COS cell lysates expressing active granzyme B, but not inactive zymogen or an active site mutant, were incubated with radiolabeled PARP, PARP was cleaved to two fragments of 89-kDa and 24-kDa - the same sizes as the fragments generated during apoptosis. An inhibitor study of the PARP cleaving activity of these lysates revealed that the mediator was an ICE/Ced-3 protease, identified as CPP32 by the use of the CPP32 inhibitor Ac-DEVD-CHO. Therefore, in this system, expression of granzyme B in COS cells resulted in the activation of their endogenous CPP32. Activation of CPP32 in active granzyme B-expressing COS cells was confirmed by Western blotting (Figure IV-5A).

Finally, a role for CPP32 activation in CTL-mediated cytotoxicity was suggested by the fact that CPP32 is cleaved in target cells following granule-mediated CTL attack (Figure IV-5B). A role for PARP activity in granule-mediated killing had previously been suggested by two groups (Redegeld *et al.*, 1992; Hayward and Herberger, 1988) who demonstrated that a PARP inhibitor could block cellular cytotoxicity. Again, it seems that PARP activity may be required for apoptosis, but activation of CPP32, which cleaves PARP and returns its activity to basal levels, is also required. Temporally, PARP activity may be required at the onset of apoptosis, perhaps to deplete NAD and ATP, but PARP must subsequently be inactivated for apoptosis to proceed.

These studies were the first to define the link between cell-mediated cytotoxicity and developmental or programmed cell death - that is, that a CTL (or NK cell) utilizes a cell's endogenous death program to cause a target cell to die. Although the role of PARP in apoptosis is controversial, CPP32 activity is responsible for the cleavage of a number of nuclear proteins during apoptosis and therefore may be involved in the induction of the nuclear events of apoptosis. This is intriguing since granzyme B has been linked primarily with DNA fragmentation during CTL-mediated cytotoxicity.

Identification of an intracellular substrate for granzyme B as a protease which is part of an enzymatic cascade implies that there may be numerous sites at which cell-mediated immunity can be inhibited within the target cell. This information is crucial to the design and development of novel immunosuppressants.

Addendum

The results presented here were subsequently confirmed by other workers (Quan *et al.*, 1996; Martin *et al.*, 1996). Furthermore, granzyme B has recently been shown to cleave the precursors of Mch3α/CMH-1/ICE-LAP3 (Gu *et al.*, 1996; Chinnaiyan *et al.*, 1996b) and FLICE/MACH (Muzio *et al.*, 1996) suggesting that multiple CPP32-related protease precursors serve as granzyme B targets. In each instance, granzyme B activates the protease by cleaving between the large and small subunits, as suggested by the data presented here. The prodomain is then removed by an autocatalytic mechanism, but its removal is not required for enzymatic activity (Gu *et al.*, 1996; Martin *et al.*, 1996).

One hypothesis resulting from these studies would be that granzyme B cleavage of CPP32 (and CPP32-related proteases) is somehow linked to the induction of target cell DNA fragmentation. Additional studies revealed that CPP32-proteases are indeed involved in the induction of target cell DNA fragmentation, and that granzyme B is the only granzyme capable of cleaving pro-CPP32, suggesting that the nonredundant role proposed for granzyme B (Heusel *et al.*, 1994; Shresta *et al.*, 1995) is mediated by the CPP32 enzymes (Chapter V; Darmon *et al.*, 1996).

CHAPTER V - CPP32 CLEAVAGE REPRESENTS A CRITICAL ROLE FOR GRANZYME B

CHAPTER V - CPP32 CLEAVAGE REPRESENTS A CRITICAL ROLE FOR GRANZYME B[3]

INTRODUCTION

Cytotoxic T lymphocytes (CTLs) represent the body's primary defense against tumourigenic and virus-infected cells, and are responsible for organ transplant rejection and autoimmune disease (reviewed by Berke, 1995; Atkinson and Bleackley, 1995; Smyth, 1995). When the T cell receptor (TCR) recognizes foreign antigen, presented in the context of the major histocompatibility complex class I molecules, the CTL induces apoptotic cell death in the target cell through one of two distinct mechanisms - Fas-based and granule-mediated cytotoxicity. During granule-mediated killing, TCR activation leads to the induction of function-related genes, including perforin and the granzymes. These are packaged into granules which are then mobilized to the surface of the CTL that is adjacent to the target cell. Exocytosis of CTL granule contents into the intercellular space between CTL and target cell allows perforin, a pore-forming protein found in the granules (Liu *et al.*, 1995), to polymerize in the target cell membrane, thereby facilitating the entry of the granzymes, a family of serine proteases (Atkinson and Bleackley, 1995; Lowin *et al.*, 1995) into the target cell, where they induce apoptosis.

Granzyme B (Cytotoxic Cell Proteinase-1/CCP1) represents a prototype of six of these serine proteases (granzymes B to G). Originally identified as a CTL-specific gene (Lobe *et al.*, 1986a, 1986b) whose expression correlated with the induction of cytotoxicity (Lobe *et al.*, 1986b), it was subsequently localized to the cytoplasmic granules of CTLs (Redmond *et al.*, 1987; Ojcius *et al.*, 1991). Quantitative PCR revealed that only granzyme B expression is correlated with the development of cytotoxicity in T cells in response to stimulation by mitogen, allogeneic cells or anti-CD3 (Prendergast *et al.*, 1992), suggesting that it may be a direct effector in the lytic process. Shi *et al.* (1992a, 1992b) have shown that fragmentin-2, a serine protease purified from rat natural killer cell granules and having homology to granzyme B, is able to induce rapid DNA fragmentation, a hallmark of apoptosis, in YAC-1 target cells in the presence of perforin. Additionally, Heusel *et al.* (1994) have shown that granzyme B is necessary for the early induction of DNA fragmentation and apoptosis in allogeneic target cells lysed by CTL (Heusel *et al.*, 1994; Shresta *et al.*, 1995),

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natural killer or lymphokine-activated killer cells (Shresta *et al.*, 1995) isolated from mice homozygous for a null mutation in the granzyme B gene.

Recent studies of apoptosis have shown that a family of cysteine proteases related to Interleukin-1β Converting Enzyme (ICE) play a key role. Originally identified as the protease responsible for producing biologically active IL-1β from the inactive precursor (Thornberry *et al.*, 1992; Cerretti *et al.*, 1992), a role for ICE in the induction of apoptotic cell death was suggested by the discovery that *ced-3*, a gene required for programmed cell death in the nematode *Caenorhabditis elegans*, had homology to ICE (Yuan *et al.*, 1993). Indeed, overexpression of ICE or *ced-3* in Rat-1 fibroblasts resulted in apoptotic death of these cells (Miura *et al.*, 1993). However, studies of mice deficient in ICE seemed to suggest that ICE does not play a unique role in apoptosis, but rather that other related proteases may be critical (Li *et al.*, 1995).

Multiple ICE-like proteases have now been isolated (reviewed by Henkart, 1996; Martin and Green, 1995), including Ich1L/Nedd-2 (Wang *et al.*, 1994b; Kumar *et al.*, 1994), TX/Ich-2/ICE_{rel}II (Faucheu *et al.*, 1995; Kamens *et al.*, 1995; Munday *et al.*, 1995), ICE_{rel}III (Munday *et al.*, 1995), Mch2α (Fernandes-Alnemri *et al.*, 1995a), Mch3α/CMH-1/ICE-LAP3 (Fernandes-Alnemri *et al.*, 1995b; Lippke *et al.*, 1996; Duan *et al.*, 1996), FLICE/MACH (Boldin *et al.*, 1996; Muzio *et al.*, 1996), and CPP32/Apopain/Yama (Fernandes-Alnemri *et al.*, 1994; Nicholson *et al.*, 1995; Tewari *et al.*, 1995a). These enzymes can be divided into three subfamilies: the ICE-like enzymes (ICE, TX/Ich-2/ICE_{rel}II, ICE_{rel}III), the CPP32-like proteases (CPP32, Ced-3, Mch2α, Mch3α/CMH-1/ICE-LAP3, FLICE/MACH) and the Nedd-2 proteins (Nedd-2, Ich-1L). Each of these proteases is synthesized as an inactive precursor requiring cleavage after Asp residues for activation. CPP32 (putative cysteine protease of 32-kDa) was identified as the protease responsible for cleavage of the nuclear protein poly(ADP-ribose) polymerase (PARP) during the induction of apoptosis (Nicholson *et al.*, 1995; Tewari *et al.*, 1995a), as well as the catalytic subunit of DNA-dependent protein kinase (DNA-PK [Casciola-Rosen *et al.*, 1996; Song *et al.*, 1996]) and the 70-kDa protein component of the U1 small ribonucleoprotein particle (U1-70kDa [Casciola-Rosen *et al.*, 1996]). These enzymes play roles in DNA and RNA turnover within the cell - U1-70kDa is part of the complex involved in RNA splicing while DNA-PK and PARP are involved in activating DNA repair in response to strand breaks. The fact that CPP32 cleaves multiple nuclear proteins seems to suggest that CPP32 is involved in inducing some of the nuclear events associated with apoptosis. Indeed, treatment of apoptotic nuclei (Nicholson *et al.*, 1995; Lazebnik *et al.*, 1994) or

whole cells (Schlegel *et al.*, 1996) with the CPP32 inhibitor Ac-DEVD-CHO prevents the nuclear changes of apoptosis. In addition, cleavage of PARP, and its consequent inactivation, may result in the release of negative regulation of the Ca^{++}/Mg^{++}-dependent endonuclease implicated in DNA fragmentation (Tanaka *et al.*, 1984; Yoshihara *et al.*, 1974, 1975) further suggesting a link between CPP32 and apoptotic DNA fragmentation.

When the ICE proteases were first discovered, it was postulated that granzyme B, which has substrate specificity requiring an Asp at P_1 (Murphy *et al.*, 1988; Odake *et al.*, 1991), could induce target cell apoptosis by cleaving and activating an ICE protease (Vaux *et al.*, 1994). Although there is currently no evidence to suggest that granzyme B cleaves and activates ICE (Chapter III; Darmon *et al.*, 1994), it has been demonstrated that CPP32 is an intracellular substrate both *in vitro* and *in vivo* for granzyme B (Chapter IV; Darmon *et al.*, 1995; Quan *et al.*, 1996; Martin *et al.*, 1996). Other workers have shown that granzyme B can cleave the CPP32-related proteases CMH-1/Mch3α/ICE-LAP3 (Gu *et al.*, 1996; Chinnaiyan *et al.*, 1996b) and FLICE/MACH (Muzio *et al.*, 1996) *in vitro* , suggesting that granzyme B may act by activating this family of proteases. The finding that CPP32 can be cleaved by granzyme B, while allowing a model for granzyme B activity to be proposed, does not prove that this is the pathway linking granzyme B to target cell DNA fragmentation. Granzyme B could have another substrate within the cell which accounts for the ability of this protease to induce fragmentation. In addition, other granzymes could also cleave and activate CPP32, although this seems unlikely since no other granzyme cleaves after Asp residues. The studies outlined in this chapter were designed to address the question of whether CPP32 is the link between granzyme B and target cell DNA fragmentation and to determine whether the defect in target cell DNA fragmentation induced by granzyme B-deficient effectors can be explained on the basis of failure to cleave CPP32 and related enzymes.

RESULTS

Generation of Cytolytic Cells Lacking Granzyme B

In these studies, cytolytic cells from C57BL/6J mice (haplotype $H\text{-}2^b$) lacking granzyme B (GB KO) and from wild type control mice (GB WT) were generated from splenocytes using irradiated Balb/c ($H\text{-}2^d$) splenocytes as stimulators, as outlined in Chapter II. These cytolytic cells were then tested for their ability to induce cytolysis (measured as ^{51}Cr release) and DNA fragmentation (measured as $[^3H]$thymidine release) from a number of target cells in order to ensure that they behaved identically to

the freshly isolated cytolytic cells described by previous workers (Heusel *et al.*, 1994; Shresta *et al.*, 1995). The results of this study are shown in Figure V-1. In panel A, ^{51}Cr release from labeled targets incubated with GB KO or GB WT effectors was measured. Four different cell lines were used, and in each case the levels of target cell cytolysis induced by the two effector cells was similar, although the GB WT cells seemed to induce slightly more ^{51}Cr release than the GB KO cells. In contrast, there is a substantial and consistent difference in target cell DNA fragmentation induced by these effectors (Figure V-1B). For three of the targets tested, the GB WT induced at least 3-fold more DNA fragmentation than the GB KO. Interestingly, the difference between the GB KO and GB WT effectors was less pronounced when YAC-1 cells were used as targets. These cells express higher levels of CPP32 than the other target cell lines used (Darmon and Bleackley, unpublished data), suggesting a correlation between CPP32 levels and the efficiency of DNA fragmentation during apoptosis.

To confirm that these cytolytic effectors utilized the granule exocytosis mechanism of killing, rather than Fas-mediated killing, calcium-dependency of cytolysis was determined. As outlined in Chapter I, the granule exocytosis mechanism requires calcium for both degranulation and perforin aggregation in the target cell membrane. In contrast, Fas-mediated cytotoxicity is calcium-independent. Therefore we performed cytotoxicity assays in the presence of a calcium chelator (EGTA). In these studies, four different ^{51}Cr-labeled target cells were incubated with the GB KO and GB WT effectors for 2 or 4 hours, in the presence or absence of EGTA. The results of these studies are shown in Figure V-2. In panel A, GB WT effectors were used, while panel B represents the GB KO effectors. In every case, the presence of EGTA suppressed cytotoxicity to less than 10% specific lysis, compared to 50-70% specific lysis in control samples. These results confirm that the cytolytic activity of the GB KO and GB WT cells is due primarily to granule exocytosis.

CPP32 is Not Cleaved in the Absence of Granzyme B

The ability of the GB KO and GB WT cells, generated above, to induce CPP32 cleavage in target cells was then assessed. Effector and target cells were incubated together for 4 hours at 37°C, the cells were lysed, and lysates were assessed for CPP32 status by Western blotting using an antibody directed against the large subunit of CPP32. The results of this experiment (Figure V-3) show that GB KO cells were unable to induce CPP32 cleavage in YAC-1 and EL4 target cells. In contrast, CPP32 was completely cleaved to the active form (p17/p12) when targets were incubated with GB WT effectors. Similar results were obtained when P815 cells were used as targets

(data not shown). These results clearly demonstrate that granzyme B is required for cleavage and activation of CPP32 during CTL-mediated killing, and confirm the previous finding that CPP32 is a cellular substrate for granzyme B (Chapter IV; Darmon *et al.*, 1995). If the incubation time between effectors and targets was increased to 24 hours, CPP32 still was not cleaved in target cells incubated with GB KO effectors, suggesting that no other granzyme is capable of CPP32 activation following CTL attack.

Inhibition of CPP32-like Enzymes Suppresses DNA Fragmentation

Together, the results thus far suggest that granzyme B is involved in inducing target cell DNA fragmentation through the cleavage and activation of CPP32 (and probably CPP32-like proteases). However, no functional relationship has been established between cleavage of CPP32-like enzymes and the induction of internucleosomal DNA cleavage. These final experiments were designed to study the possible role of CPP32 (and CPP32-like proteases) as a bridge between granzyme B and the induction of target cell DNA fragmentation.

