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MECHANISMS OF LYSIS BY CYTOTOXIC T CELLS

by

ERIC A. ATKINSON

A THESIS SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled "**MECHANISMS OF LYSIS BY CYTOTOXIC T CELLS**" submitted by Eric A. Atkinson in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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Date: 15 / 1 / 96

ABSTRACT

Cytotoxic T lymphocytes (CTL) kill their target cells in one of two ways. Tumour and virus infected cells are eliminated by exocytosing the contents of CTL cytolytic granules towards them in a calcium dependent process. Notable granule proteins involved in causing the destruction of the target cell are the pore forming protein perforin, and a family of serine proteinases, the granzymes. CTLs can also kill other activated T cells in a calcium-independent process in order to down-regulate an immune response. This second mechanism of CTL cytolysis involves ligation of a death protein termed Fas on the target cell by the Fas ligand on the killer cell. Both pathways impinge on a pre-existing suicide pathway in the target cell and cause death via apoptosis, a process which involves DNA fragmentation.

In order to explore the function of granzyme B, I have constructed gene targeting vectors designed to knockout the granzyme B gene in mouse embryonic stem cells. A number of correctly targeted ES cells were identified by Southern blot analysis, but production of mutant mice was not performed due to their generation by another lab.

I have also explored the contribution that the T cell protein tyrosine kinase $p59fyn^T$ makes towards CTL effector function using fyn-deficient mice. This kinase was found to not be required for generation of cytolytic potential or for the induction of the granzyme B and perforin message in splenocytes stimulated with either anti-CD3 antibody or alloantigen. However, it did appear to influence the lifespans of the activated lymphocytes.

Further evaluation of the role of p59fyn in controlling lymphocyte lifespans led to the demonstration that fyn physically interacts with Fas. Also, fyn was shown to be involved in lymphocyte death caused by Fas ligation. These findings represent an important advancement in our understanding of how Fas signals cell death, as the signal transduction mechanism utilized by Fas has been heretofore unknown.

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LIST OF ABBREVIATIONS

ATP	Adenosine Triphosphate
CD	Cluster of Differentiation
CDK	Cyclin Dependent Kinase
CDL	Cytotoxic T Cell
cDNA	Complementary DNA
ConA	Concanavalin A
Cr	Chromium
DAG	1,2-Diacylglycerol
DMSO	Dimethyl Sulfoxide
E:T	Effector to Target Ratio
ECL	Enhanced Chemiluminescence
EDTA	Ethylenediamine tetraacetic acid
ES Cell	Embryonic Stem Cell
FITC	Fluoroscein Isothiocyanate
GANC	Gancyclovir
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
HRP	Horse Radish Peroxidase
HSV	Herpes Symplex Virus
ICE	Interleukin 1-B Converting Enzyme
IL-2	Interleukin 2
IP3	Inositol-1,3,5-Tris Phosphate
ITAM	Immunereceptor Tyrosine Activation Motif
kDa	Kilodalton
LIF	Leukemia Inhibitory Factor
MHC	Major Histocompatability Complex
MLC	Mixed Lymphocyte Culture

MLR	Mixed Lymphocyte Reaction
MOPS	3-(N-morpholine)propanesulfonic acid
neo	Neomycin Phosphotransferase
NGF	Nerve Growth Factor
NK Cell	Natural Killer Cell
PAGE	Polyacrylamide Gel Electrophoresis
PARP	Poly(ADP-ribose) Polymerase
PBL	Peripheral Blood Lymphocytes
PBMC	Peripheral Blood Mononuclear Cells
PBS	Phosphate Buffered Saline
PCD	Programmed Cell Death
PCR	Polymerase Chain Reaction
PEL	Peritoneal Exudate Lymphocyte
PGK	Phosphoglycerol Kinase
PKC	Protein Kinase C
PLC	Phospholipase C
PMA	Phorbol-12-myristate-13-acetate
PTK	Protein Tyrosine Kinase
RBL	Rat Basophilic Leukemia
SDS	Sodium Dodecyl Sulphate
SH2	Src Homology 2 Domain
SH3	Src Homology 3 Domain
TCR	T Cell Receptor
Th	Helper T Cell
TK	Thymidine Kinase
TNF	Tumour Necrosis Factor
TNFR	TNF Receptor

UV	Ultraviolet
α	alpha
β	beta
γ	gamma
δ	delta
ε	epsilon
η	eta
ζ	zeta
leu/L	leucine
phe/F	phenylalanine
tyr/Y	tyrosine
X	any amino acid

CHAPTER ONE: INTRODUCTION

A version of this chapter has been submitted for publication. E.A. Atkinson and R.C. Bleackley, 1996. Critical Reviews in Immunology.

I. INTRODUCTION

Detection and elimination of virus-infected and tumour cells is an important function of the immune system. This role is performed by specialized cytotoxic lymphocytes. How these professional killers effect the destruction of their targets has been an area of intensive investigation for a number of years. Recent experiments have greatly increased our understanding of this process and have clarified some contentious issues in the field. This review will attempt to describe our current view of how cytotoxic lymphocytes function.

II. GRANULE EXOCYTOSIS AS A MECHANISM FOR CYTOTOXIC LYMPHOCYTE FUNCTION

A. Physical Characteristics of Cytotoxic Lymphocytes

Two major effectors of cellular cytotoxicity are natural killer (NK) cells and CD8+ cytotoxic lymphocytes (CTL). Although these two cell types recognize their targets in different ways - NK cells via a MHC-inhibited manner that is still ill-defined, and CTL via T cell receptor/CD8 recognition of specific antigen in the context of class I MHC molecules - it has long been believed that the killing mechanism itself is fundamentally the same. Both cell types contain dense cytoplasmic granules that have characteristics of both secretory granules and lysosomes. NK cells are constitutively cytotoxic and always show a large granular lymphocyte morphology (Timonen et al., 1981; Trinchieri, 1989). In contrast, resting CTL are small, agranular cells that concomitantly acquire cytolytic potential and cytoplasmic granules upon exposure to antigen or interleukin-2 (Dennert and Podack, 1983).

Early suggestions that secretion and degranulation might be important in cytotoxic lymphocyte function came from the observations that upon target cell binding, the granules, microtubule-organizing centers and Golgi apparatus of the killer cells reorient themselves towards the point of cell-cell contact (Yannelli et al., 1986; Geiger et al., 1982; Kupfer and Dennert, 1984). This led to the formulation of the granule

exocytosis model of cytotoxic lymphocyte function, which provided a dominant paradigm for much of the work in this field (Henkart and Henkart, 1982). It was suggested that cytotoxic lymphocytes produce potent lytic molecules and store them in their granules. When a target cell is bound via specific recognition receptors and additional adhesion molecules, a tight junction is formed between the two cells. The cytotoxic cell then exocytoses its granules towards the target cell, and the released lytic mediators inflict a "lethal hit" on the target. This model was strongly supported by the demonstration that cytoplasmic granules isolated from NK cells and CTL are cytolytic to a number of tumour cell lines, as well as to red blood cells (Millard et al., 1984; Henkart et al., 1984; Podack and Konigsberg., 1984). In addition, electron microscopic analysis of target cells exposed to cytolytic lymphocytes and purified lytic granules revealed the presence of ring-shaped pore structures in their membranes, suggesting the deposition of a membrane-damaging protein similar to the complement membrane attack complex. (Dourmashkin, et al., 1980; Podack and Dennert, 1983; Dennert and Podack, 1983; Podack and Konigsberg, 1984).

B. Granule Components Involved in Cytotoxicity

1. Perforin

Fractionation of granule components from both NK cells and CTL led to the isolation of a single lytic protein that was responsible for the membrane perforating activity (Masson and Tschopp, 1985; Liu et al., 1986;, Podack et al., 1985; Zalman et al., 1987). This protein, termed cytolysin or, more commonly, perforin, is a 65 kDa polypeptide that binds to lipid bilayers and inserts into them, aggregating to form pores, in a strictly calcium-dependent manner (Podack et al., 1985; Yue et al., 1987;, Tschopp et al., 1989, Young et al., 1987, Blumenthal et al,. 1984; Ishiura et al., 1990) . It has been suggested that perforin damage alone might cause target cell death via unregulated membrane permeability leading to colloid osmotic lysis. Although this may happen at

the high effector to target ratios used *in vitro*, it seems unlikely to occur *in vivo* where target cells would predominate under most circumstances.

It has long been known that target cell death is accompanied by DNA fragmentation (Russel, 1983; Cohen et al., 1985; Cohen, 1991; Golstein et al., 1991), similar to that observed during programmed cell death or apoptosis (Kerr et al., 1972; Wyllie, 1980). Indeed, DNA fragmentation is observed well before membrane breakdown can be detected by release of ^{51}Cr-labelled cytoplasmic proteins. However, exposure of a variety of cell lines to purified perforin leads to cell lysis in the absence of DNA fragmentation (Duke et al., 1989). This suggests that additional lytic factors may cooperate with perforin to ensure target cell death. Although the fact that cytolytic lymphocytes and isolated granules can lyse red blood cells, enucleated cells, and lightly fixed cells (Siliciano and Henney, 1978; Bubbers and Henney, 1975) would suggest a lack of target cell nuclear involvement in the lytic process, it must be remembered that *in vivo*, cytotoxic lymphocytes may encounter greater resistance to their arsenal than is commonly displayed in *in vitro* systems. For instance, a single killer cell may have to destroy a number of individual target cells, and the limited membrane damage due to secretion of perforin from a single killer cell may be easily repaired by many metabolically active cells. Complete poly-perforin channels of 10-20 nM diameter can be seen on target cells exposed to high levels of perforin. These tubular pores are of sufficient size to allow proteins and dyes to pass through them (Blumenthal et al., 1984; Peters et al., 1990). However, the amount of perforin that inserts into a target cell's membrane under normal physiological conditions may not be sufficient to aggregate into these large functional channels. Perforin lesions may be comprised of one or a few aggregated perforin molecules. In this case, it would be expected that the target cell would be able to repair simple, limited perforin damage by endocytosing the affected area of the membrane.

2. Granzymes

There is now considerable evidence that additional cytolytic granule proteins do indeed cooperate with perforin in target cell destruction. A family of granule-localized serine esterases, commonly refered to as granzymes (Table 1-I), has been identified by a number of labs using different approaches (Masson and Tschopp, 1987; Gershenfeld and Weissman, 1986; Lobe et al., 1986; Bleackley et al., 1988) . A number of lines of circumstantial evidence suggested a role for the granzymes in the cytotoxic process. This includes the co-localization with perforin to cytotoxic granules, induction of transcription upon antigen engagement or other methods of T cell activation, induction kinetics that just precede the acquisition of cytotoxic potential, and the observations that protease inhibitors affected the function of cytolytic lymphocytes (Bleackley et al., 1988; Chang and Eisen, 1980; Redelman and Hudig, 1980; Hudig et al., 1981; Hudig et al., 1991). However, until recently, direct evidence for a role in target cell lysis has been lacking.

It has now been convincingly demonstrated that at least some of the granzymes are involved in the initiation of target cell DNA fragmentation. Using fractionated cytolytic granules and searching for an activity that could induce DNA fragmentation in target cells permeabilized with purified perforin, 3 different "fragmentin" activities were found (Shi et al., 1992a; Shi et al., 1992b). These fragmentins were shown to be homologous to members of the granzyme family. An important role for perforin activity in granzyme-induced DNA breakdown was indicated by the absolute requirement for perforin in this assay. Incubation of the target cells in the fragmentin solution had no effect on target cell membrane permeability or DNA fragmentation. An alternative but complementary approach has led to similar findings. Rat basophilic leukemia cells (RBL) transfected with granzyme A or granzyme B, either alone or in conjunction with perforin, have also led to the demonstration of DNA fragmentation in response to these serine esterases (Shiver and Henkart, 1991; Shiver et al., 1992; Nakajima and Henkart, 1994; Nakajima et al., 1995). RBL are mast-like, granule-containing cells that can be

Table 1-I

Mouse Granzymes

Proteinase	Synonym	Mass (kDa)	Reference
Granzyme A	HF	35 (R) 60 (NR)	Pasternack & Eisen, 1985 Masson *et al.*, 1986a Masson *et al.*, 1986b Gershenfeld & Weissman, 1986
	SE1 CTLA-3 TSP-1		Young *et al.*, 1986 Brunet *et al.*, 1986 Simon *et al.*, 1986
Granzyme B	CCP1	29	Lobe *et al.*, 1986a Lobe *et al.*, 1986b Lobe *et al.*, 1988 Masson & Tschopp, 1987
	SE2 CTLA-1		Young *et al.*, 1986 Brunet *et al.*, 1986
Granzyme C	CCP2	27	Lobe *et al.*, 1986a Lobe *et al.*, 1986b Lobe *et al.*, 1988 Jenne *et al.*, 1988
Granzyme D	CCP5	35-40	Bleackley *et al.*, 1988a Bleackley *et al.*, 1988b Jenne *et al.*, 1988 Prendergast *et al.*, 1991
Granzyme E	CCP3	35-50	Bleackley *et al.*, 1988a Bleackley *et al.*, 1988b Jenne *et al.*, 1988 Prendergast *et al.*, 1991
	MCSP-2		Kwon *et al.*, 1988
Granzyme F	CCP4	35-45	Bleackley *et al.*, 1988a Bleackley *et al.*, 1988b Jenne *et al.*, 1988 Prendergast *et al.*, 1991
	MCSP-3		Kwon *et al.*, 1988
Granzyme G	CCP6	33	Masson & Tschopp, 1987 Jenne *et al.*, 1989

induced to degranulate upon binding IgE-coated targets. Only cells transfected with both perforin and granzyme plasmids showed tumour cell lysis that was accompanied by DNA fragmentation. Cells transfected with granzyme cDNA alone are not lytic, and cells transfected with perforin cDNA alone are lytic, although relatively weakly, but kill in the absence of DNA fragmentation. Interestingly, cells transfected with both perforin and granzyme cDNAs are also more potent in causing ^{51}Cr release, an indicator of cell lysis. This would suggest that DNA fragmentation, induced by one or more granzymes, plays an important role in target cell destruction. Alternatively, the granzymes may have additional roles in target cell destruction that are unrelated to the breakdown of nuclear DNA. The role that DNA fragmentation plays in target cell death, as in other instances of apoptotic death, is still a matter of contention, and will be discussed later in this review.

Finally, a convincing role for granzyme B, the most abundant granule-localized serine protease and the most potent fragmentin, has been demonstrated by ablation of its gene via gene targetting in embryonic stem cells and generation of granzyme B^{null} mice (Heusel et al., 1994). CTL from granzyme B-deficient mice show a reduced efficiency at lysing target cells as measured by ^{51}Cr release. In addition, target cell DNA fragmentation is significantly reduced. Interestingly, however, DNA fragmentation does occur, but with reduced kinetics. Longer incubation times result in a significant level of DNA fragmentation being observed and a complete rescue of ^{51}Cr release. The mediators responsible for the remaining DNA fragmentation inducing activity are not known, but it is reasonable to suggest that additional granzyme members, especially those for which fragmentation activity has already been described, could contribute towards this process. In this respect it is noteworthy that the lytic activity of a mouse CTL line could be downregulated by transfection of an antisense granzyme A construct (Talento et al., 1992). Further confirmation of a role for granzyme B in target cell death is the observation that antisense granzyme B also reduces the lytic activity of a cytotoxic lymphocyte cell line (Bochan et al., in press).