In these experiments two peptide aldehyde inhibitors were used. Peptide aldehydes are potent, reversible inhibitors of cysteine proteases and undergo nucleophilic addition to the catalytic cysteine to form a thiohemiacetal. The potency of these inhibitors was originally attributed to their ability to mimic the transition state during amide bond hydrolysis (Westerik and Wolfenden, 1972), however, the crystal structure of ICE with its inhibitor Ac-YVAD-CHO shows that the inhibitor binds in a non-transition-state conformation with the oxyanion of the thiohemiacetal being stabilized by the active site histidine (Walker *et al.*, 1994; Wilson *et al.*, 1994). Ac-DEVD-CHO has previously been shown to inhibit CPP32 (Nicholson *et al.*, 1995) CMH-1/Mch3α/ICE-LAP3 (Fernandes-Alnemri *et al.*, 1995b; Lippke *et al.*, 1996), FLICE (Muzio *et al.*, 1996) and may inhibit Mch2α (Fernandes-Alnemri *et al.*, 1995a), suggesting that it might inhibit all CPP32-like proteases. In contrast, Ac-DEVD-CHO cannot inhibit ICE (Nicholson *et al.*, 1995). In these studies, ^{51}Cr and [^{3}H]thymidine release from labeled L1210-Fas cells was measured in the presence or absence of this inhibitor, using the cytotoxic T cell clone CTL21.9 (which kills using the granule pathway) as effector. Results of this study are shown in Figure V-4. Similar results were obtained using EL4 and YAC-1 as targets (data not shown). Although Ac-DEVD-CHO had no apparent effect on ^{51}Cr release from labeled targets (Figure V-4A), it dramatically reduced [^{3}H]thymidine release (Figure V-4B) from 75% to 8% in this study. These data suggest that CPP32 cleavage and activation by

granzyme B during CTL-mediated cytotoxicity plays a critical role in the induction of target cell DNA fragmentation. These results cannot be due to inhibition of granzyme B itself. Using [^{35}S]Met-labeled CPP32 precursor as an indicator of granzyme B activity, it was found that the presence of 200 μM of this inhibitor with granzyme B in an *in vitro* cleavage assay failed to suppress granzyme B activity (data not shown).

Having established that Ac-DEVD-CHO affects only [^{3}H]thymidine release, and not ^{51}Cr release, we wondered whether ICE-like proteases also contribute to target cell DNA fragmentation and/or membrane damage. In these studies the prototypic ICE inhibitor Ac-YVAD-CHO was used. This inhibitor has been shown to inhibit ICE (Thornberry *et al.*, 1992) and TX/Ich-2/ICE$_{rel}$II (Kamens *et al.*, 1995) but does not inhibit CPP32-like proteases (Nicholson *et al.*, 1995; Lippke *et al.*, 1996). The results demonstrated that ICE-like proteases play no role in the induction of target cell DNA fragmentation during granule-mediated killing. In a comparison between the effects of Ac-DEVD-CHO and Ac-YVAD-CHO on [^{3}H]thymidine release from labeled targets, Ac-YVAD-CHO had no effect on [^{3}H]thymidine release during granule-mediated killing, even at the highest concentration used (100 μM, Figure V-5). However, Ac-DEVD-CHO had a striking effect on [^{3}H]thymidine release, with maximal inhibition achieved by 50 μM, but clear inhibition even at 5 μM (Figure V-5). These results suggest that ICE-like proteases play no role in the induction of target cell DNA fragmentation, while CPP32-like proteases are required for this process. It was also found that ICE-like proteases play no role in the induction of target cell membrane damage. Ac-YVAD-CHO exerted no effect on ^{51}Cr release from labeled targets when added to the assay at a final concentration of 40 μM (data not shown). These results are consistent with those of other workers who showed that inhibition of macrophage ICE activity by YVAD-chloromethylketone, as demonstrated by suppression of mature IL-1β release, had no effect on ^{51}Cr release in response to CTL attack (Nett-Fiordalisi *et al.*, 1995). Taken together, these inhibitor studies suggest the involvement of CPP32-like, but not ICE-like, proteases in the induction of DNA fragmentation during granule-mediated cytotoxicity. Furthermore, neither of these protease families appears to play a significant role in the induction of membrane damage following CTL attack.

FIGURE V-1: Cytolytic activity of cell lines derived from granzyme B deficient mice and control mice.

Labeled target cells were incubated with cytolytic cells derived from mice lacking granzyme B (GB KO) or from control mice (GB WT) at an effector-to-target ratio of 5:1 in the presence of 2 μg/ml ConA for 2 hours at 37°C, and then ^{51}Cr (A) or [^{3}H]thymidine (B) release was measured. Mean and standard deviation of triplicate samples is shown.

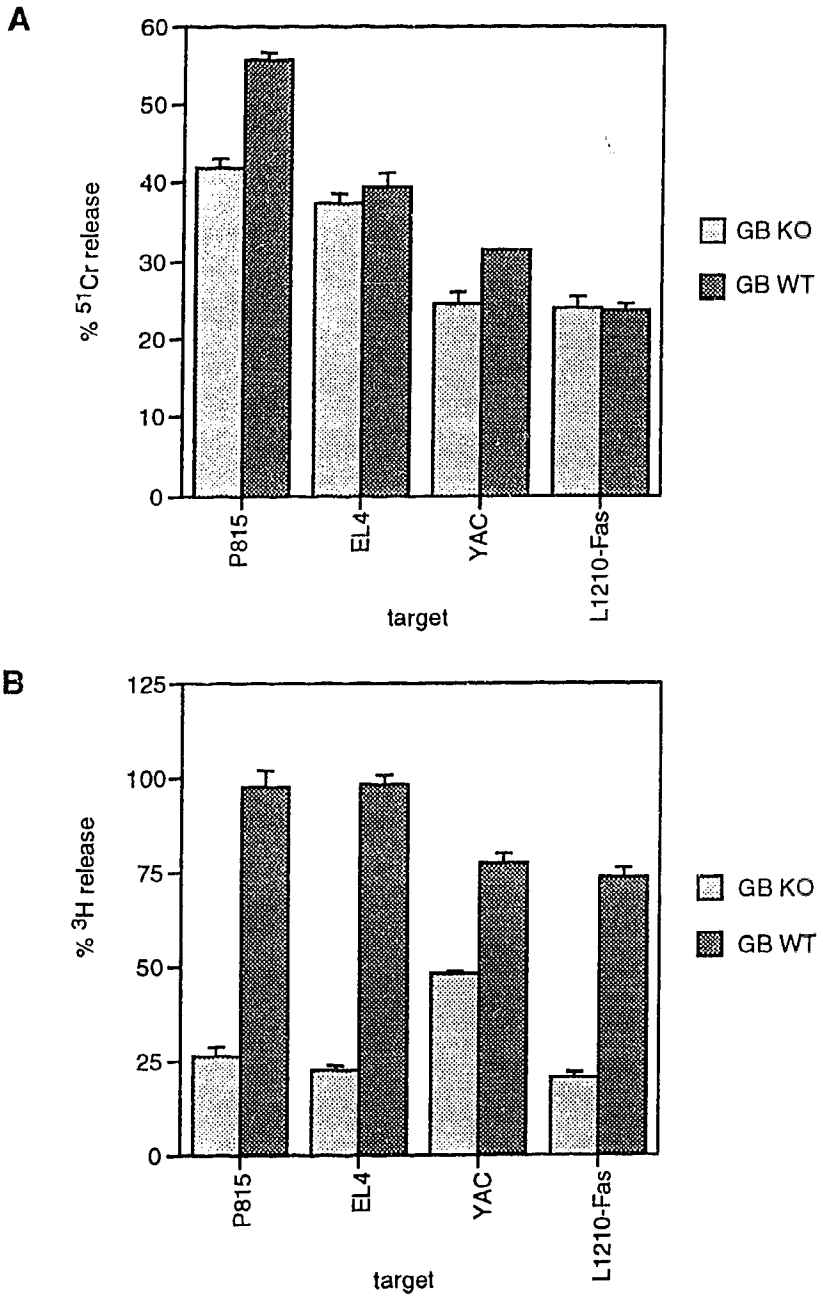
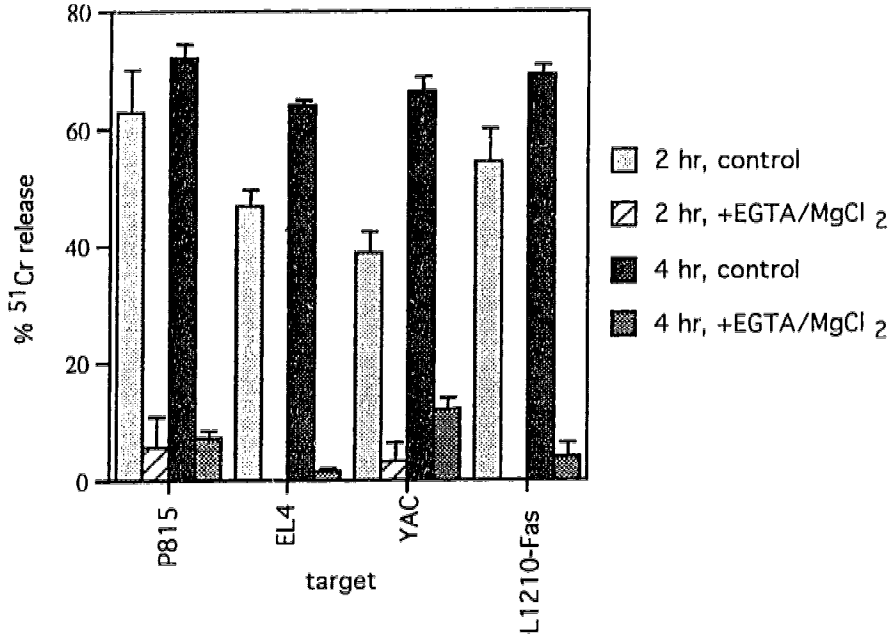

A
60
50
40
30
20
10
0
% 51Cr release
P815
EL4
YAC
L1210-Fas
target
GB KO
GB WT
B
125
100
75
50
25
0
% 3H release
P815
EL4
YAC
L1210-Fas
target
GB KO
GB WT

FIGURE V-2: Calcium-dependency of cytolytic activity of GB KO and GB WT cells.

Labeled target cells were incubated with cytolytic cells derived from control mice (GB WT; A) or from mice lacking granzyme B (GB KO; B) at an effector-to-target ration of 5:1 in the presence of 2 μg/ml ConA, with or without 2 mM EGTA and 4 mM $MgCl_2$ at 37°C for 2 hours or 4 hours. Mean and standard deviation of triplicate samples is shown.

A. GB WT


80
60
40
20
0
% 51Cr release
P815
EL4
YAC
L1210-Fas
target
2 hr, control
2 hr, +EGTA/MgCl 2
4 hr, control
4 hr, +EGTA/MgCl 2

B. GB KO

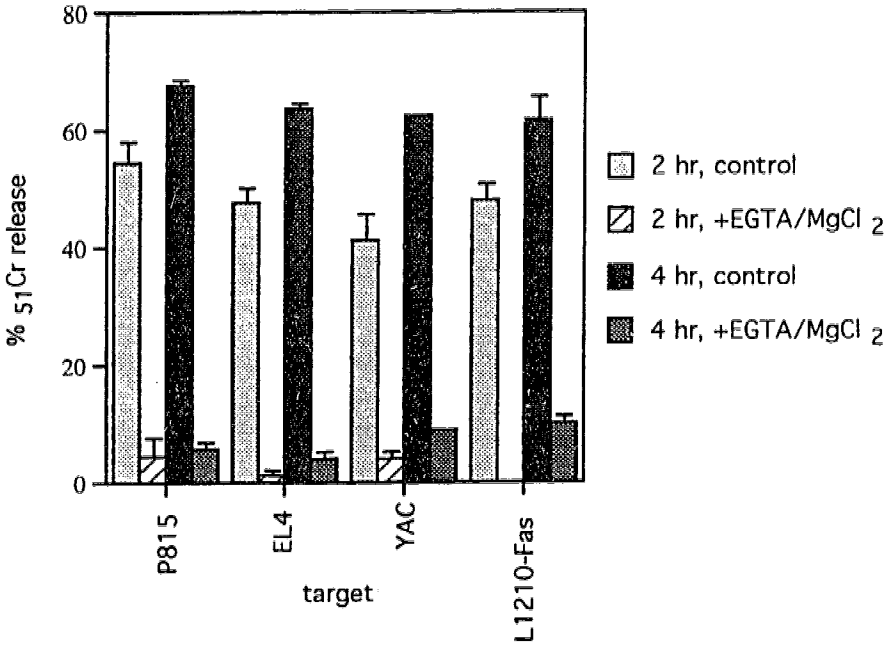

80
60
40
20
0
% 51Cr release
P815
EL4
YAC
L1210-Fas
target
2 hr, control
2 hr, +EGTA/MgCl 2
4 hr, control
4 hr, +EGTA/MgCl 2

FIGURE V-3: CPP32 is not cleaved in the absence of granzyme B.
YAC-1 or EL4 target cells (10^6) were incubated with effector cells lacking granzyme B (GB KO) or control cells (GB WT) at an effector-to-target ratio of 5:1 for 4 hours at 37°C. Cells were then lysed and lysates were resolved by SDS-PAGE. CPP32 was detected using an antibody directed against the large subunit of CPP32.
p32 - full length CPP32; p17 - large subunit of active CPP32.

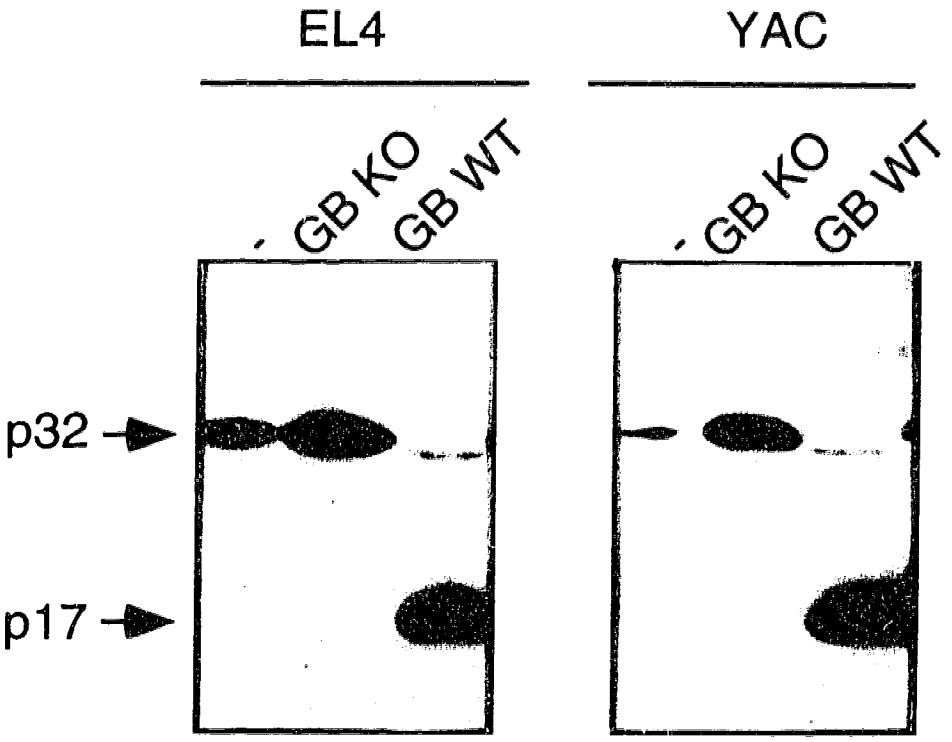
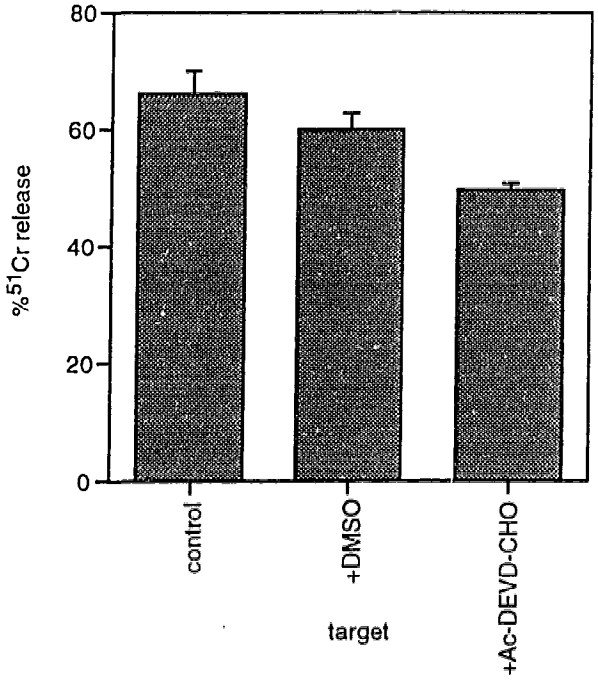

EL4
YAC
-
GB KO
GB WT
-
GB KO
GB WT
p32
p17

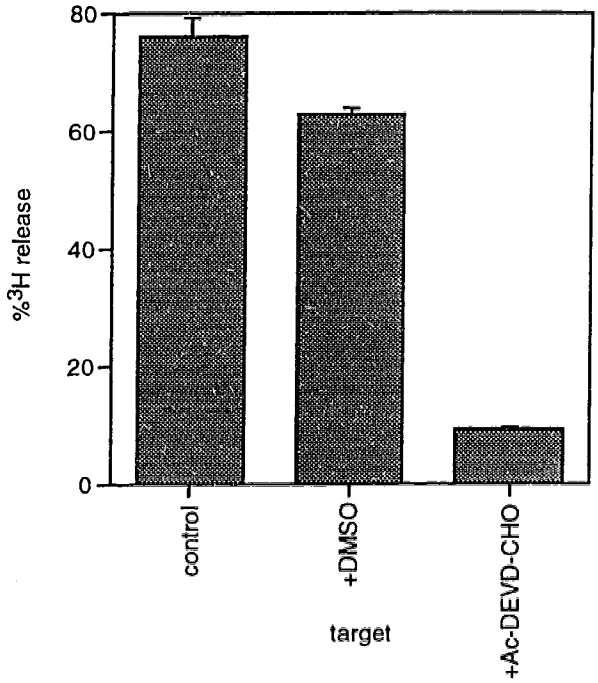
FIGURE V-4: CPP32 is involved in the induction of target cell DNA fragmentation.