3. Other Granule Proteins

Additional cytotoxic granule proteins may play a role in the granule exocytosis pathway of cellular cytotoxicity. A 15 kDa granule-associated protein termed TIA-1 was first defined by a monoclonal antibody raised against cytolytic granules (Anderson et al., 1990). Expression of TIA-1 is restricted to CTL and NK cells, and is upregulated upon cellular activation via mitogens, cytokines, or stimulation of the T cell receptor (Anderson et al., 1990; Cesano et al., 1993). This protein belongs to the RRM family of RNA binding proteins, and has been shown to induce DNA fragmentation in permeabilized but not intact cells (Tian et al., 1991). How or if this protein contributes towards granule-mediated target cell death is not yet known, although its association with granules and nucleolytic activity is certainly suggestive of a potential role in this process.

Another granule protein that has received attention recently is calreticulin. Calreticulin is a 46 kDa (60 kDa in SDS-PAGE) protein that is ubiquitously expressed and, due to a COOH-terminal KDEL ER retention signal, had been widely thought of as a resident protein of the endoplasmic reticulum (reviewed in Michalak et al., 1992). Calreticulin was first implicated in CTL function by the observation that it is upregulated upon cellular activation (Burns et al., 1992). Subsequently, it was shown that it was identical to a 60 kDa granule protein that copurifies with perforin (Dupuis et al., 1993). Concomitantly, we had localized calreticulin to the cytoplasmic granules in activated CTL by immunocytochemistry. How this protein manages to evade the ER and make its way to the granules is not known, as calreticulin in granules still seems to possess the KDEL ER retention signal (R.C. Bleackley and M. Michalak, unpublished). Whether or not calreticulin functions in granule-mediated cytotoxicity is not presently known, but its tight binding to perforin is highly suggestive. Perhaps it may function as a chaperone for perforin through the exocytic pathway, or as a buffer for calcium, protecting perforin from premature activation (Burns et al., 1994; Dupuis et al., 1993; Bleackely et al., 1995). Alternatively, it could play a more direct role in target cell destruction if it gains access to

the target cell cytoplasm, and thus contribute towards the effects of the target cell calcium fluctuations seen upon CTL attack. Also, we and another group have shown that calreticulin can bind to nuclear receptors and modulate steroid dependent transcription (Burns et al., 1994b; Dedhar et al., 1994). It is possible that this activity could be exploited to contribute towards the induction of a suicide program in the target cell.

Other granule-associated molecules listed in Table 1-II are thought to play roles directly or indirectly in the killing process. There is good evidence for the function of some of these molecules, e.g. chondroitin sulphate acting as an inhibitor of granzymes/perforin. (Stevens et al., 1989) Others, however, require further characterization.

4. Granule Targeting

During the initial stages of CTL stimulation, signals transduced from the TCR and cytokine receptors result in the activation of genes that encode the molecules that are involved in killing (reviewed in Hadad et al., 1993). When the activated CTL binds to a target these cytolytic effector molecules are "delivered" to the offending antigenic cell. In the granule-mediated mechanism the genes that are activated include perforin, granzymes, proteoglycan and calreticulin. All of these molecules bear a signal sequence that directs the nascent peptides to the endoplasmic reticulum. Ultimately they are sequestered in the cytoplasmic granules but the mechanism that controls this intracellular targeting remains unclear (Griffith, 1995). Granzymes A and B appear to be sorted, at least in part, by the mannose-6-phosphate receptor (Griffiths & Isaaz, 1993). However, additional mechanisms are suspected to operate for the granzymes, and perforin definitely appears to use a different pathway. Obviously CTL need to have evolved mechanisms to protect themselves from potentially lethal molecules such as perforin and granzymes as they are being sorted. The latter are made with a dipeptide extension that abrogates enzymatic activity. Either just before or perhaps upon packaging the zymogens are activated most likely by DPPI/cathepsin C. Once within the granule the acidic pH and the

Table 1-II

Proteins Found in Lytic Granules

Protein	Function	Reference
Perforin (cytolysin)	Pore forming protein	Millard *et al.*, 1984 Henkart *et al.*, 1984 Masson & Tschopp, 1985 Liu *et al.*, 1986 Podack *et al.*, 1985 Zalman *et al.*, 1987
Granzymes	Proteolytic enzymes	See Table 1
DPPI	Granzyme activation	Smyth *et al.*, 1995
Chondroitin sulphate	Complex with other granular proteins	Serafin *et al.*, 1986 Stevens *et al.*, 1989
Calreticulin	Binds perforin	Dupuis *et al.*, 1993
	Calcium binding protein	Burns *et al.*, 1992 Michalak *et al.*, 1992
TIA-1	RNA binding	Tian *et al.*, 1991
Leukalexin	TNF-like	Liu *et al.*, 1987
Leukophysin	Granule mobility	Abdelhaleem *et al.*, 1991
Mannose-6-phosphate receptor	Protein trafficking	Burkhardt *et al.*, 1990

proteoglycan likely inhibit their lethal activities. Again the situation with perforin is less clear. Some investigators have suggested that perforin is made in an inactive form while others have disputed this claim. Another possibility is that calreticulin acts as a chaperone for perforin as it moves through the ER and Golgi.

Once the molecules are sequestered in the granules they await the signal for regulated secretion that is transmitted when a target cell is engaged (Atkinson et al., 1990; Anel et al., 1994) The vectoral release of the granule proteins appears to be an effective mechanism to ensure a high concentration of lytic effectors in the vicinity of the target while sparing nearby non-antigenic cells. The granules appear to move along microtubules (Burkhardt et al., 1993) and are driven by a kinesin motor whose activity is regulated by phosphorylation (McIlvain et al., 1994). Defective secretion of cytolytic components in Chediak-Higashi syndrome has recently been proposed to result from incorrect vesicle fusion in hemopoietic cells including CTL/NK (Baetz, 1995).

C. Mechanisms of Perforin/Granzyme-Induced Apoptosis

1.ICE-Family Proteases

Precisely how the granzymes contribute towards target cell destruction and act to induce DNA fragmentation is not known, although this is currently under intense investigation. Considerable attention has been given to the unique substrate specificity of granzyme B (Murphy et al., 1988). Studies with synthetic peptide analogues have demonstrated this serine esterase to have Asp-ase activity (Odake et al., 1991; Poe et al., 1991), and this has now been confirmed by site-specific mutagenesis studies (Caputo et al., 1994). The only other known mammalian proteases to cleave after aspartate residues are those within a growing family of proteases, the prototype of which is the cysteine-protease interleukin-1ß converting enzyme (ICE) (see Kumar, 1995 for review). ICE gained prominence in the cell death field after it was shown to be a homologue of the *Caenorhabditis elegans* gene ced3, (Yuan et al., 1993) which is vital to programmed cell death in this nematode (Ellis and Horvitz, 1986; Yuan and Horvitz, 1990). ICE and ced3

appear to be functionally interchangeable, since overexpression of either gene in mammallian cells results in death by apoptosis (Miura et al., 1993). The rare Asp-ase activity shared by ICE and granzyme B is intriguing and has caused speculation that they may share a similar function or substrates, or impinge on the same cell death pathway. Since ICE is activated by cleavage of pro-ICE at Asp residues, one hypothesis is that granzyme B may act to proteolytically cleave the ICE precursor and generate the active enzyme, which would then initiate the apoptotic program. We have not found ICE to be a substrate for granzyme B *in vitro*, (Darmon et al., 1994); however other groups have apparently obtained results to the contrary. The reasons for these conflicting results are presently unclear.

Cell-free systems employed to study apoptosis have revealed that one of the earliest detectable events in this process is the cleavage of the DNA repair enzyme poly(ADP-ribose) polymerase, or PARP (Lazebnik, et al., 1994). How cleavage of PARP influences apoptosis is not yet known, but inhibition of DNA repair, loss of negative regulation of a Ca^{2+}/Mg^{2+}-dependent endonuclease implicated in DNA fragmentation, and effects of NAD and ATP stores in the cell have been implicated. The reader is referred to Earnshaw (1995) for a recent discussion of the possible role of PARP in apoptosis. Upon induction of apoptosis, PARP is cleaved at an Asp-containing site by an enzyme activity that resembles, but is distinct from ICE (Lazebnik et al., 1994). This ICE-like enzyme was termed prICE (for protein resembling ICE), and recently, two groups have identified it as the cysteine protease CPP-32 (Nicholson et al., 1995; Tewari et al., 1995). Like ICE, CPP-32 requires proteolytic activation by cleavage at an Asp residue, but unlike ICE, it is not capable of autocatalytic cleavage. We have now demonstrated that granzyme B can cleave and activate CPP-32 directly, revealing that this important ICE-like protease is a key substrate for granzyme B and may be a critical mediator in granzyme B-initiated apoptosis (Darmon et al., 1995). However, because PARP-deficient mice appear to develop normally (Wang et al., 1995), CPP-32 cleavage

of PARP cannot be a universally required step in all form of apoptosis. It is likely that other events occur in parallel, and therefore additional important cellular substrates for granzyme B and CPP-32 also exist. These results also suggest that developmental programmed cell death (PCD in Figure 1-1) and CTL-mediated lysis intersect at CPP-32. As already stated, the activator of CPP32 in the CTL system is granzyme B, but its identity in PCD awaits discovery. In certain examples of PCD, new transcription and translation is required, possibly for the production of this enigmatic activator.

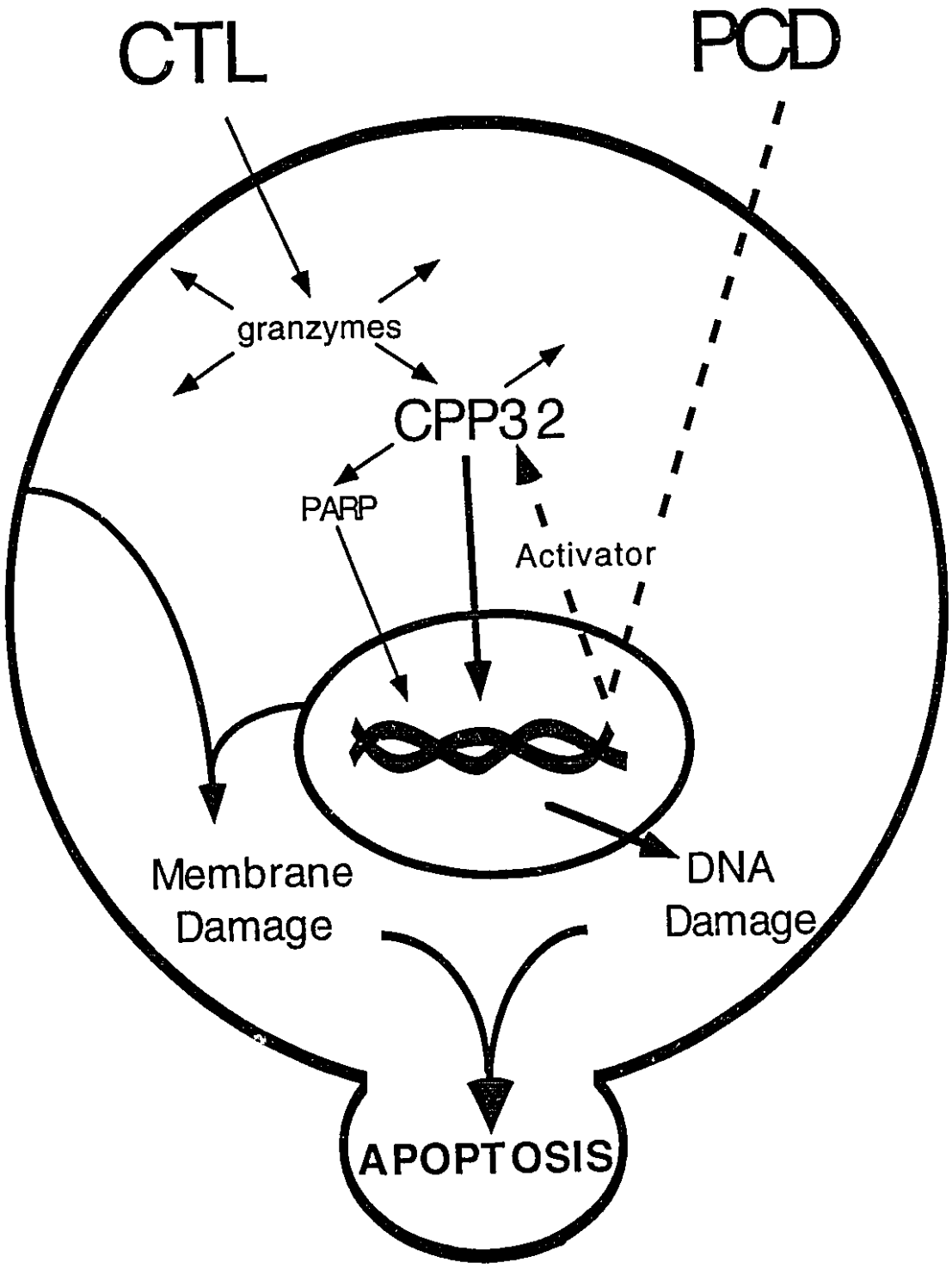
Since the ICE/ced 3 family of Asp-specific proteases continues to grow, and since each appears to require proteolytic activation at Asp-containing sequences, it is possible that granzyme-mediated cleavage of different family members may occur simultaneously in a single cell, or selectively in different cell types. These other proteases may have additional substrates that affect the apoptotic pathway in different ways. It is tempting to speculate that granzyme B activates an ICE-family proteolytic cascade within a target cell that contains these enzymes in their proenzyme forms. This would result in a massive amplification of the death signal initiated by a very small amount of granzyme B, and would resemble other biological systems such as the complement and blood clotting cascades. It is also worth keeping in mind that there are multiple granzymes in CTL and it is possible that more than one ICE-family member may be activated by each, leading to parallel pathways to death (Macen et al., submitted). As this is a rapidly progressing area of research, new insight into the roles of ICE-related proteases in cell-mediated cytotoxicity and apoptosis will likely be forthcoming.

2. Activation of $p34^{cdc2}$

Apoptosis triggered by perforin and granzyme B/fragmentin 2 has recently been shown to result in the activation of the cyclin dependent kinase (CDK) $p34^{cdc2}$(Shi et al., 1994). This kinase is an important member of a larger CDK family and is involved in regulating the G2/M transition of the cell cylce (see Forsburg and Nurse, 1991 for review). Premature activation of $p34^{cdc2}$ has been shown to result in mitotic catastrophe,

FIGURE 1-1 CTL-Mediated Killing and Programmed Cell Death Intersect at a Cysteine Proteinase, CPP32.

CTL induce target cell death by cleavage and activation of CPP32. In developmental programmed cell death (PCD), an activator is produced, possibly requiring transcription and translation, that also cleaves CPP32. One substrate for CPP32 has been identified as poly(ADP ribose) polymerase, but it is likely others contribute toward the apoptotic events that occur as a consequence of CPP32 activation.