Labeled L1210-Fas targets were incubated with CTL21.9 effectors at an effector-to-target ratio of 5:1 for 4 hours at 37°C in the presence of Ac-DEVD-CHO (40 μM) or DMSO alone as a control, and then ^{51}Cr (A) or [^{3}H]thymidine (B) release was measured. Mean and standard deviation of triplicate samples is shown.

A


80
60
40
20
0
%51Cr release
control
+DMSO
+Ac-DEVD-CHO
target

B


80
60
40
20
0
%3H release
control
+DMSO
+Ac-DEVD-CHO
target

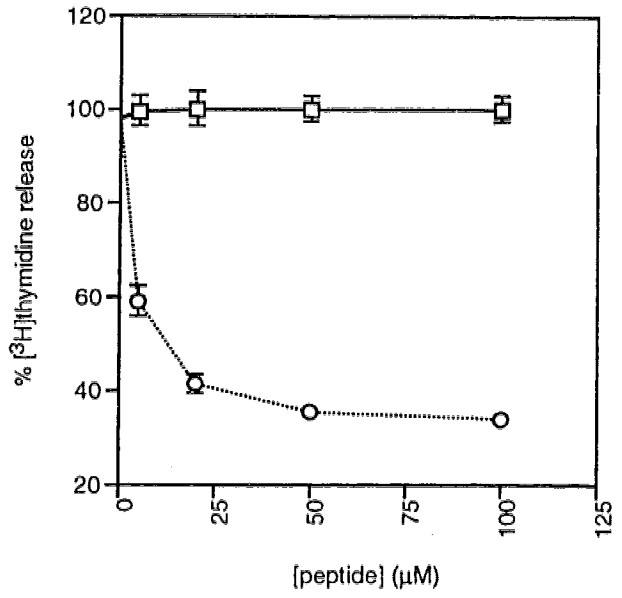


Figure V-5: Inhibition of [^{3}H]thymidine release during granule-mediated cytotoxicity by Ac-DEVD-CHO but not Ac-YVAD-CHO.

Labeled L1210-Fas target cells were incubated with GB WT effectors at an effector-to-target ratio of 5:1 for 4 hours at 37°C in the presence of the indicated concentration of Ac-YVAD-CHO (squares) or Ac-DEVD-CHO (circles), and then [^{3}H]thymidine release was measured. ConA was included at a final concentration of 2 μg/ml. Mean and standard deviation of triplicate samples is shown.

DISCUSSION

The work presented in this chapter has defined a chain of events occurring during CTL-mediated killing and resulting in target cell DNA fragmentation. Using cytolytic cells derived from mice deficient in granzyme B, it has been shown that CPP32 is not cleaved during CTL-mediated cytotoxicity in the absence of granzyme B. This implies that no other granzymes are able to activate the CPP32 enzymes, a result which is not particularly surprising since other granzymes do not share granzyme B's substrate specificity and likely could not generate active protease. We have previously demonstrated results to this effect in Chapter IV (Darmon *et al.*, 1995). There it was shown that while FPLC fractions containing granzyme A could cleave pro-CPP32 in an *in vitro* assay, the fragment generated was not of correct size to generate active enzyme.

In addition, a functional relationship between activation of CPP32-like proteases and DNA fragmentation has been demonstrated through the use of the peptide aldehyde inhibitors Ac-DEVD-CHO and Ac-YVAD-CHO. Based on these studies, and work presented in the previous chapter, the current model for CTL-induced target cell DNA fragmentation suggests that granzyme B enters the target cell during CTL attack and cleaves the CPP32 precursor, as well as the precursors for CPP32-related enzymes, to produce active protease(s). The CPP32-like enzymes then go on to induce target cell DNA fragmentation by cleaving nuclear proteins through a mechanism which remains to be elucidated. Multiple substrates for CPP32 have been identified, including the nuclear proteins PARP (Nicholson *et al.*, 1995; Tewari *et al.*, 1995a), U1-70kDa, and DNA-PK (Casciola-Rosen *et al.*, 1996; Song *et al.*, 1996), and the cytoplasmic proteins D4-GDI (Na *et al.*, 1996) and the SREBPs (Wang *et al.*, 1995a, 1996). PARP may negatively regulate the Ca^{++}/Mg^{++}-dependent endonuclease involved in mediating DNA fragmentation, suggesting that PARP cleavage and inactivation by CPP32 may lead to activation of the Ca^{++}/Mg^{++}-dependent endonuclease, resulting in DNA fragmentation (Tanaka *et al.*, 1984; Yoshihara *et al.*, 1974, 1975). This model for linking CPP32 to DNA degradation remains to be confirmed. Alternatively, by cleaving PARP and DNA-PK, CPP32 could simply cripple a cell's DNA repair system, thereby allowing DNA fragmentation to proceed.

Interestingly, although no CPP32 cleavage occurred when target cells were incubated with GB KO effectors for up to 24 hours, DNA fragmentation still occurred, although at limited levels. This suggests that a second granule protein is capable of inducing target cell DNA fragmentation but that this protein, possibly a granzyme, is not as efficient as granzyme B. This protein does not appear to induce fragmentation through the activation of CPP32 since even after 24 hours no CPP32 was cleaved when

GB KO cells were used as effectors, implying the existence of an alternate pathway to DNA fragmentation. Interestingly, after 24 hours, CPP32 appeared as a larger band (that is, larger even than pro-CPP32), suggesting that CPP32 may undergo post-translational modification during apoptosis, which limits its activity. Similar results were found upon incubating target cells with effectors which kill through the Fas pathway (Figure VI-1). Furthermore, this modification is not activation-dependent since it occurred in targets of both GB WT (active CPP32) and GB KO (inactive CPP32) cells. This implies that the posttranslational modification is not a result of negative feedback, but rather is a natural event during the course of apoptosis. Although the nature of this modification is unknown, it is not ubiquitinated (Darmon, Gwozd, Ellison, and Bleackley, unpublished data).

Granzyme B had previously been implicated as playing a role in cytolysis as well as DNA fragmentation. However, the difference in the induction on ^{51}Cr release from labeled target cells between GB KO and GB WT effectors is relatively small compared to the difference in ability to induce [^{3}H]thymidine release. These results are similar to others found by Heusel *et al.* (1994) and Shresta *et al.* (1995). However, this does not suggest that granzyme B does not play a role in the induction of membrane damage, only that this role for granzyme B is not unique. Possibly granzyme B, as well as other granzymes, can induce membrane damage in target cells. In the absence of granzyme B, these other granzymes may be able to substitute for granzyme B activity, resulting in data which suggests no role for granzyme B in membrane events. A prime candidate for the granzyme inducing membrane damage is granzyme A. It is the other predominant granzyme, it is conserved between mice and humans, and it is found, along with granzyme B, in *ex-vivo*-derived murine CTL (Garcia-Sanz *et al.* 1990; Ebnet *et al.*, 1991). Additionally, a role for granzyme A in cytolysis has previously been suggested by the results of Shiver *et al.* (1992) who showed that coexpression of granzyme A and perforin in RBL mast cells increased target cytolysis induced by mast cells above the level seen when cells expressing perforin alone were used as effectors. It remains to be seen whether this represents an *in vivo* role for granzyme A, although it has been reported that CTLs from mice deficient in granzyme A are as cytolytic as CTLs from wild type mice (Ebnet *et al.*, 1995). However, if both granzyme A and granzyme B are able to induce target cell cytolysis then granzyme B should substitute for granzyme A activity in this system. If granzyme A has no unique role in CTL-mediated cytotoxicity then the granzyme A knockout mice would be expected to show no distinct phenotype, which has been demonstrated (Ebnet *et al.*, 1995).

Interestingly, granzyme B has been shown to be necessary for cytolysis mediated by NK cells (Shresta *et al.*, 1995). It is possible that this difference between CTLs and NK cells is due to the presence of an additional component in CTL granules which is missing in NK granules. This hypothesis proposes that in CTLs, the lack of granzyme B has no dramatic effect on induction of membrane damage because the presence of this other component masks the requirement for granzyme B. However, in NK cells where this component is not present, the role of granzyme B in cytolysis is unveiled. The nature of this component, if it exists, is unknown.

The lack of effect of Ac-YVAD-CHO on either ^{51}Cr or [^{3}H]thymidine release seems to suggest that ICE-like proteases are not required for granule-mediated cytotoxicity. It has recently been shown that during Fas-mediated killing, ICE-like proteases may be responsible for the activation of CPP32-like proteases (Enari *et al.*, 1996). It is therefore likely that during granule-mediated killing, granzyme B replaces the function of ICE-like proteases by directly activating the CPP32 proteins. This confirms the hypothesis first presented in Chapter III (Darmon *et al.*, 1994) suggesting that granzyme B and ICE share similar cellular substrates. ICE has previously been shown to be capable of generating active CPP32 from pro-CPP32 (Tewari *et al.*, 1995a).

The results presented here in Chapters IV and V have identified an intracellular substrate for granzyme B, and have demonstrated a functional relationship between cleavage of this substrate and the induction of target cell DNA fragmentation. This was the first identification of a granzyme B substrate which is relevant to cell death. Furthermore, the discovery that an ICE/Ced-3 protease is a substrate has demonstrated that a CTL utilizes a target cell's intrinsic death program to induce target cell death, and has united the fields of programmed cell death and cell-mediated cytotoxicity.

CHAPTER VI - ICE-CED-3 PROTEASES IN FAS-MEDIATED CYTOTOXICITY

CHAPTER VI - ICE/CED-3 PROTEASES IN FAS-MEDIATED CYTOTOXICITY[4]

INTRODUCTION

Cytotoxic T lymphocytes (CTLs) represent the body's primary defense against tumourigenic and virus-infected cells, and are responsible for organ transplant rejection and autoimmune disease (reviewed by Berke, 1995; Atkinson and Bleackley, 1995; Smyth, 1995). Recognition of a target cell bearing a foreign antigen through the T cell receptor (TCR) results in the induction of apoptotic death in the target cell and can occur through two distinct mechanisms - granule- and Fas-mediated cytotoxicity. During granule-mediated killing, TCR activation results in mobilization of lytic granules within the T cell cytoplasm toward the target cell. Exocytosis leads to the release of granule contents - including a family of serine proteases (the granzymes (Atkinson and Bleackley, 1995; Lowin *et al.*, 1995)) and the pore-forming protein perforin (Liu *et al.*, 1995) - into the intercellular space. Polymerization of perforin in the target cell membrane facilitates granzyme entry into the target cell, where they induce apoptosis. In Fas-mediated cytotoxicity, TCR engagement results in upregulation of Fas ligand (FasL) levels on the surface of the T cell. FasL then ligates the Fas receptor on the target cell thus setting in motion a series of events that results in death of the Fas-bearing cell (Nagata and Golstein, 1995). The exact nature of the signal generated is unknown although acidic sphingomyelinase, ceramide and protein phosphorylation are all believed to play a role (reviewed in Chapter I). Regardless of the signal involved, apoptosis is induced in the target cell.

Only recently have some of the mechanisms involved in apoptosis come to light. Much of this work has revolved around a family of cysteine proteases related to Interleukin-1β Converting Enzyme (ICE). Originally identified as the protease responsible for producing biologically active IL-1β from pro-IL-1β (Thornberry *et al.*, 1992; Cerretti *et al.*, 1992), a role for ICE in apoptosis was suggested by the discovery that *ced-3*, a gene isolated from the nematode *Caenorhabditis elegans* as being essential for programmed cell death during development, had homology to ICE (Yuan *et al.*, 1993). Multiple members of the ICE/Ced-3 family of cysteine proteases have since been isolated (reviewed by Henkart, 1996; Martin and Green, 1995), including Ich1 (Wang *et al.*, 1994b), the human homologue of the murine Nedd-2

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[4] A version of this chapter has been accepted for publication: Darmon, A.J., and Bleackley, R.C. (1996) *J. Biol. Chem.* in press

(Kumar *et al.*, 1994), TX/Ich-2/ICE_{rel}II (Faucheu *et al.*, 1995; Kamens *et al.*, 1995; Munday *et al.*, 1995), ICE_{rel}III (Munday *et al.*, 1995), Mch2α (Fernandes-Alnemri *et al.*, 1995a), Mch3α/CMH-1/ICE-LAP3 (Fernandes-Alnemri *et al.*, 1995b; Lippke *et al.*, 1996; Duan *et al.*, 1996), FLICE/MACH (Boldin *et al.*, 1996; Muzio *et al.*, 1996) and CPP32/Apopain/Yama (Fernandes-Alnemri *et al.*, 1994; Nicholson *et al.*, 1995; Tewari *et al.*, 1995a). These enzymes can be divided into three subfamilies based on sequence homology: the ICE-like enzymes (ICE, TX/Ich-2/ICE_{rel}II, ICE_{rel}III), the CPP32-like proteases (CPP32, Ced-3, Mch2α, Mch3α/CMH-1/ICE-LAP3, FLICE/MACH) and the Nedd-2 proteins (Nedd-2, Ich-1L). Each of these proteases is synthesized as an inactive precursor requiring cleavage after Asp residues for activation. CPP32 (putative cysteine protease of 32-kDa) was identified as the protease responsible for cleavage of the nuclear protein poly(ADP-ribose) polymerase (PARP) during the induction of apoptosis (Nicholson *et al.*, 1995; Tewari *et al.*, 1995a). It has been shown that CPP32 is involved in granule-mediated cytotoxicity since it is an intracellular substrate for granzyme B (Chapter IV and V; Darmon *et al.*, 1995, 1996), an enzyme critical for the early induction of DNA fragmentation in target cells (Shi *et al.*, 1992a, 1992b; Heusel *et al.*, 1994; Shresta *et al.*, 1995).

Interest in ICE/Ced-3 proteases as mediators of Fas-induced apoptosis began when it was shown that the cowpox virus-encoded serpin CrmA, which had been shown to inhibit ICE (Komiyama *et al.*, 1994; Ray *et al.*, 1992), could suppress Fas-induced cell death (Tewari and Dixit, 1995; Enari *et al.*, 1995b; Los *et al.*, 1995). At this point, the inhibitory properties of CrmA were attributed to its ability to inhibit ICE. Subsequent studies of CTL-mediated cytotoxicity indicated that CrmA could inhibit cell-mediated killing when expressed in target cells, and that, although CrmA had previously been shown to inhibit granzyme B (Quan *et al., 1995*), the inhibition during CTL-mediated killing was due to inhibition of the Fas pathway (Tewari *et al.*, 1995c). Using thymocytes derived from mice deficient in ICE, Kuida *et al.* (1995) showed that these cells were resistant to apoptosis induced by anti-Fas antibody.

Other studies using the potent tetrapeptide aldehyde inhibitor of ICE Ac-YVAD-CHO ($K_{i,ICE}$ = 0.76 nM [Thornberry *et al.*, 1992]) further suggested a role for ICE and related enzymes in Fas-induced apoptosis. Enari *et al.* (1995b) demonstrated that this peptide could suppress Fas-induced apoptosis on whole cells. Using other peptides based on this sequence, other workers have utilized a cell-free system to show that while cytosolic extracts from anti-Fas-treated cells can induce apoptotic changes in freshly-isolated non-apoptotic nuclei, this apoptotic activity could be suppressed by addition of the peptide (Enari *et al.*, 1995a; Martin *et al.*, 1995a).

Other evidence supports a role for ICE/Ced-3 family members in Fas-induced cell death. In studies of cells expressing CrmA, it was found that CrmA could specifically inhibit cleavage of U1-70kDa, a CPP32 substrate (Casciola-Rosen *et al.*, 1996) during Fas-mediated apoptosis (Tewari *et al.*, 1995b). Interestingly, CrmA has much lower affinity for CPP32 than for ICE ($K_{i,ICE} < 4$ pM [Komiyama *et al.*, 1994]; $K_{i,CPP32} = 0.56$ μM [Fernandes-Alnemri *et al.*, 1995b]), suggesting that CrmA could not be acting directly on CPP32. Finally, it was recently reported that the ICE/Ced-3 proteases ICE-LAP3 and CPP32 are cleaved and activated during anti-Fas-induced apoptosis (Duan *et al.*, 1996; Schlegel *et al.*, 1996).

The final set of experiments reported here constitute work done to elucidate the role of ICE/Ced-3 proteases, particularly CPP32, during Fas-mediated cytotoxicity.

RESULTS

CPP32 is Not Always Cleaved During Fas-Mediated Cytotoxicity

Based on the previous studies demonstrating a role for ICE/Ced-3 proteases during granule-mediated killing (Darmon *et al.*, 1994, 1995, 1996; Chapters III, IV, V) it was interesting to determine the roles of these proteases during Fas-mediated killing. The first question was whether CPP32 is cleaved during Fas-induced apoptosis. EL4 and YAC-1 (mouse lymphoma) target cells were incubated with activated PMM-1 cells (a T cell hybridoma derived from peritoneal exudate lymphocytes [Kaufmann *et al.*, 1981]) for 4 or 24 hours. These effectors kill exclusively through the Fas mechanism, as demonstrated by the fact that killing is calcium-independent (Garner *et al.*, 1994). Cell lysates generated following this incubation were then assessed for CPP32 status by SDS-PAGE separation followed by Western blotting using an antibody directed against the large subunit of CPP32. The results, shown in Figure VI-1A, indicated that when YAC-1 cells are incubated with effectors for 4 hours, a band appears at 17-kDa. This band was not present in the time zero sample and represents cleavage of the CPP32 precursor (p32) to the active form (p17/p12). This cleavage product was not apparent in EL4 lysates, even after 24 hour incubation with effector cells, and the cleavage in YAC-1 lysates was partial at best. Similar studies using a variant of L1210 transfected with murine Fas cDNA (L1210-Fas [Rouvier *et al.*, 1993]) as targets revealed that CPP32 was not cleaved during Fas-induced killing of these cells (data not shown). Concurrent with this study, DNA fragmentation (assessed as [^{3}H]thymidine release) was measured from YAC-1 and EL4 targets incubated with PMM-1 effectors and was found to be normal

(Figure VI-1B). Taken together, these results suggest that CPP32 may be cleaved in some cells during Fas-mediated killing, but is not essential for DNA fragmentation since [^{3}H]thymidine release is still apparent in the absence of CPP32 cleavage (EL4 and L1210-Fas cells). These results are in direct contrast to the studies of granule-mediated killing, where CPP32 was completely cleaved in both EL4 and YAC-1 cells exposed to effectors for 4 hours (Chapter IV; Darmon *et al.*, 1995). It should be noted that DNA fragmentation during Fas-mediated cytotoxicity is consistently more pronounced in YAC-1 cells, where CPP32 is partially cleaved, than in EL4, in which no cleavage of CPP32 is apparent (Darmon and Bleackley, unpublished data). Thus, cleavage of CPP32 may affect the efficiency of DNA fragmentation.

Effect of Peptide Inhibitors on Fas-Mediated Cytotoxicity

In the next series of experiments a battery of peptide inhibitors, which were synthesized for their ability to inhibit ICE/Ced-3 proteases, were used. The structures and properties of these inhibitors are summarized in Table VI-1. Briefly, inhibitor 1 consists of the sequence AD and is an inhibitor of ICE/Ced-3 proteases. Inhibitor 2 contains the prototypic sequence of an ICE/Ced-3 inhibitor, VAD, with a modified amino terminal phenylalanine residue which lacks an amino group. Inhibitors 3 and 4 are very good inhibitors of ICE itself and contain the YVAD sequence. Inhibitor 4 is the Ac-YVAD-CHO inhibitor often used to study ICE/Ced-3 proteases ($K_{i,ICE}$ = 0.76nM [Thornberry *et al.*, 1992]). Inhibitor 5 consists of the peptide YVKD with a biotinylated lysine residue. Inhibitor 6 was originally identified as a CPP32 inhibitor with the structure Ac-DEVD-CHO ($K_{i,CPP32}$ = 0.2 nM [Nicholson *et al.*, 1995]). Inhibitor 7 is the reduced aldehyde of inhibitor 4 and acts as a negative control. Although K_i values for inhibition of CPP32 by these peptide inhibitors were unavailable except for inhibitor 4 (Ac-YVAD-CHO) and inhibitor 6 (Ac-DEVD-CHO), and the lab is not equipped to determine these kinetic values, an estimate of the ability of these inhibitors to suppress CPP32 activity was produced. Purified human CPP32 was preincubated with varying concentrations of the inhibitors, and then assessed for its ability to cleave [^{35}S]PARP. The results of this study are summarized in Table VI-2, and clearly indicate that none of the inhibitors used can suppress CPP32 activity at concentrations similar to inhibitor 6 ($K_{i,CPP32}$ = 0.2 nM [Nicholson *et al.*, 1995]). The best inhibitor of CPP32 in this group, inhibitor 2, could only suppress CPP32 activity at a concentration of 100 nM, far above the K_i of inhibitor 6.

Initial studies using these inhibitors simply addressed their effect on ^{51}Cr release (as a measure of cytolysis) from labeled target cells, using PMM-1 cells (which kill through the Fas pathway) as effectors. The results using L1210-Fas as target cells are shown in Figure VI-2. Similar results were obtained using EL4 cells as targets (data not shown). In these experiments, only inhibitor 2 had any significant effect on target cell ^{51}Cr release induced by ligation of the Fas receptor. Inhibitor 6, the inhibitor of CPP32-like enzymes, had no effect on Fas-mediated killing of L1210-Fas. This is consistent with the data showing that CPP32 is not activated in these cells during Fas-mediated cytotoxicity (Figure VI-1). Likewise, the inhibitor of proteases related to ICE, inhibitor 4, also had no effect on ^{51}Cr release.

One possible explanation for the failure of some of these inhibitors to affect ^{51}Cr release is that they fail to enter the target cell. Previous studies using inhibitor 6 have reported poor membrane permeability (Nicholson *et al.*, 1995), although subsequent studies from this group indicated that inhibitor 6 could indeed enter the target cell (Schlegel *et al.*, 1996). Although membrane permeability of all the inhibitors could not be assessed, we have data which suggests that at least three of the inhibitors enter the target cell. In a whole cell assay for IL-1β release (Thornberry *et al.*, 1992), it has been found that although the IC_{50} values for inhibitors 2 and 4 are relatively high compared to the K_i value for ICE ($IC_{50,\#2}$ = 3 μM; $IC_{50,\#4}$ = 2.5 μM) they are still below the concentration used in these studies (40 μM). In addition, our finding that inhibitor 6 enters target cells during granule-mediated killing (Chapter V) suggests that this inhibitor is also cell permeable. This is comparable with results of Schlegel *et al.* (1996) who have used this inhibitor in whole cells to inhibit apoptosis. Since we can only be certain that inhibitors 2, 4, and 6 can enter the target cell, only these inhibitors were used in subsequent studies.

Studies using these inhibitors addressed their effect on ^{51}Cr or [^{3}H]thymidine release (as a measure of membrane integrity or DNA fragmentation, respectively) from labeled target cells, using PMM-1 cells (which kill through the Fas pathway) as effectors. The results using L1210-Fas as target cells are shown in Figure VI-3, A and B. Similar results were obtained using EL4 cells as targets (data not shown). In these experiments, only inhibitor 2 had a significant effect on target cell ^{51}Cr and [^{3}H]thymidine release induced by ligation of the Fas receptor. Intriguingly, inhibitor 6, the CPP32 inhibitor, had no effect on either chromium or thymidine release, suggesting no role for CPP32 in cell death following ligation of Fas. This result is consistent with our finding that CPP32 is not cleaved efficiently in these cells during Fas-induced apoptosis (Figure VI-1A).

FIGURE VI-1: Cleavage of CPP32 and [^{3}H]thymidine Release During Fas-Mediated Cytotoxicity

A. EL4 and YAC-1 target cells were incubated with PMM-1 effector cells at an effector-to-target ratio of 5:1 at 37°C for 4 or 24 hours. CPP32 status in cell lysates was assessed by Western blotting using an antibody directed against the p17 subunit of CPP32.

B. EL4 and YAC-1 target cells were labeled and incubated with PMM-1 effectors at an E:T ratio of 5:1 for 4 or 24 hours at 37°C, at which point [^{3}H]thymidine release was determined. Mean and standard deviation of triplicate samples is shown.

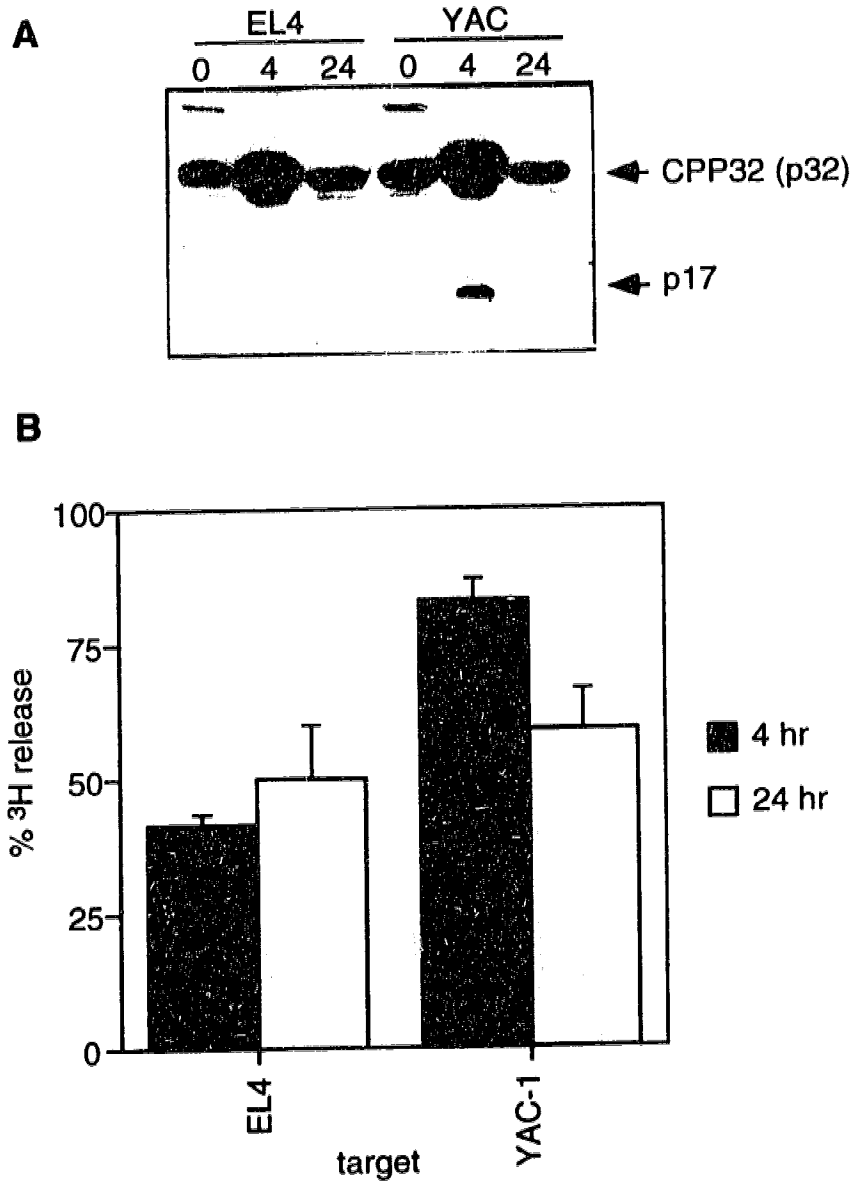

A
EL4
YAC
0 4 24 0 4 24
CPP32 (p32)
p17
B
100
75
50
25
0
% 3H release
4 hr
24 hr
EL4
YAC-1
target

TABLE VI-1: SUMMARY OF STRUCTURES AND PROPERTIES OF PEPTIDE INHIBITORS USED TO STUDY FAS-MEDIATED CYTOTOXICITY.