CTL
PCD
granzymes
CPP32
PARP
Activator
Membrane
Damage
DNA
Damage
APOPTOSIS

a phenomenon which shares several characteristics with programmed cell death (Forsburg and Nurse, 1991). Interestingly, inhibition of $p34^{cdc2}$ activity, either by addition of a synthetic substrate peptide or at the non-permissive temperature in a $p34^{cdc2}$ temperature-sensitive cell line, resulted in inhibition of granzyme B-mediated DNA fragmentation (Shi et al., 1994). Furthermore, expression in BHK cells of wee1, a kinase that negatively regulates $p34^{cdc2}$, conferred resistance to apoptosis induced by perforin and granzyme B (Chen et al., 1995). These results suggest that $p34^{cdc2}$ activity is required for granzyme B-mediated apoptosis and that granzyme B may initiate apoptosis via a mechanism that results in the premature activation of the kinase. The generality of this mechanism has been questioned, however, as apoptosis caused by other agents in the same $p34^{cdc2}$ temperature-sensitive cell line was not consistently observed to require $p34^{cdc2}$ (Martin et al., 1995). The fact that $p34^{cdc2}$ belongs to a CDK family with multiple members complicates this issue and it remains possible that different members are activated by different apoptotic stimuli, resulting in a similar fate for the cell. Regardless of the universality of a CDK-inititated mitotic catastrophe in all forms of apoptosis, $p34^{cdc2}$ activation does appear to play a role in granzyme B-mediated cell death (Shi et al., 1994; Chen et al., 1995). It remains to be seen just how granzyme B activates this kinase, as $p34^{cdc2}$ does not appear to be a substrate for the protease.

3. The Perforin "Channel" Revisited

There is a tendency to assume that the granzymes function in the interior of the target cell. In addition to being intuitively reasonable, this idea is supported by a number of observations. First, target cell loading with the protease inhibitor aprotinin inhibited lysis and DNA release due to perforin and granzyme A (Nakajima et al., 1994). While this may indeed reflect an inhibition of the granzymes after entry into the cell, it does not rule out the possibility that other endogenous proteases are activated as a result of the cytotoxic lymphocyte lethal hit, and that these endogenous proteases contribute towards

target cell death. Since apoptosis initiated by non-granule mediated stimuli was not assessed in these studies, this remains a distinct possibility.

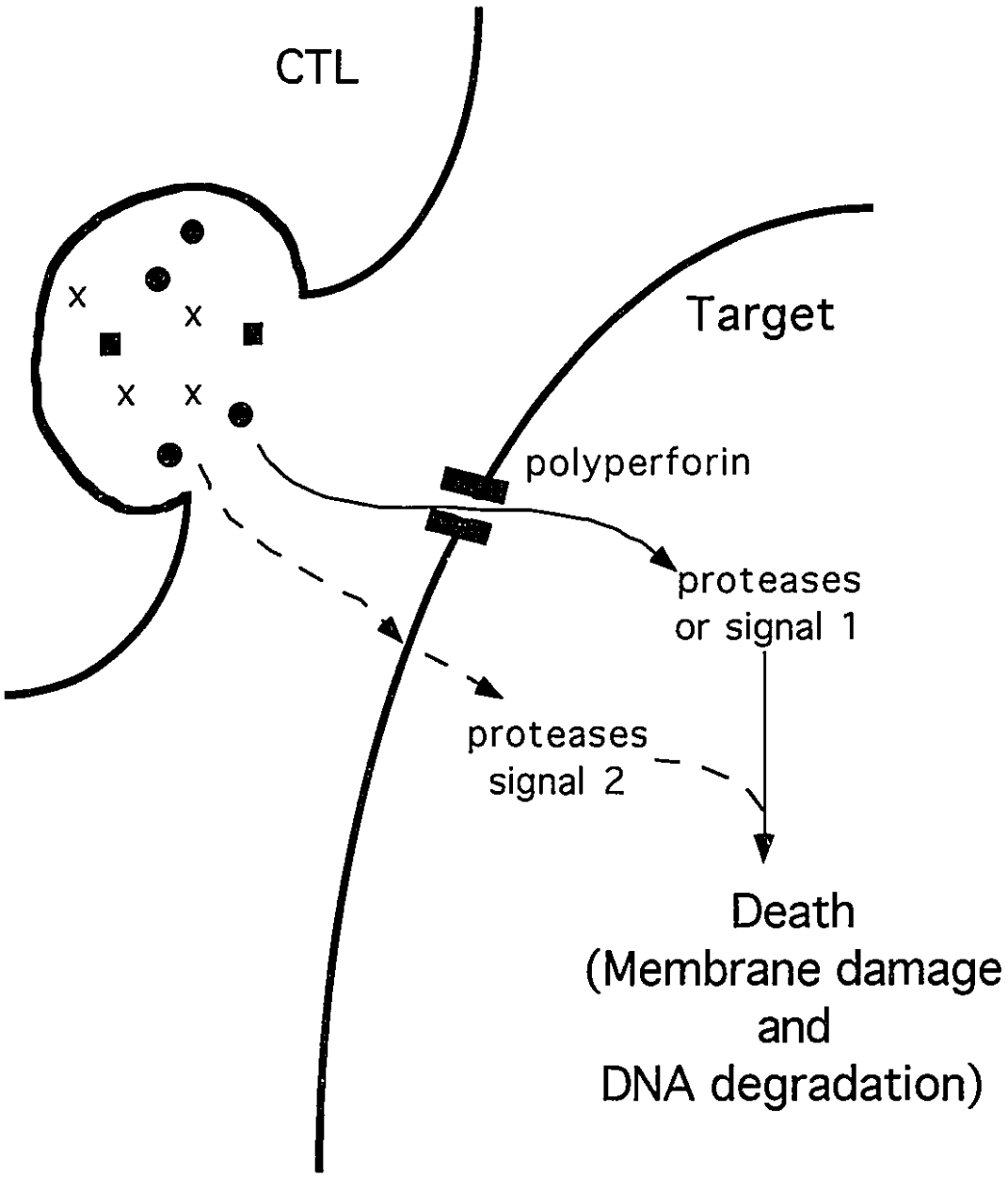
A second line of evidence that has suggested that the granzymes function within the target cell is the absolute requirement for perforin in granzyme-mediated DNA fragmentation. This is true both *in vitro*, in the perforin/fragmentin assay (Shi et al., 1992a and 1992b), and *in vivo*, in the RBL perforin/granzyme transfectants (Shiver et al., 1991 and 1992). An obvious interpretation of this finding is that perforin insertion into the target cell membrane allows entry of the granzymes (and possibly other granule components). This could occur via a number of mechanisms, including simple passage through large perforin channels, as depicted in the solid line in Figure 1-2. Although the poly-perforin channels seen with the electron microscope are large enough for macromolecules to pass through, it is unclear, as discussed earlier, if they form *in vivo*. In addition, the intracellular events such as DNA fragmentation often occur before the obvious signs of macromolecular leakage which would occur through these putative channels in the membrane if they existed. It seems more likely that a small amount of perforin inserted into the target membrane facilitates the entry of granzymes via another mechanism.

The hypothesis that has gained the most support from workers in the field involves pinocytosis of the perforin lesions as the target cell attempts to repair its damaged membrane. This would result in additional granule components being taken up into the cell. However, if this is the entry route for lytic mediators, it is not clear how they would escape from the resulting lysosome to gain free access to cytoplasmic or nuclear substrates.