Inhib. #	Structure	Inhibition of ICE[a]	Inhibition of CPP32[a]	Features	Reference
1	i-C_4H_9C(=O)-Ala-AspC(=O)CH_2O-2,6-BTBz[b]	5000 $M^{-1}s^{-1}$	ND[c]	competitive, irreversible	N.A. Thornberry, unpublished
2	Ph$(CH_2)_2$C(=O)-Val-Ala-AspC(=O)CH_2O-Ph$(F)_5$	1,100,000 $M^{-1}s^{-1}$	ND	competitive, irreversible	Thornberry *et al*, 1994
3	Ac-Tyr-Val-Ala-AspC(=O)CH_2O-2,4,6-TMBz[d]	1,200,000 $M^{-1}s^{-1}$	ND	competitive, irreversible	N.A. Thornberry, unpublished
4	Ac-Tyr-Val-Ala-AspC(=O)H	0.76 nM	>10,000 nM	competitive, reversible	Thornberry *et al*, 1992
5	Ac-Tyr-Val-Lys(N_ε-biotinylated)-AspC(=O)CH_2O-2,6-DMBz[e]	500,000 $M^{-1}s^{-1}$	ND	competitive, irreversible	Thornberry *et al*, 1994
6	Ac-Asp-Glu-Val-AspC(=O)H	ND	0.2 nM	competitive, reversible	Nicholson *et al*, 1995
7	Ac-Tyr-Val-Ala-AspCH_2OH	>10,000 nM	ND	competitive, reversible	N.A. Thornberry, unpublished

[a] inhibition constants: k (second order rate constant) for inhibitors 1, 2, 3, 5; K_i for remaining inhibitors

[b] 2,6-BTBz = 2,6-bis-(trifluoromethyl)benzoyl

[c] ND - not determined

[d] 2,4,6-TMB = 2,4,6-trimethylbenzoyl

[e] 2,6-DMBz = 2,6-dimethylbenzoyl

TABLE VI-2: INHIBITION OF CPP32 ACTIVITY BY PEPTIDE INHIBITORS

Inhibitor number	Concentration of Inhibitor[1]				
	10 μM	1 μM	100 nM	10 nM	1 nM
1	+	+	+/-	+/-	+/-
2	+	+	+	-	-
3	+	+	-	-	-
5	+	+	+/-	-	-
7	-	-	-	-	-

[1] symbols used:

+ : inhibition of PARP cleavage

- : no inhibition of PARP cleavage

+/- : partial inhibition of PARP cleavage

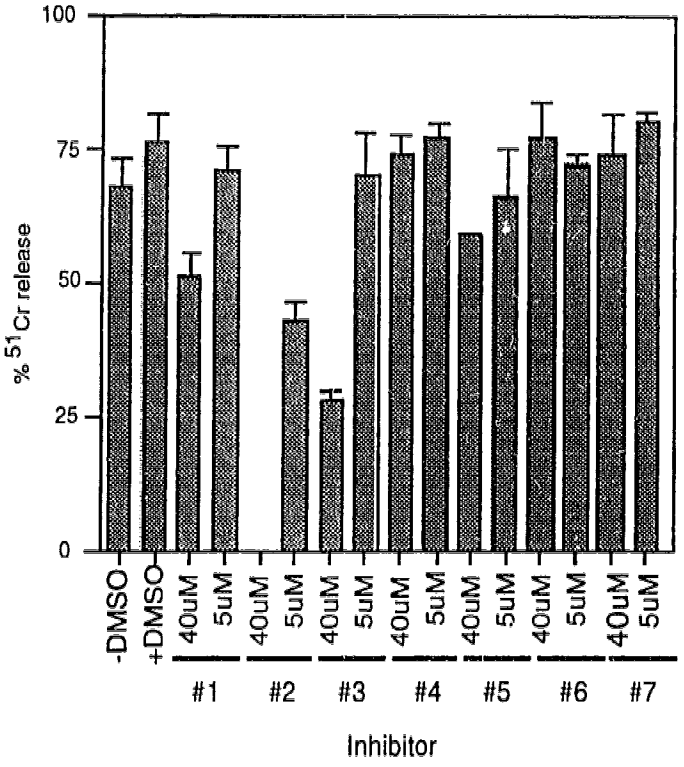


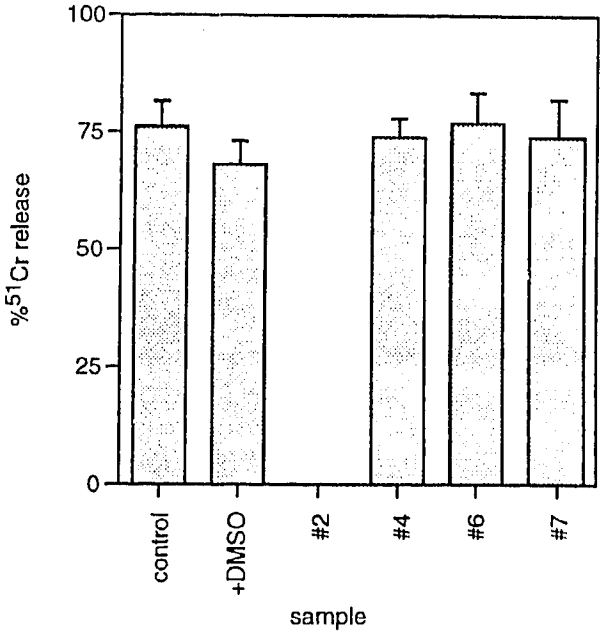
FIGURE VI-2: Effect of ICE Peptide Inhibitors on ^{51}Cr Release During Fas-Mediated Cytotoxicity

Lysis of chromium-labeled L1210-Fas target cells by PMM-1 cells added at an E:T ratio of 2.5:1, in the absence and presence of peptide inhibitors. Inhibitors were used at final concentrations of 40 μM and 5 μM, DMSO alone was added to the cells as a control for this effect, - DMSO is in the absence of any additional component to the assay. After 4 h, the percentage of chromium released was determined. Mean and standard deviation of triplicate samples is indicated.

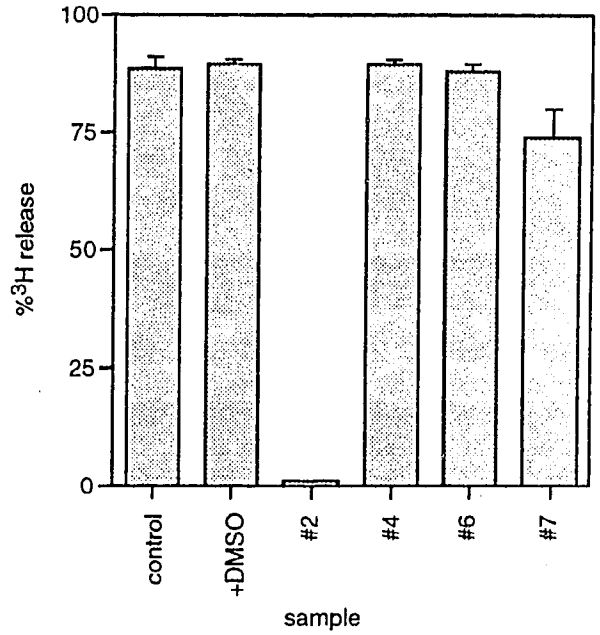
FIGURE VI-3: Effect of Four Peptide Inhibitors on ^{51}Cr and [^{3}H]thymidine Release from L1210-Fas Cells During Fas-Mediated Cytotoxicity

L1210-Fas target cells were labeled and incubated with PMM-1 effectors at an E:T ratio of 2.5:1 in the presence of the inhibitors indicated (final concentration 40 μM) for 4 hours at 37°C, at which point ^{51}Cr release (A) or [^{3}H]thymidine release (B) were determined. DMSO - in the presence of DMSO alone; control - nothing additional added to the assay. Mean and standard deviation of triplicate samples is indicated.

A.


100
75
50
25
0
%51Cr release
control
+DMSO
#2
#4
#6
#7
sample

B.


100
75
50
25
0
%3H release
control
+DMSO
#2
#4
#6
#7
sample

For comparison, the effect of these inhibitors on Fas-induced death of YAC-1 cells, in which CPP32 is cleaved following Fas ligation (Figure VI-1A) was studied. Again, labeled target cells were incubated with PMM-1 effectors in the presence or absence of the inhibitors, and then ^{51}Cr or [^{3}H]thymidine release were measured. The data, presented in Figure VI-4, C and D, clearly shows that inhibitor 2 has a significant effect on both ^{51}Cr and [^{3}H]thymidine release from these cells following Fas ligation. Additionally inhibitor 6 (the CPP32 inhibitor) also suppressed [^{3}H]thymidine release from YAC-1 cells. This is in contrast to the results using inhibitor 6 with L1210-Fas cells (Figure VI-3, A and B). When taken together with the results from Figures VI-1 and VI-3, A and B, these studies lead to the conclusion that CPP32 is not essential for Fas-induced cell death since it is not always cleaved during Fas-mediated apoptosis. In cells which exhibit no CPP32 cleavage, the CPP32 inhibitor (inhibitor 6) affects neither ^{51}Cr nor [^{3}H]thymidine release. However, there is a correlation between CPP32 cleavage and inhibition of DNA fragmentation by inhibitor 6, suggesting that CPP32 may be involved in Fas-induced cell death in some cells. Additionally, a second ICE/Ced-3 protease seems to play a significant role in inducing both cytolysis and DNA fragmentation in response to Fas ligation. This protease would be inhibited by inhibitor 2, since inhibitor 2 suppresses both ^{51}Cr and [^{3}H]thymidine release from labeled EL4 and L1210-Fas cells treated with PMM-1 effectors. The identity of this protease is currently unknown.

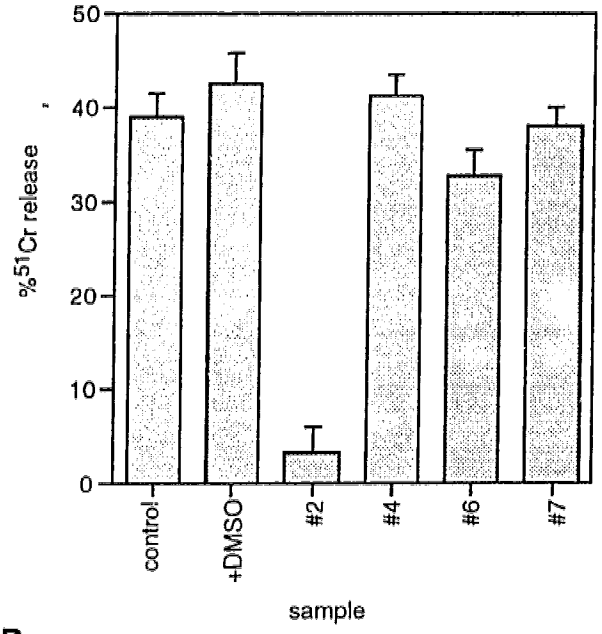
ICE is Not Activated During Fas-Mediated Apoptosis

Although there was no apparent effect of inhibitor 4 (Ac-YVAD-CHO; $K_{i,ICE}$ = 0.76 nM [Thornberry *et al.*, 1992]) on ^{51}Cr release during Fas-mediated killing, it was still possible that the results using inhibitor 2 could be explained by inhibition of ICE itself, since other investigators have previously found that an "ICE" activity is involved in Fas-mediated cytotoxicity (Enari *et al.*, 1995a, 1995b; Los *et al.*, 1995), a result which has been confirmed in this lab using lysates from anti-Fas treated cells in a fluorogenic assay (Darmon and Bleackley, unpublished data). To evaluate ICE activity, cell lysates from YAC-1 cells treated with anti-Fas antibody were tested for their ability to cleave radiolabeled pro-IL-1β to produce the 17.5-kDa IL-1β. To date, ICE is the only known ICE/Ced-3 protease capable of activating pro-IL-1β. [^{35}S]pro-IL-1β, which translated as a doublet due to the use of an alternate initiation codon, was incubated overnight with lysates from YAC-1 cells treated with anti-Fas antibody for up to 8 hours. The results, shown in Figure VI-5A, revealed that treatment of YAC-1 cells with anti-Fas did not result in the appearance of any pro-IL-1β-cleaving

FIGURE VI-4: Effect of Four Peptide Inhibitors on ^{51}Cr and [^{3}H]thymidine Release from YAC-1 Cells During Fas-Mediated Cytotoxicity.

YAC-1 target cells were labeled and incubated with PMM-1 effectors at an E:T ratio of 5:1 in the presence or absence of the inhibitors indicated (final concentration 40 μM) for 4 hours at 37°C, at which point ^{51}Cr release (A) or [^{3}H]thymidine release (B) were determined. DMSO - in the presence of DMSO alone, control - nothing additional added to the assay. Mean and standard deviation of triplicate samples is indicated.

A.


50
40
30
20
10
0
%51Cr release
control
+DMSO
#2
#4
#6
#7
sample

B.

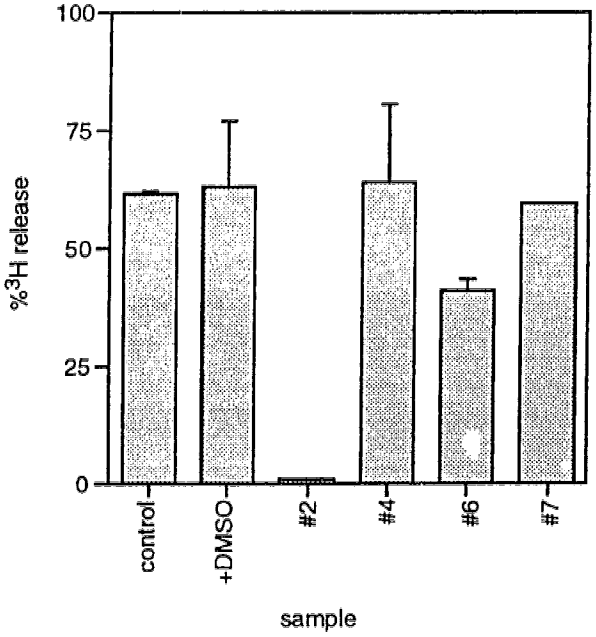
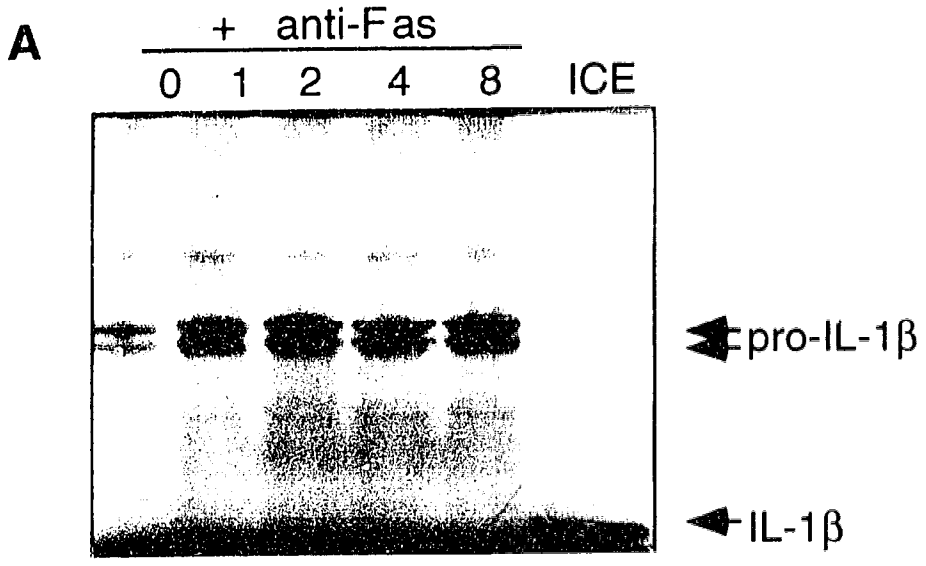
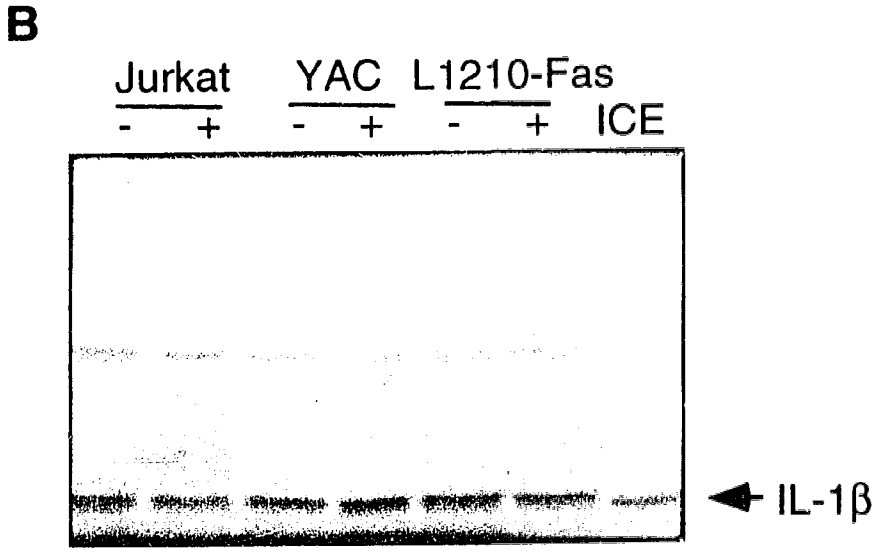

100
75
50
25
0
%3H release
control
+DMSO
#2
#4
#6
#7
sample

FIGURE VI-5: ICE is not activated during Fas-mediated cytotoxicity.

A. Lysates from YAC-1 cells treated with anti-Fas antibody for 0 to 8 hours were tested for their ability to cleave [^{35}S]pro-IL-1β to produce 17.5-kDa IL-1β. Cleavage reactions were incubated at 37°C overnight, then analyzed by SDS-PAGE. ICE - incubation with 15 ng purified human recombinant ICE.

B. Lysates from L1210-Fas, YAC-1 and Jurkat cells treated with (+) or without (-) anti-Fas antibody for 8 hours were supplemented with 45 ng human recombinant ICE then tested for their ability to cleave [^{35}S]pro-IL-1β as above.


A
+ anti-Fas
0 1 2 4 8 ICE
pro-IL-1β
IL-1β


B
Jurkat YAC L1210-Fas
- + - + - + ICE
IL-1β

activity. Similar results were obtained using lysates from both L1210-Fas and human Jurkat cells treated with anti-Fas (data not shown). The lack of ICE activity was not due to the presence of a cellular inhibitor of ICE since supplementing the lysates with purified recombinant human ICE resulted in cleavage of pro-IL-1β (Figure VI-5B), detected by appearance of the 17.5-kDa mature IL-1β. Therefore, the increased "ICE" activity seen during Fas-mediated apoptosis is not due to activation of ICE itself, and the inhibitory action of inhibitor 2 cannot be explained by inhibition of ICE.

DISCUSSION

The experiments outlined in this chapter have shown that while CPP32 may be cleaved and activated in some cells during Fas-mediated cytotoxicity, this cleavage is not required for cell death since other cells, in which CPP32 is not cleaved, are able to undergo apoptosis in response to Fas ligation. However, it has been demonstrated that another ICE/Ced-3 protease involved in Fas-mediated cytotoxicity is critical for the induction of both the membrane and DNA events of Fas-induced apoptosis. The specific protease involved has not been identified, but does not appear to be ICE (since no ICE activity is detected in lysates from cells treated with anti-Fas antibody) or CPP32 (since inhibitor 2 cannot suppress CPP32 activity to a great extent, and inhibitor 6 cannot suppress ^{51}Cr release). It cannot be ruled out that inhibitor 2 suppresses multiple ICE/Ced-3 proteases however. Therefore, the identification of the target(s) of inhibitor 2 is essential to a further understanding of the induction of cell death following Fas ligation.

On the surface, these results seem to be in contrast to recent work suggesting that CPP32 is a key mediator of Fas-induced cell death (Schlegel *et al.*, 1996). However, the two studies are actually complementary. The data presented here has shown that CPP32 is cleaved in some cell types, and when cleaved, its activity is involved in the induction of target cell DNA fragmentation. However, in other cell types CPP32 activity is not involved in the induction of apoptotic cell death. Schlegel *et al.* (1996) used Jurkat cells treated with anti-Fas antibody to show cleavage and activation of CPP32. However, Jurkat cells, like YAC-1 cells, may simply represent another cell line in which CPP32 is cleaved during Fas-mediated apoptosis. The use of one cell line does not suggest that CPP32 is cleaved in all cells and a more extensive survey of cell lines, like the one presented here, reveals that CPP32 cleavage is not a universal feature of Fas-induced apoptosis.

Another difference between these two studies is the mechanism of induction of apoptosis - while Schlegel *et al.* cross-linked the Fas receptor using an antibody, the studies presented here utilized CTLs which kill through the Fas pathway. Obviously, when whole cells are used to induce death, other cell surface receptors besides Fas may also be cross-linked and transduce signals. These additional signals may modulate a cell's response to cell death induced by Fas ligation, in much the same way that TCR/CD3 recognition of a target cell also requires CD4 or CD8 recognition of the MHC. Therefore, studies utilizing only anti-Fas antibody should be interpreted with caution because, physiologically, other cell surface receptors may also be involved in transducing the death signal. The studies presented here, using whole cells to induce Fas killing, are likely more physiologically relevant than studies utilizing anti-Fas.

One intriguing result of this study is that DNA fragmentation occurs in the absence of CPP32 and related protease activity. This suggests that other ICE/Ced-3 proteases may be able to substitute for CPP32 activity during Fas-mediated cytotoxicity. Alternatively, CPP32-like activity may be a consequence rather than a requirement for DNA fragmentation. This CPP32-independent pathway to DNA fragmentation has previously been revealed in the studies of granule-mediated cytotoxicity (Chapter V; Darmon *et al.*, 1996). In these studies it was found that DNA fragmentation could still proceed in the absence of granzyme B (which activates CPP32 and related enzymes) or in the presence of Ac-DEVD-CHO (inhibitor 6 in this study), although with reduced efficiency. Therefore, it seems that the CPP32 proteases are involved solely in the induction of rapid DNA fragmentation, but DNA fragmentation can still occur in the absence of these enzymes.

Interestingly, Enari *et al.* (1996) have recently shown that ICE-like proteases are activated prior to CPP32-like proteases during Fas-mediated apoptosis. These results suggest that ICE-like proteases are responsible for the activation of CPP32-like enzymes. These data confirm the hypothesis presented here regarding the existence of an ICE/Ced-3 protease which controls both cytolytic and nucleolytic events of apoptosis. Combining the two studies results in a model for Fas-mediated cytotoxicity in which a protease related to ICE (but not ICE) activates CPP32 (and related enzymes) to induce rapid DNA fragmentation, and also cleaves substrates required for membrane damage. The identification of this central mediator will therefore be essential to a further understanding of Fas-mediated killing.

Addendum

Recently, two groups have reported the identification of an ICE/Ced-3 protease, named FLICE/MACH, which is able to associate with FADD/MORT1, which in turn associates with Fas (Boldin *et al.*, 1996; Muzio *et al.*, 1996). The suggestion is that this protease represents the "top" of the proteolytic cascade activated by Fas ligation. That is, Fas ligation leads to activation of FLICE/MACH, which proceeds to induce the nuclear and cytoplasmic events of apoptosis. Although it is currently unknown whether this is indeed the case, FLICE/MACH represents an excellent candidate for the protease inhibited by inhibitor 2 in the studies here. Further studies of FLICE/MACH and its inhibition profile are therefore crucial to a better understanding of the proteases activated during Fas-mediated apoptosis.

CHAPTER VII - SUMMARY, DISCUSSION AND FUTURE DIRECTIONS

CHAPTER VII - SUMMARY, DISCUSSION AND FUTURE DIRECTIONS

I. SUMMARY

A. GRANULE-MEDIATED CYTOTOXICITY

The studies outlined here have greatly expanded our understanding of CTL-mediated cytotoxicity via the granule exocytosis mechanism. At the outset, it was unknown how granzyme B, a key mediator of CTL cytotoxicity, could induce target cell DNA fragmentation, and it was presumed that this protease activated an endonuclease (Smyth *et al.*, 1994). Our studies have shown that granzyme B cleaves and activates one ICE/Ced-3 protease (CPP32; Chapter IV) but not another (ICE; Chapter III) during CTL-mediated cytotoxicity. CPP32 in turn cleaves the nuclear proteins PARP, DNA-PK, and U1-70kDa as well as other substrates (such as the SREBPs and D4-GDI) and induces DNA fragmentation (Chapter V). Thus, the mechanism of action of granzyme B has been much more clearly defined, and while not completely understood, it is now known that CTLs utilize a cell's endogenous death program in the induction of apoptosis.

B. FAS-MEDIATED KILLING

The work outlined here has also expanded our knowledge of the role of ICE/Ced-3 proteases during Fas-mediated cytotoxicity. Like granule-mediated killing, ICE does not seem to play a role in Fas-induced apoptosis (Chapter VI). In contrast to granule-mediated killing, CPP32 does not appear to be involved in Fas-induced cell death in all target cells. However, the evidence presented here suggests a critical role for another ICE/Ced-3 protease, which is neither ICE nor CPP32, in the induction of both the cytoplasmic and nuclear events of apoptosis (Chapter VI). The identification of this protease will be critical to a further understanding of Fas-mediated cytotoxicity, and apoptosis in general.

II. DISCUSSION

A. ICE/Ced-3 PROTEASES AND CTL-MEDIATED CYTOTOXICITY

The fields of apoptosis and CTL-mediated cytotoxicity have progressed in leaps and bounds in the past few years. The demonstration that the ICE/Ced-3 proteases are key apoptotic mediators has led to the identification of multiple substrates for these enzymes, and has explained multiple facets of immunology, from CTL-mediated cytotoxicity (cleavage of these proteases during induction of target cell

death) to autoimmunity (exposure of cryptic determinants of autoantigens due to proteolysis). As more and more of these proteases are being identified, the mechanisms involved in mammalian apoptosis are becoming increasingly complex - multiple proteases are able to cleave the same substrates making identification of the role of a particular protease extremely difficult. In addition, there is evidence of hetero-oligomerization between family members. With the added fact that some family members are alternately spliced to generate inactive proteins, and the knowledge that multiple family members are expressed within the same cell, this suggests a number of mechanisms by which ICE/Ced-3 protease activity may be regulated *in vivo*. Firstly, different hetero-oligomers may have differing substrate specificities or affinities. For example, an active tetramer derived from Mch3α/CMH-1/ICE-LAP3 and CPP32 precursors may have a slightly different activity than a tetramer derived solely from CPP32 precursors. Secondly, alternately spliced, inactive isoforms may differentially regulate the activity of active family members - Mch3α may have a greater affinity for, and therefore be better regulated by, Mch3β than CPP32, for example. Thirdly, the ability of ICE/Ced-3 proteases to autoactivate may differ between family members - ICE may be more autocatalytic than CPP32. In addition, a proteolytic cascade may be in effect - some ICE/Ced-3 family members can activate other family members and thereby amplify a diversity of apoptotic stimuli.

The other key apoptotic mediators which have been identified, those proteins related to Bcl-2, undergo similar types of regulation - there is hetero-oligomerization between family members, and different proteins are alternately spliced to generate differentially active forms - Bcl-xL is a protector while Bcl-xS is a "killer".

Given all of the above, it is extremely difficult to decipher the role of any one protein - ICE/Ced-3 protease or Bcl-2 family member - in any particular form of apoptosis. In order to draw conclusions, therefore, it seems that both *in vitro* and *in vivo* studies must be performed. Such is the case with the work presented here on granule-mediated killing. It has been shown that granzyme B can cleave the precursor of CPP32 in *in vitro* studies using granzyme B derived from a "real" source (MTL 2.8.2) and recombinant granzyme B expressed in COS cells. In addition, using cytolytic effectors lacking granzyme B, it has been shown that granzyme B is the only granzyme capable of cleaving pro-CPP32 during granule-mediated killing, and that this cleavage by granzyme B is functionally related to the ability to induce rapid DNA fragmentation. Therefore, based on both *in vitro* and *in vivo* studies, it is safe to conclude that CPP32 represents an intracellular granzyme B substrate.

Two other members of the CPP32 family add complexity to the study of this protease - both Mch2α and Mch3α/CMH-I/ICE-LAP3 have been shown to cleave PARP to fragments of sizes similar to those generated by CPP32, suggesting that the PARP-cleaving activity seen during apoptosis may not be attributable solely to CPP32 (Fernandes-Alnemri *et al.*, 1995a, 1995b; Duan *et al.*, 1996; Lippke *et al.*, 1996). Indeed, both CPP32 and Mch3α have similar kinetic constants for cleaving a synthetic substrate containing the DEVD sequence, and both are equivalently inhibited by Ac-DEVD-CHO ($K_{i,CPP32}$ = 0.2 nM; $K_{i,Mch3\alpha}$ = 1.8 nM [Nicholson *et al.*, 1995; Fernandes-Alnemri *et al.*, 1995b]) suggesting that both CPP32 and Mch3α may cleave PARP during apoptosis. Interestingly, CPP32 is able to cleave and activate the Mch3α precursor, but not *vice versa*, suggesting that Mch3α activity may depend, at least in part, on the activity of CPP32, and further suggesting the existence of a proteolytic cascade. Therefore, *in vivo*, an apoptotic stimulus may result in activation of CPP32 and/or Mch3α and both of these proteases may cleave PARP (and other cellular substrates). The demonstration that Mch3α subunits can form active complexes with CPP32 subunits (Fernandes-Alnemri *et al.*, 1995b) further complicates this picture. The high homology between these two proteases suggests that pro-Mch3α may also act as a granzyme B substrate, as well as pro-CPP32, a hypothesis which has recently been confirmed (Gu *et al.*, 1996; Chinnaiyan *et al.*, 1996b). Interestingly, granzyme B appears to cleave pro-Mch3α more efficiently than it cleaves pro-CPP32, implying that Mch3α may represent a "better" substrate than pro-CPP32. In addition, granzyme B has also been found to cleave and activate the related protease FLICE/MACH (Muzio *et al.*, 1996). Taken together, these studies suggest that the precursors of proteases related to CPP32 are the primary targets of granzyme B during granule-mediated cytotoxicity.

Interestingly, while granzyme B seems uniquely involved in the induction of target cell DNA fragmentation, it does not appear to play a unique role in the induction of membrane damage. However, the demonstration of synergy between granzymes A and B (Nakajima *et al.*, 1995a) has suggested that these proteases act coordinately to induce membrane damage. Therefore, in the absence of granzyme B, granzyme A may mediate all the membrane damage seen and thereby mask the role of granzyme B. Indeed, this seems to be the case since GB KO effectors were initially reported to have substantially reduced DNA fragmentation but only slightly reduced cytolysis (Heusel *et al.*, 1994), suggesting that granzyme B plays no autonomous role in inducing membrane damage. In contrast, in NK cells granzyme B seems to play a unique role in the induction of membrane damage (Shresta *et al.*, 1995).

Furthermore, CTLs derived from granzyme A deficient mice are reported to be indistinguishable from CTLs derived from control mice (Ebnet *et al.*, 1995). In this instance, if granzyme B and granzyme A share common cellular substrates for inducing membrane damage then granzyme B activity could mask the effect of knocking out granzyme A activity. In order to confirm this hypothesis, mice deficient in both granzyme A and granzyme B should be generated. If granzymes A and B share common substrates then it would be likely that CTLs derived from the double knock-out mice would be unable to induce both ^{51}Cr and [^{3}H]thymidine release. Of course, a role for perforin, or other granule proteins, in the induction of the cytoplasmic events of apoptotic death cannot be ruled out.

Any role for granzyme A in the induction of DNA fragmentation and/or membrane damage is not likely to be mediated by the ICE/Ced-3 proteases, however, since granzyme A does not have the correct substrate specificity for activation of these enzymes (granzyme A cleaves after Arg/Lys). Indeed, our data using granzyme A revealed that although granzyme A can cleave pro-CPP32, the fragment generated is not of correct size to yield active enzyme (Chapter IV, Darmon *et al.*, 1995). However, the results of Chapter V have revealed the existence of a pathway to DNA fragmentation which is independent of both granzyme B and CPP32-like activity. It is therefore likely that this could represent a pathway regulated by granzyme A.