The idea that perforin acts to somehow allow the proteases and other granule molecules into the cell is supported by the observation that granzyme A could initiate DNA breakdown in detergent-solubilized and perforin-treated cells but not in cells with intact membranes (Hayes et al., 1989). However, it has also been found that

FIGURE 1-2 Granule-Mediated Target Cell Lysis.

Upon binding of a CTL to a target, the cytoplasmic granules are vectorally released. Perforin damages the membrane of the target and may thus facilitate uptake of proteases (granzymes) - solid line alone. Alternately, perforin may contribute one signal (eg. ion channel changes) to the death pathway, but in order for apoptosis to occur, a second signal contributed by the granzymes is required - solid and dashed lines.


CTL
Target
polyperforin
proteases
or signal 1
proteases
signal 2
Death
(Membrane damage
and
DNA degradation)

microinjection of granzyme B directly into the cytoplasm of a cell does not lead to death as it does in the presence of perforin (R.C. Bleackley and A.H. Greenberg, unpublished). It has therefore not been formally excluded that the site of action of some of the granzymes is on the outside rather than the inside of the target cell.

The observation that purified granzymes are not able to induce lysis, or DNA fragmentation, when incubated with intact targets would initially suggest that they do not act to signal apoptosis by acting at the cell membrane, perhaps by cleaving certain receptors. However, this could still be the case, if a second signal, provided by perforin, is necessary for the signals generated by granzyme action to be manifested. This is depicted in Figure 1-2, where perforin insertion into the membrane constitutes signal 1 and granzymes (acting either on the cell membrane or intracellularly) provides signal 2. In combination, they result in death. It is known that membrane attack by perforin results in a rapid calcium spike in target cells (Allbriton et al., 1988). It would appear that this increase in intracellular calcium is important to cell death, since suboptimal doses of perforin and a calcium ionophore can synergize to cause ^{51}Cr release from cells (Kraut et al., 1990). It is conceivable that granzyme cleavage of cell surface receptors could generate signals, including additional calcium fluxes, that could synergize with the calcium spike initiated by perforin to effect cell death. In this regard, it is interesting that granzyme A has been implicated in cleavage and signalling of the thrombin receptor, an extracellular receptor that is known to increase intracellular calcium upon activation (Suidan et al., 1994). Clearly, identification of physiological granzyme substrates is essential in defining the exact role these proteases play in initiating target cell destruction.

The search for additional physiological granzyme B substrates is currently being undertaken by a number of labs. Using far-western protein overlay techniques and confocal microscopy, our lab and Trapani's have identified a 80 kDa nuclear protein that is bound by granzyme B (Pinkoski and Bleackley, submitted). The identity of this protein is not yet known, but its cellular localization makes it an interesting candidate for a

mediator of granzyme B-induced DNA breakdown. We are currently working to generate sequence information about this potential granzyme B substrate.

4. Bcl-2 and Target Cell Survival

The influence of the cell survival protein Bcl-2 on cytotoxic granule-mediated apoptosis of target cells is somewhat unclear. It has been reported that expression of this protein either blocks (Chiu et al., 1995), or has no effect (Vaux et al., 1992) on target cell death caused by CTL. Additionally, *bcl-2* expression has been reported to inhibit apoptosis caused by purified cytotoxic granules (Chiu et al., 1995). Since there are a number of other structurally and functionally related proteins in the Bcl-2 family (see Nunez and Clarke, 1994), and since their activity is significantly modulated by other proteins that interact with them, discrepancies in results in these experiments may be due to differences in the balance of the various proteins in different cell types. At least in some cases, then, it appears that intracellular levels of this protein family may affect target cell death caused by granule protein components.

5. The Forgotten Effector

Much attention has been focussed on the events that occur within the target cell after delivery of the lytic effector molecules by the CTL or NK cell. It must not be forgotten, however, that the cytotoxic cell must resist the lethal molecules that it has deposited, detach from the target, and go on to kill again.

The debate concerning the sensitivity of CTL/NK cells to their lytic mediators has raged for some time. In comparison to conventional hemopoietic targets, CTL are certainly less sensitive to the granule components (Kranz and Eisen, 1987). However, when compared to fibroblasts the difference is minimal. The difficulties that many investigators have experienced with transfecting DNA into CTL could be interpreted as an indication of some special membrane properties that allow the killer to survive (Frégeau and Bleackley, 1991). Some investigators have provided evidence for protective molecules (Mueller and Tschopp, 1995). Others have suggested that the vectoral

exocytosis mechanism could allow the effector to be protected by a "layer" of proteoglycan originating from the inside face of the granule but translocated to the outer membrane of the killer cell as a result of the exocytic process (see Martz, 1995 for review).

Detachment and the ability to kill again have received very little attention. There has been recent evidence that once the granule supply of lytic molecules is depleted, a non granule-mediated secretory pathway could be brought into play (Isaaz et al., 1995). Work on detachment is just beginning, as it is only recently that we have started to appreciate that this is a mechanism that may well be an actively signaled event (Mescher et al., 1991).

III. FAS-BASED CYTOTOXICITY

The granule exocytosis pathway of cellular cytotoxicity is dependent upon extracellular calcium at two levels. First, calcium is required for the process of degranulation, and chelation of this divalent cation blocks both target cell lysis and release of granule proteins such as serine esterases and proteoglycan. Secondly, the action of perforin, including the binding of and insertion into cell membranes, as well as its aggregation is strictly calcium-dependent. For some time, the validity and/or universality of the granule exocytosis pathway was challenged, based upon a number of observations that could not be adequately explained by the model. The lack of DNA fragmentation seen with purified perforin lysis of targets was initially a concern, although the contribution of the granzymes towards this process was unclear at the time. More importantly, some CTL appeared to be potent killer cells in the absence of detectable granules, perforin, or serine proteases (Allbritton et al., 1988, Berke and Rosen, 1988; Berke et al., 1993). Although this has been a highly contentious issue, and some reports of the lack of perforin may have suffered from employing assays with inadequate sensitivity, we have shown by the highly sensitive polymerase chain reaction that cytolytic lymphocytes can indeed be potent killers in the absence of perforin and

granzymes (Helgason et al., 1992). The most damaging evidence that seemed to question the universality of a granule exocytosis/perforin-dependent pathway of target cell destruction, however, were reports of significant levels of ^{51}Cr release in the absence of external calcium, and without the normal release of granule marker proteins (Ostergaard et al., 1987; Trenn et al., 1987). Recently, this phenomenon has been discovered to be due to the action of a transmembrane death-signaling protein termed the Fas antigen (See Nagata and Golstein, 1995 for review).

A. Identification, Cloning and Expression of Fas

The Fas antigen (also known as APO-1 and CD95) was originally identified as a cell surface protein that signalled cell death when exposed to anti-Fas antibody (Trauth et al., 1989; Yonehara et al., 1989). Molecular cloning of Fas/APO-1 revealed it to be a type I membrane glycoprotein with a single transmembrane domain dividing the molecule into extracellular and intracellular domains (Itoh et al., 1991; Watanabe-Fukunaga et al., 1992; Oehm et al., 1992). Sequence homology within the extracellular domain identified Fas as a member of the superfamily that includes the receptors for tumour necrosis factor and nerve growth factor. Fas is expressed on a wide variety of cell types, with the highest levels of expression being seen in the thymus, heart, liver, and ovary (Watanabe-Fukanaga et al., 1992). Fas is also present at low levels on mature T cells, and its expression is dramatically upregulated upon T cell activation (Trauth et al., 1989; Owen-Schaub et al., 1992; Miyawaki et al., 1992; Drappa et al., 1993). It is also present on a number of tumour cell lines, especially those of lymphoid origin. The ability of Fas to transmit a death signal into the cell can be impressively demonstrated by conferring apoptotic sensitivity to anti-Fas antibody upon transfection of a Fas-negative cell with a Fas-encoding plasmid (Itoh et al., 1991).