Unfortunately, the studies of Fas-mediated cytotoxicity are less clear cut than those of granule-mediated killing - only *in vivo* data is available and is somewhat confusing. In some cell lines, no evidence of CPP32 cleavage was seen, while in others, partial cleavage was seen. In no cell line was CPP32 completely cleaved as it is in granule-mediated killing. However, it is possible that Mch3α/CMH-1/ICE-LAP3 or a related protease was activated in these cells and was responsible for DNA fragmentation. This is unlikely however, since in cells in which CPP32 was not cleaved the DEVD-CHO inhibitor (inhibitor 6; Chapter VI; Darmon and Bleackley, 1996) had no effect on DNA fragmentation. Since Mch3α/CMH-1-ICE-LAP3 and CPP32 are equivalently sensitive to this peptide aldehyde (Fernandes-Alnemri *et al.*, 1995b), this suggests that neither CPP32 nor Mch3α are involved in the induction of DNA fragmentation during Fas-mediated cytotoxicity. Therefore, in cells in which CPP32 is not cleaved, another pathway seems to be responsible for the induction of DNA fragmentation, which is independent of these enzymes. Obviously further work is required to resolve this second mechanism.

One intriguing result from the Fas experiments was the finding that an ICE/Ced-3 protease is involved in the induction of both the cytoplasmic and nuclear

events of apoptosis in response to Fas ligation. Although the identification of this protease is unknown, this data suggests that this protease may be at the top of the cascade which culminates in target cell DNA fragmentation (possibly through activation of CPP32/Mch2α/Mch3α/CMH-1/ICE-LAP3) and the cytoplasmic changes (possibly through cleavage of a cytoplasmic protein) seen during target cell apoptosis. Identification of this protease is therefore critical to further elucidating the individual roles of the ICE/Ced-3 proteases during apoptosis, as well as the mechanisms involved in Fas-mediated cytotoxicity.

Importantly, two groups have recently reported the identification of an ICE/Ced-3 protease, named FLICE/MACH, which interacts with activated Fas as part of the death-inducing signaling complex (Boldin *et al.*, 1996; Muzio *et al.*, 1996). This protease contains a domain homologous to a domain found in FADD/MORT1, a protein which also contains a death domain through which it interacts with Fas. Therefore, FADD/MORT1 seems to act as an adaptor molecule, allowing FLICE/MACH to interact with Fas. Since FLICE/MACH associates with the Fas protein following Fas ligation, it is ideally suited to being an initiator of apoptosis. It remains to be seen whether FLICE/MACH subsequently activates other ICE/Ced-3 proteases and whether it is inhibited by inhibitor 2, but its discovery is very exciting given our studies using inhibitors which demonstrate the existence of a central ICE/Ced-3 protease.

B. A MODEL FOR CTL-MEDIATED CYTOTOXICITY

The results outlined above lead to the following model for CTL-mediated cytotoxicity, shown in Figure VII-1. During granule-mediated cytotoxicity, granzymes A and B enter the target cell, probably through channels formed by perforin. Granzyme B cleaves and activates CPP32, and probably other related family members, which then go on to cleave PARP, DNA-PK, U1-70kDa, SREBPs, D4-GDI, and possibly other cellular substrates. CPP32 may also be able to activate other family members in a proteolytic cascade. Granzyme B may also have other cellular substrates involved in the induction of target cell membrane damage. Identical or similar substrates may be cleaved by granzyme A. Together these cleavages result in the induction of target cell apoptosis. Although other granule proteins may be involved in the induction of cell death, it seems that granzyme A and B may be the primary mediators of granule-mediated killing. In addition to the pathway regulated by granzyme B, an alternate pathway to DNA fragmentation exists

which is independent of granzyme B/CPP32-like activity, and may be regulated by granzyme A.

During Fas-mediated cytotoxicity, a key ICE/Ced-3 protease, possibly FLICE/MACH, is activated and seems responsible for inducing both DNA and membrane damage. This protease may proceed to cleave and activate a CPP32 family member and induce DNA fragmentation. This protease likely also cleaves other cellular substrates and induces the morphological changes of apoptosis. Additionally, the alternate pathway to DNA fragmentation which is apparent in granule-mediated killing also seems to be involved during Fas-mediated killing and does not require the CPP32 proteases.

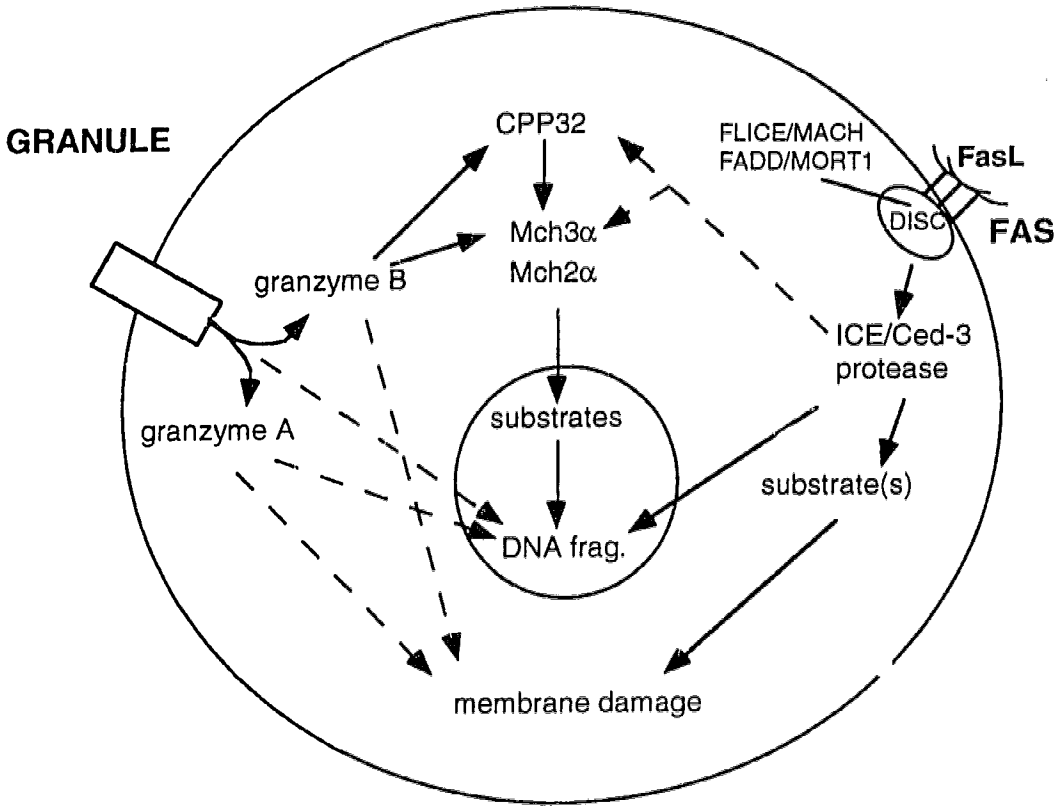
One intriguing possibility for regulating the CPP32-independent pathway to DNA fragmentation are the RNA-binding proteins TIA-1 and TIAR. The 15-kDa isoform of TIA-1 is localized to the lytic granules of CTLs (Anderson *et al.*, 1990; Cesano *et al.*, 1993) and has been found to induce DNA fragmentation in digitonin-permeabilized cells (Tian *et al.*, 1991), while TIAR translocates from the nucleus to the cytoplasm during Fas-mediated killing (Taupin *et al.*, 1995). Furthermore, a kinase activated following Fas ligation specifically phosphorylates the 40- and 53-kDa isoforms of TIA-1 (Tian *et al.*, 1995). Thus, one idea is that these proteins could initiate a chain of events during apoptosis which results in target cell DNA fragmentation. Alternatively, these proteins may interfere with a cell's splicing and/or translation reactions and therefore selectively produce apoptosis-inducing forms of the ICE/Ced-3 proteases and the Bcl-2 family. Thus, a critical question in determining the role of these proteins will be the identification of the RNA sequences to which they bind.

FIGURE VII-1: Model for CTL-Mediated Cytotoxicity.

Pathways which are based on experimental evidence are indicated by solid lines, proposed pathways by dashed lines.

During granule-mediated cytotoxicity, the granule proteins enter the target cell through perforin pores. Granzyme B cleaves CPP32 (and possibly related family members), which may or may not activate other ICE/Ced-3 family members, and eventually leads to cleavage of PARP, U1-70kDa and DNA-PK. Granzyme A may induce membrane damage by cleaving an as-yet-unidentified cellular substrate. Granzyme B may also be able to induce membrane damage. There is also an alternate pathway to DNA fragmentation which is granzyme B and CPP32 independent.

During Fas-mediated apoptosis, ligation of Fas results in initiation of a death signal, leading to the activation of an ICE/Ced-3 protease, possibly FLICE/MACH. This protease is involved in the induction of both the nuclear and cytoplasmic changes of apoptosis. The protease may cleave and activate members of the CPP32 family of ICE/Ced-3 proteases, and may have cellular substrates which are involved in the induction of membrane damage. In addition, a pathway which is independent of the CPP32 proteases exists, which results in DNA fragmentation.


GRANULE
CPP32
FLICE/MACH
FADD/MORT1
FasL
DISC
FAS
Mch3α
Mch2α
granzyme B
ICE/Ced-3
protease
granzyme A
substrates
substrate(s)
DNA frag.
membrane damage

C. IMPLICATIONS OF ACTIVATION OF CPP32-LIKE PROTEASES

One important area of investigation in the future will be how activation of CPP32 (and related proteases) is related to the induction of DNA fragmentation and apoptosis. Although this functional relationship has been demonstrated in both granule- and Fas-based cytotoxicity, and a number of substrates for the CPP32 proteases have been identified, the mechanism of induction of DNA fragmentation, and relationship to apoptosis, is unknown. The following is a summary of some of the potential consequences of cleavage and activation of the CPP32 proteases during apoptosis, by examining the roles of cleavage of proteins which have been identified as substrates for these enzymes.

1. PARP

The first substrate identified for CPP32 was poly(ADP-ribose) polymerase (PARP). PARP is a 113-kDa nuclear protein which transfers the ADP-ribose moiety from nicotinamide adenine dinucleotide (NAD) in a DNA-dependent manner to a selected number of protein acceptors involved in chromatin architecture and DNA metabolism (heteromodification) as well as to itself (automodification) (reviewed by Lindahl *et al.*, 1995; de Murcia and Ménissier de Murcia, 1994). In this capacity, PARP is involved in the maintenance of genome integrity and chromatin structure (Ding *et al.*, 1992; Smulson *et al.*, 1994; Ding and Smulson, 1994) as well as in DNA repair (Satoh and Lindahl, 1992).

PARP itself is a multifunctional enzyme and can be divided into three domains (Kameshita *et al.*, 1984): a 46-kDa amino terminal domain containing two zinc-finger-like motifs (Ménissier de Murcia *et al.*, 1989; Gradwohl *et al.*, 1990) and a nuclear localization signal (Schreiber *et al.*, 1992); a 22-kDa central automodification domain containing the glutamic acid residues which act as acceptors of poly(ADP-ribose); and a 54-kDa carboxy terminal NAD-binding domain which contains all the sequences required for PARP catalytic activity.

During "normal" cellular proliferation, PARP scans the cellular DNA through its DNA binding domain in order to maintain genome stability. Upon finding strand breaks, PARP binds the DNA through its zinc-fingers. This results in the activation of PARP through a conformational change (Ohgushi *et al.*, 1980). PARP proceeds to ADP-ribosylate surrounding proteins, especially histones, as well as itself. It is currently believed that the key acceptor of ADP-ribose is actually PARP itself, however, it is unknown whether one PARP molecule ADP-ribosylates autocatalytically, or whether a second PARP molecule is recruited to act as acceptor

(Lindahl *et al.*, 1995). The accumulation of negative charges on PARP and the chromosomal acceptor proteins, due to ADP-ribosylation, reduces the affinity of these proteins for the DNA. PARP and the chromosomal proteins fall off the DNA, allowing the DNA repair machinery access to the damaged site (Satoh and Lindahl, 1992). The binding of PARP to DNA strand breaks could also slow down the progress of the DNA replication fork causing DNA polymerases to stall close to strand breaks occupied by PARP. It is also possible that PARP could interact with components of the replication machinery since PARP has been found to copurify with DNA replicative forks, topoisomerase I (Ferro *et al.*, 1983) and DNA polymerase α (Simbulan *et al.*, 1993). By stalling the replication process, PARP enables a cell to repair damaged DNA before it is transmitted to future generations.

During apoptosis, PARP is activated, presumably as a consequence of the DNA strand breaks generated during DNA fragmentation. It has been proposed that the activation of PARP by DNA strand breaks contributes to the cellular depletion of NAD and ATP which occurs during apoptosis (Berger *et al.*, 1987; Wielckens *et al.*, 1987; Tanizawa *et al.*, 1989; Denisenko *et al.*, 1989; Wielckens and Delfs, 1986). However, Kaufmann *et al.* (1993) found that in cells undergoing chemotherapy-induced apoptosis, PARP was proteolytically processed to two fragments of Mr 89-kDa and 24-kDa. The 89-kDa fragment, which contains the catalytic domain, retains only basal level activity. To reconcile this apparent paradox - that is, that PARP activation contributes to NAD depletion and that PARP is cleaved to a less active form during apoptosis - these workers examined NAD levels and PARP status in cells undergoing apoptosis. They found that the majority of cellular NAD was depleted prior to PARP cleavage, that is, while PARP was still in the active 113-kDa form. Thus, it seems that PARP is initially activated at the onset of apoptosis and contributes to NAD depletion and then is subsequently inactivated by proteolysis.

PARP activity may also be linked to the regulation of DNA fragmentation occurring during apoptosis. In 1980, Wyllie reported that treatment of rat thymocytes *in vitro* with glucocorticoid could induce DNA fragmentation. Unlike the "smear" of DNA seen during necrosis, DNA from apoptotic cells had a unique appearance - the DNA fragmented into segments whose sizes were multiples of 180-200 bp, suggesting cleavage only of the linker DNA between nucleosomes. DNA fragmentation has since become a hallmark of apoptosis and is found under almost all conditions, suggesting the presence of an endogenous endonuclease found in multiple cell types (Shimizu *et al.*, 1990; Cohen and Duke, 1984; Odaka *et al.*, 1990).

The earliest detectable change in an apoptotic cell is a rapid, sustained increase in intracellular calcium which suggested that the endonuclease may be calcium-dependent. Hewish and Burgoyne (1973a, 1973b) described an endogenous endonuclease requiring calcium and magnesium for activity and cleaving DNA internucleosomally. This Ca^{++}/Mg^{++}-dependent endonuclease is constitutively present within the cell (Cohen and Duke, 1984; McConkey *et al.*, 1989) but maintained in an inactive state. Although several attempts have been made, the endonuclease(s) responsible for DNA fragmentation are yet to be identified although one intriguing report attributes nuclease activity to the amino acid isomerases, the cyclophilins (Montague *et al.*, 1994). A recent report has revealed the isolation of an inducible Ca^{++}/Mg^{++}-dependent endonuclease from lymphocytes (Khodarev and Ashwell, 1996), but it is unclear whether this endonuclease is constitutively expressed.

Earlier studies have found that the Ca^{++}/Mg^{++}-dependent endonuclease is maintained in a repressed state by ADP-ribosylation (Yoshihara *et al.*, 1974, 1975; Tanaka *et al.*, 1984; Rice *et al.*, 1992; Nelipovich *et al.*, 1988). Cleavage of PARP during apoptosis, resulting in decreased levels of activity, may therefore release the inhibition of this endonuclease, since the ADP-ribose moieties on the endonuclease could be rapidly removed by poly(ADP-ribose) glycohydrolase. However, if PARP activation maintains the Ca^{++}/Mg^{++}-dependent endonuclease in an inactive state, how is this endonuclease initially activated to cause the strand breaks which activate PARP? It now seems that DNA fragmentation occurs in two distinct phases - initially, the DNA is cleaved to produce large (50-300 kb) molecular weight fragments. This cleavage occurs by excision of chromosomal DNA loops (Lagarkova *et al.*, 1995). Subsequently, these large fragments are broken down into oligonucleosomal sized pieces. It is therefore likely that the initial endonuclease activated, which produces the large fragments, is Ca^{++}-dependent (Zhivotovsky *et al.*, 1994) but is not regulated by ADP-ribosylation. A likely candidate is DNase γ (Peitsch *et al.*, 1993a; Tanuma and Shiokawa, 1994; Shiokawa *et al.*, 1994) although it has recently been suggested that the DNA fragmentation observed during apoptosis is actually due to the accumulation of frequent single-strand breaks rather than due to double strand breaks (Peitsch *et al.*, 1993b). The strand breaks generated by the endonuclease could lead to activation of PARP. PARP could deplete the cell's NAD and ATP levels, prior to being proteolytically processed. Proteolytic processing of PARP would allow activation of the Ca^{++}/Mg^{++}-dependent nuclease, which then allows internucleosomal DNA fragmentation to proceed.

It has also been proposed that modification of histone H1 by PARP may alter chromatin structure by either altering nucleosome structure or by opening up the chromatin (by having PARP preferentially bind histone H1) (Poirier *et al.*, 1982). Indeed, poly(ADP-ribosyl)ation of histone H1 has been found to correlate with DNA fragmentation during apoptosis (Yoon *et al.*, 1996) suggesting that this may indeed be a second role for PARP activation during apoptosis, although the effect of proteolysis on this role is currently unknown. One possibility is that the proteolysis which releases the zinc fingers from the automodification and catalytic domains allows the zinc fingers to remain bound to the DNA and thereby maintain the chromatin in an open conformation.

Alternatively, inactivation of PARP by proteolysis, mediated by CPP32 and its related proteases, may simply cripple a cell's DNA repair system, thereby allowing DNA fragmentation to proceed.

2. DNA-PK

DNA-dependent protein kinase is actually a heterotrimer consisting of the Ku heterodimer (p70 and p80 subunits) and the catalytic subunit, DNA-PK_{CS} (p350). The catalytic subunit contains a region of homology to phosphatidylinositol kinases (Hartley *et al.*, 1995; Poltoratsky *et al.*, 1995) and is a serine/threonine kinase whose activity is regulated by association with Ku.

The function of DNA-PK has recently become evident through the merging of two distinct fields - those studying DNA double-strand-break (DSB) repair and those studying V(D)J recombination, the process by which B and T lymphocytes are able to generate antigen receptors with diverse specificity. These fields are common in that both processes require the repair of DSBs through a process of recombination. Indeed, the necessity of DNA-PK in these diverse processes is evidenced by mice with severe combined immunodeficiency (SCID). These mice lack functional B and T lymphocytes due to the inability to carry out V(D)J recombination. It is now believed that a mutation in the p350 subunit of DNA-PK is responsible for the SCID phenotype (Kirchgessner *et al.*, 1995; Blunt *et al.*, 1995; Boubnov and Weaver 1995; Peterson *et al.*, 1995; Miller *et al.*, 1995; reviewed by Jackson and Jeggo, 1995).

When DSBs occur in DNA, these are recognized by the Ku heterodimer. The Ku70 subunit actually recognizes the DNA ends (Zhang and Yaneva, 1992), while Ku80 possesses DNA helicase activity, and may assist in unwinding DNA in preparation for repair by recombination (Tuteja *et al.*, 1994). Once the Ku heterodimer is bound to the DNA, it forms a p350 binding site. The p350 catalytic

subunit then joins the complex and is activated (Suwa *et al.*, 1994). Active, heterotrimeric DNA-PK then phosphorylates surrounding DNA-bound proteins, including transcription factors (such as Fos and Jun [Abate *et al.*, 1993; reviewed by Finnie *et al.*, 1993]), and RNA polymerase I. Phosphorylation of RNA polymerase I specifically represses promoter-directed transcription initiation (Labhart *et al.*, 1995; Kuhn *et al.*, 1995). In this manner, DNA-PK may regulate gene expression by regulation of transcriptional activity thereby ensuring DNA repair prior to gene expression. DNA-PK is also able to autophosphorylate, an event which leads to its inactivation (Chan and Lees-Miller, 1996). In this regard, DNA-PK is similar to PARP. Its activity is activated by DNA strand breaks, and its activity is responsible for its subsequent inactivation (much the same way that PARP binding to DNA activates the enzyme, it ADP-ribosylates itself, and then falls off the DNA as a result).

DNA-PK also appears to be directly involved in the repair of DSBs. Although the exact mechanism of action is currently unknown, one possibility is that DNA-PK functions by maintaining the ends of a strand break in close association until they can be religated (Jeggo *et al.*, 1995). In addition to this structural role, DNA-PK may phosphorylate and activate enzymes involved in the repair/recombination processes. Finally, recombination/repair may be enhanced by the inhibition of transcription factors in the area.

During apoptosis, DNA-PK is proteolytically processed by CPP32 (and probably related proteases [Casciola-Rosen *et al.*, 1995; 1996; Song *et al.*, 1996]). The target of this proteolytic event is the p350 subunit and proteolysis removes the amino terminal domains containing a leucine zipper motif from the carboxy terminal kinase domain.

The role of proteolysis of p350 during apoptosis is currently unknown although it is interesting to note that proteolysis separates key functional domains, as it does in PARP cleavage. It is known that proteolysis reduces the kinase activity of DNA-PK$_{CS}$ rendering it inefficient at DSB repair (Casciola-Rosen *et al.*, 1996). Thus, this may represent a second mechanism by which the CPP32 enzymes cripple a cell's DNA repair system, thereby allowing DNA fragmentation to proceed. Further studies should help illuminate the role of this proteolysis in the induction of apoptosis.

3. U1-70kDa

The U1 small nuclear ribonucleoprotein is essential for the splicing of pre-mRNA and functions by recognizing the 5' splice site in conjunction with a family of Ser/Arg rich proteins (the SR proteins) which may bridge the 5' and 3' splice sites (Tarn and Steitz, 1995). Individual SR proteins have distinct abilities to promote the interaction of U1 with alternative 5' splice sites suggesting they play a distinct role in regulating 5' splice site selection (Zahler and Roth, 1995). The activity of U1 depends on both the RNA and protein components of the particle (reviewed by Lamond, 1993). U1-70kDa is functionally important in the splicing reaction, and its activity depends upon its state of phosphorylation. Thus, when U1-70kDa is thiophosphorylated, its activity is inhibited and splicing cannot occur (Tazi *et al.*, 1993). Like PARP and DNA-PK, U1-70kDa is one of a series of autoantigens which are proteolytically processed during apoptosis by CPP32 (Casciola-Rosen *et al.*, 1994b, 1995), and possibly other, related proteases. Proteolysis releases the RNA-binding domain of U1-70kDa from the distal Arg-rich region (Query *et al.*, 1989; Casciola-Rosen *et al.*, 1996), again resulting in the separation of key functional domains.

The effect of proteolytic cleavage of U1-70kDa on its activity, and splicing in general, is currently unknown. Overexpression of the carboxy terminal domain of U1-70kDa has been shown to have a dominant negative effect on mRNA splicing and transport to the cytoplasm (Romac and Keene, 1995). This carboxy terminal region contains Ser/Arg-rich regions. Interestingly, one of the fragments generated by CPP32 cleavage of U1-70kDa contains a Ser/Arg-rich region and therefore release of this fragment would likely have similar effects to overexpression of the carboxy terminus. Since repair pathways depend on new mRNA synthesis, cleavage of U1-70kDa during apoptosis may cripple repair by inhibiting RNA splicing. Additionally, five members of the SR family of proteins contain a DXXD sequence, which is potentially recognized by CPP32, just upstream of their SR domains (Casciola-Rosen *et al.*, 1996). These proteins may also be cleaved by CPP32 resulting in the liberation of multiple SR domains during apoptosis which could abolish RNA splicing. Further work is required to more clearly define the role of proteolysis of U1-70kDa in apoptosis.

4. SREBPs

Sterol-regulated proteolytic cleavage of SREBPs represents a central mechanism of control of cholesterol levels in animal cells. These membrane-bound

transcription factors consist of an amino terminal domain containing sequences of a transcription factor belonging to the basic helix-loop-helix-leucine zipper family (Yokoyama *et al.*, 1993; Hua *et al.*, 1993). Following this domain are two hydrophobic sequences which are responsible for membrane attachment to the nuclear and ER membranes. Finally, the proteins have long carboxy terminal extensions (Sato *et al.*, 1994; Hua *et al.*, 1995). In the cell, the protein is oriented such that both the amino and carboxy termini are in the cell's cytoplasm, and the loop between the transmembrane domains is found in the lumen of the ER.

In sterol-depleted cells a protease cleaves the SREBPs at a site just prior to the first transmembrane domain, thereby releasing the amino terminus (transcription factor domain). The freed transcription factor can then translocate to the nucleus, where it activates transcription of the low density lipoprotein receptor (Yokoyama *et al.*, 1993), farnesyl diphosphate synthase (Ericsson *et al.*, 1996), 3-hydroxy-3-methylglutaryl coenzyme A reductase (Vallet *et al.*, 1996) and acetyl coenzyme A carboxylase (Lopez *et al.*, 1996) genes. As sterols accumulate within the cell, cleavage of the SREBPs is reduced and any active SREBP in the nucleus is degraded.

Although the protease responsible for SREBP activation under normal conditions is unknown, recent data has shown that both CPP32 and Mch3α/CMH-1/ICE-LAP3 cleave the SREBPs at a similar site to that used by the natural activator of SREBPs during apoptosis induced by staurosporine, etoposide or anti-Fas antibody (Wang *et al.*, 1996; Pai *et al.*, 1996). The site has the sequence $^{D}/_{S}$EPD-S which is quite similar to the cleavage site recognized in PARP (DEVD). The role of this cleavage, if any, during apoptosis is unknown. One possibility is that this represents a fortuitous coincidence. That is, following CPP32 activation in the cytosol, CPP32 travels to the nucleus. If SREBPs are at the nuclear membrane, and contain a putative cleavage site for CPP32, it could be that they are cleaved by CPP32 as it enters the nucleus, and cleavage plays no role in apoptosis. Alternatively, sterols may play an as yet undetermined role in apoptosis, and CPP32 may facilitate this role by activating genes (by cleaving SREBPs) which control sterol metabolism. Further work in this area will hopefully shed some light on this intriguing discovery.

III. FUTURE DIRECTIONS

Of critical importance in the future will be the determination of the commitment step during apoptosis. Numerous events during apoptosis have been identified, but the truly essential event remains elusive. Although a number of reports have detailed activation of CPP32 (and related proteases) during apoptosis in a

variety of systems, the work outlined here has demonstrated that CPP32 activation may not be critical to all forms of cell death although it clearly plays a role in granule-mediated cytotoxicity. The results have shown that both membrane damage and, to some extent, DNA fragmentation can occur in the absence of granzyme B (which activates the CPP32 proteases during granule-mediated killing) and in the presence of an inhibitor of CPP32 proteases. Additionally, the data have shown that CPP32 is not activated during Fas-mediated killing in all target cells, and represents the first example of a system where CPP32 is not active during apoptosis. Based on these studies, it seems safe to say that while CPP32 activation may be required for the *rapid* induction of DNA fragmentation during apoptosis, both DNA fragmentation and apoptosis can occur in the absence of CPP32. Thus, the identification of the "commitment" event is essential to a further understanding of both apoptosis and CTL-mediated cytotoxicity. One approach to this problem is the study of cells in which proteins implicated in cell death have been knocked out. For example, if inhibition of DNA repair processes is a critical event in apoptosis, then studies of cells lacking certain repair pathways shoudl reveal this. A PARP "knockout" mouse has already been generated (Wang *et al.*, 1995b) and the SCID mouse lacks DNA-PK_{CS}. If inhibition of repair is critical, and cleavage of PARP and DNA-PK_{CS} by CPP32-like proteases contributes to this, then cells derived from these mice should be more susceptible to death since one of their repair pathways is already deficient. As more "knockout" mice are generated, the role of ICE/Ced-3 protease cleavages can be easily tested.

Alternatively, a cell may possess multiple, parallel pathways which culminate in cell death rather than one central death pathway. This stands to reason when the importance of programmed cell death is considered. After all, this is the mechanism by which organisms get rid of unwanted cells, cells which have developed improperly, viral-infected cells, tumourigenic cells, and is also involved in the maintenance of tissue homeostasis. In this regard, cell death is as important to an organism as cell division. Therefore, it is likely that any one cell can undergo cell death through a number of pathways rather than one. As an example, consider the removal of viral-infected cells, mediated by CTLs. If only one cell death pathway exists, a virus would simply need to overcome that one pathway in order to circumvent a host's immune system. However, if the host possesses multiple mediators of cell death then it can ensure that viral infection can be dealt with. Indeed, although some viruses are able to synthesize inhibitors of ICE (and related enzymes), CTLs may be able to bypass the viral inhibition by possessing multiple

lytic mediators with multiple cellular targets. If there are actually parallel pathways to apoptosis, which seems likely given the importance of this process, then a crucial area of future research will be to determine which mediators belong to which pathway, and how the various pathways intersect. Again, the use of knockout mice will contribute to these studies.

Another important aspect to consider would be the identification of the initial event during Fas-mediated apoptosis. Although much effort has been expended on the role of ICE/Ced-3 proteases during Fas-mediated killing, it remains unclear how the protease cascade becomes activated. A likely candidate is FLICE/MACH, but further studies are required to more clearly define the role of this protease. The death signal generated by Fas ligation is also known to contain a number of components, including protein kinases and ceramide, and seems to be initiated by a signaling complex associated with Fas. How are these mediators linked to Fas ligation and ICE/Ced-3 protease activity? The finding that an ICE-like enzyme is responsible for the generation of ceramide during death induced by the *Drosophila* death domain-containing protein REAPER (Pronk *et al.*, 1996) suggests that an ICE-like activity may initiate ceramide release but the mechanism involved here remains to be determined.

The studies presented here have shown that a second pathway to DNA fragmentation exists, which is not mediated by CPP32 proteases. This pathway is utilized in both granule- and Fas-mediated cytotoxicity and may be a characteristic of apoptosis in general. The elucidation of the nature of this pathway, and the proteins involved, will be crucial not only to the study of CTL-mediated cytotoxicity, but also to apoptosis.

Finally, further elucidation of the individual roles of the ICE/Ced-3 and Bcl-2 family members will need to be carried out. One problem with the study of these proteins is the nature of their interaction - within the families there are numerous oligomerizations possible, with the result that overexpression or knock-out studies may only indicate the result of disrupting cellular equilibrium rather than the actual role of the family member of interest. In addition, the use of peptide aldehyde inhibitors cannot distinguish between individual family members - both Mch3/CMH-1/ICE-LAP3 and CPP32 are equivalently inhibited by Ac-DEVD-CHO. Thus, an alternate method of studying these proteins must be developed before further elucidation of the events involved in apoptosis is possible. One possibility is to study these enzymes in isolation in yeast. Studies have reported that overexpression of BAK in yeast results in cell cycle arrest (S.N. Farrow, personal communication),

suggesting that this may be a mechanism of studying these proteins. However, further characterization of yeast cell biology is required before this becomes a general technique.

IV. CONCLUSIONS

In conclusion, in a few short years, it has been demonstrated that the mechanisms used by CTLs to induce target cell death are identical to those used during physiological cell death. These findings hold exciting potential for the isolation of novel immunosuppressants, however, the mechanisms involved during CTL-mediated cytotoxicity and target cell apoptosis must first be more extensively characterized.

CHAPTER VIII - BIBLIOGRAPHY

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