B. Involvement of Fas in Cellular Cytotoxicity

The demonstration of a role for Fas in cellular cytotoxicity came first from elegant experiments conducted by Golstein and colleagues. After extensive serial cloning of a

cytolytic hybridoma and selection for high cytotoxic activity, they showed an absolute requirement for a functional Fas antigen on the surface of targets killed by these cells (Rouvier et al., 1993). Furthermore, the potent calcium-independent killing activity of in vivo primed peritoneal exudate lymphocytes (PEL), cells which had long been used to challenge the universality of a perforin/granule exocytosis pathway of cytotoxicity, was also shown to be due to the presence of Fas on the target cells (Rouvier et al., 1993).

C. Identification, Cloning and Characteristics of the Fas Ligand

The Fas-dependent sensitivity of the target cells to this pathway of cytolysis suggested the presence of a Fas ligand on the surface of the cytolytic hybridomas. Using a soluble chimeric molecule consisting of the extracellular domain of Fas fused to a human IgG Fc region as a marker for Fas ligand surface expression, a variant of the cytolytic hybridoma was isolated that appeared to express a very high level of Fas ligand. Interestingly, the cytolytic activity of this variant was correspondingly greater than the parental cell line. This high Fas ligand expressing cell facilitated the isolation of purified Fas ligand and the molecular cloning of the cDNA (Suda et al., 1993).

Fas ligand is a type II membrane protein with a Mr of approximately 40,000. Sequence homology in its extracellular region places it in the tumour necrosis factor family (Suda et al., 1993). Fas ligand appears to be uniquely expressed on activated T cells, although the testis of mouse and rat, but not human, show expression as well. In T cells, Fas ligand is quickly induced upon TCR stimulation, addition of IL-2, or pharmacologically by the combination of the protein kinase C-activating tumour promoter PMA and the calcium ionophore ionomycin (Suda et al., 1993; Suda et al., 1995). Although $CD8^+$ splenocytes express Fas ligand more abundantly than $CD4^+$ splenocytes, all $CD4^+$ Th1 and Th0 clones tested, and some Th2 clones could be induced to express functional Fas ligand (Suda et al., 1995). Furthermore, most if not all cytotoxic activity of these T helper cells could be attributed to the action of the Fas ligand (Suda et al., 1995; Stalder et al., 1994).

D. General Features of The Fas Death Signal

1. Requirement for Fas Cross-linking

In solution, Fas ligand trimerizes as does TNF, and so probably acts to cluster Fas on the target cell (Tanaka et al., 1995). Indeed, it has been shown that efficient killing with anti-Fas antibodies requires cross-linking, (Dhein et al., 1992) which is a similar requirement for many receptor-induced signals. It is apparent that Fas engagement and cross-linking, either by anti-Fas antibodies or by Fas ligand transduces a signal into the target cell that activates a suicide program. This program is pre-existing, as it does not require macromolecular synthesis (Yonehara et al., 1989; Itoh et al., 1991).

2. Importance of the Cytoplasmic Death Domain

The exact nature of the death signal generated by Fas is unknown at present. Neither the molecular events immediately proximal to Fas cross-linking nor the mediators of the actual suicide program have been elucidated. However, considering how recently the involvement of Fas in apoptosis, and in particular in cellular cytotoxicity, has been appreciated, remarkable progress has been made towards the characterization of this death pathway. An important contribution towards this end has been the realization that Fas not only shares homology with the members of the TNF receptor family in its extracellular, ligand binding domain, but it also shares a region of homology with the cytotoxic type 1 TNF receptor (TNF-R1) in its cytoplasmic domain. This stretch of approximately 80 amino acids is required for the delivery of the suicide signals from both Fas and TNF-R1 and has been dubbed the "death domain" (Itoh and Nagata, 1993; Tartaglia et al., 1993). Interestingly, the non-cytotoxic members of the TNFR family, including the type II TNF receptor, do not possess a homologous region. However, the Drosophila protein reaper, which is extremely important for programmed cell death in this organism, also shares homology to the death domains (Golstein et al., 1995).

The similarities between TNF-R1 and Fas, such as their regions of extracellular and intracellular homology (including the death domains), the trimerization of their

ligands, and their ability to induce cell death, are offset by some surprising differences. Most noteable are the differences in the kinetics of killing. Target cell DNA fragmentation and other apoptotic events can be easily detected within 2 hours of Fas cross-linking, whereas TNF-RI dependent killing occurs much more slowly (Clement and Stamenkovic, 1994). Also, apoptosis signaled by Fas is not susceptible to some kinds of pharmacological inhibition that TNF-R1-induced apoptosis is (see Schulze-Osthoff, 1994). Domain-swapping experiments have indicated that differences between Fas and TNF-R1 signaled apoptosis are due to their cytoplasmic, and not ligand-binding, domains (Clement and Stamenkovic, 1994).

E. Potential Transducers and Modulators of the Proximal Fas Death Signal

1. Death Domain-Associated Proteins

In an attempt to identify proteins that interact with the cytoplasmic region of Fas and which may therefore be important in Fas signal transduction, a number of groups have utilized the yeast two-hybrid system. Using this approach, three different Fas-associating proteins have recently been identified (Boldin et al., 1995a; Chinnaiyan et al., 1995; Stanger et al., 1995; Sato et al., 1995). Two of these proteins, FADD/MORT1 (Chinnaiyan et al., 1995) and RIP (Stanger et al., 1995), contain regions with homology to the death domain. The functions of both these proteins are unknown, although RIP possesses an amino-terminal protein kinase domain. It is not known if this protein does indeed possess protein kinase activity, as it lacks the invariant DFG motif in the activation loop of all other kinases. The physiological importance of these two proteins interacting with Fas in the 2-hybrid system is also not known. Although overexpression of FADD/MORT1 and RIP results in apoptosis, interaction with Fas in a physiologically relevant context has not been demonstrated. Since it has been shown that death domains dimerize (Boldin et al., 1995b), and interaction of FADD/MORT1 and RIP with Fas most likely occurs via their death domains, it is possible that these proteins were simply "fished out" fortuitously due to their possession of this domain. Under normal circumstances it is

possible that they never have the opportunity to interact with Fas. Demonstration of this interaction in Fas-bearing cells is clearly important, and future work on these molecules, under more physiological conditions, will likely be very illuminating. They remain, however, important potential mediators of the Fas death signal.

2. Tyrosine Phosphorylation

The third protein that was isolated and identified by the two-hybrid system to interact with Fas is a protein tyrosine phosphatase dubbed FAP-1, (Sato et al., 1995) which was previously identified in basophils and named PTP-BAS (Maekawa et al., 1994). Although direct physiological relevance of this interaction is also lacking, the association of FAP-1 with Fas is intriguing for a number of reasons. The fact that a tyrosine phosphatase interacts with Fas is very interesting, because tyrosine phosphorylation of a number of cellular proteins has been demonstrated in Jurkat cells upon exposure to a cross-linking anti-Fas antibody, and tyrosine kinase inhibitors inhibit anti-Fas killing of these same cells (Eischen et al., 1994). It is also very interesting that FAP-1 has been shown to interact with the COOH terminus of Fas, because this region has been shown to be a negative regulatory region of Fas (Itoh et al., 1993). Therefore, if tyrosine phosphorylation is necessary for the Fas death signal, a tyrosine phosphatase tethered to the negative regulatory region of Fas could conceivably be involved in down-regulating this signal. In this respect, it is noteworthy that gene transfer of FAP-1-encoding DNA into a T cell line reduced Fas-induced apoptosis (Sato et al., 1995).

We have also obtained evidence recently that tyrosine phosphorylation may be an important event in Fas signal transduction. We have observed that alloantigen-stimulated T lymphocytes from FynT-deficient mice have elevated lifespans in culture and reduced levels of apoptosis (Atkinson et al., submitted). Therefore, this tyrosine kinase may play an important role in relaying or modulating the death signal initiated by Fas ligation. Interestingly, we have not obtained consistent results regarding tyrosine kinase inhibitor inhibition of Fas-mediated killing. In fact, in some cases we observe a dose-dependent

enhancement of Fas-induced DNA fragmentation in target cells treated with various tyrosine kinase inhibitors (Atkinson and Bleackley, unpublished observations). This may indicate a complex signalling cascade with tyrosine kinases having a role at various places along the pathway.

F. Potential Distal Mediators of Fas-Induced Apoptosis

1. Ceramide

Engagement of Fas has been shown to result in the activation of an acidic sphingomyelinase and the generation of ceramide (Cifone et al., 1993). TNF-RI has also been shown to be functionally coupled with a sphingomyelinase (Kim et al., 1991; Dressler et al., 1992; Schutze et al., 1992). Ceramide production has been implicated in a number of different apoptotic systems, and cell-permeable synthetic ceramides can induce apoptosis and DNA fragmentation in a number of different cell types (Obeid et al., 1993). Therefore, ceramide production may be an important mediator of Fas-induced death, at least in some cell types.

2. ICE-Family Proteases

As is the case in granule-mediated cytotoxicity, recent evidence has focused interest on a potential role for proteinases, including ICE or ICE-like proteinases in Fas-induced cell death. A number of groups have shown that the ICE-inhibiting viral serpin gene product crmA, as well as synthetic ICE inhibitors also suppress Fas-mediated apoptosis (Enari et al., 1995; Los et al., 1995; Tewari and Dixit, 1995). This feature is also shared with TNF-signalled apoptosis (Enari et al., 1995; Tewari and Dixit, 1995). Furthermore, ICE-like proteolytic activity was shown to be induced after Fas ligation, using cleavage of a fluorogenic ICE substrate as a read-out (Los et al., 1995). Microinjection of ICE or crmA cDNAs directly into the nuclei of Fas-bearing cells enhanced or supressed the apoptotic activity of an anti-Fas antibody, respectively (Los et al., 1995). Consistent with this observation, gene transfer into Fas-sensitive cells of ICE cDNA increased, while crmA or anti-sense ICE cDNAs decreased Fas-induced apoptosis

(Enarl et al., 1995; Los et al., 1995; Tewari and Dixit, 1995). These observations are all in accord with signals generated as a result of Fas ligation converging on an ICE-dependent cell death pathway that is common to a wide variety of apoptotic systems, including programmed cell death pathways in evolutionarily diverse organisms, and the induced cell death caused by cytolytic lymphocyte granule components.

G. Control of Fas-Based Cytotoxicity

1. Simple Expression vs. Functional Expression of Fas

Fas transduces a death signal in the absence of macromolecular synthesis, indicating that the death program it activates is pre-existing in the cell. Furthermore, transfection of non-Fas bearing cells with a Fas-encoding cDNA converts the cell from being insensitive to Fas-ligating molecules to being sensitive. This indicates that even cells which do not normally express Fas possess the latent suicide program that Fas accesses to cause apoptosis. However, there are numerous indications that simple expression of surface Fas is not in and of itself sufficient to render a cell entirely Fas-sensitive. For instance, most resting mature T cells express low levels of Fas on their surface, although they are not Fas-sensitive (Owen-Schaub et al., 1992). Indeed, sometimes ligation of Fas can cooperate with TCR-derived signals to stimulate T cells as opposed to killing them (Alderson et al., 1993). Even after upregulation of Fas expression after T cell activation, there is a lag period before the cells become sensitive to Fas ligation (Owen-Schaub et al., 1992). It is possible that Fas is at times functionally uncoupled from its normal signal transduction pathway, or that signalling molecules required for Fas signal transmission are upregulated with slower kinetics than Fas. The idea of signal transduction molecules being a limiting factor in Fas activity is supported by the observation that isolation of CD40/Fas chimera-transfected clones bearing low or intermediate levels of the chimeric protein show a higher level of Fas-sensitivity than clones expressing high levels (Clement et al., 1994). This observation could be explained

if Fas-associating signalling molecules are being functionally squelched by an overabundance of Fas.

2. Effect of Bcl-2

As is the case with granule-mediated cytotoxicity, the effect of bcl-2 on Fas-based cytotoxicity is somewhat confusing. While at least one group has reported that expression of bcl-2 does not block Fas-based apoptosis (Chiu et al., 1995), others have demonstrated partial inhibition of Fas-induced death by bcl-2 (Itoh et al, 1993). Furthermore, a much greater degree of inhibition of Fas-based apoptosis was achieved when target cells overexpressed both bcl-2 and the bcl-2 binding protein BAG-1 (Takayama et al., 1995). This points more strongly to a functional role in this pathway for bcl-2 inhibition and highlights the difficulty in elucidating the effect of the bcl-2 family members when other members may also play a prominent role.

Interestingly, many cells that constitutively express surface Fas are sensitive to Fas-crosslinking by anti-Fas alone, whereas some cells, including some Fas-negative cells that have been transfected with a Fas expression vector, require the presence of the RNA polymerase inhibitor actinomycin D to exhibit Fas sensitivity (Enarl et al., 1995; Los et al., 1995). These observations suggest that in some cells, the suicide program is actively inhibited. Although the nature of this inhibition is unknown, the observed suppression of Fas-induced apoptosis by bcl-2 and bcl-2 binding proteins make these molecules possible contenders as mediators of this inhibition. We have also observed some differences in certain pharmacological effects on targets killed via anti-Fas versus a cytotoxic lymphocyte Fas-dependent pathway (Atkinson and Bleackley, unpublished observations). It seems possible, therefore, that Fas-ligand bearing killer cells may also contribute other factors that influence Fas-dependent cell death.

3. Control of Fas Ligand Expression

Cytotoxic lymphocytes killing through a Fas-based pathway may simply require surface expression of the Fas ligand to carry out this effect. The fact that transfection of a

fibroblast cell line with Fas ligand cDNA converts these cells into Fas-dependent killers dramatically illustrates this point (Suda et al., 1993). In addition, upregulation of the Fas ligand on cytotoxic lymphocytes upon treatment with PMA and ionomycin allows these cells to kill a variety of Fas-bearing target cells in a MHC unrestricted manner (Rouvier et al., 1993; Garner et al., 1994; Walsh et al., 1994). If it is the case that simple expression of Fas ligand is all that is necessary for a cell to be able to kill a Fas-positive cell, the question of specificity in Fas-dependent cellular cytotoxicity becomes an issue, since Fas is expressed on a wide variety of normal cell types. While in some cases, Fas may be functionally inactive, this is not the case for a number of important cell types, as injection of anti-Fas into mice is lethal, with massive liver injury occurring (Ogasawara et al., 1993). Clearly, an activated, Fas ligand bearing lymphocyte must not be allowed to destroy each and every Fas-positive cell it comes in contact with by random chance. At present, it is unclear how specificity is maintained, although control of Fas ligand expression on the killer cell may be important. Although some cytotoxic lymphocytes can kill indiscriminately via a Fas-dependent pathway when they have been stimulated by PMA and ionomycin, these same cells kill Fas-positive targets in an antigen/MHC restricted manner when stimulated more physiologically by exposure to specific alloantigen (Garner et al., 1994). It is therefore very likely that normal stimulation of these cells via signals generated physiologically by the TCR complex leads to expression of Fas ligand that is not "functional" autonomously. This could be because of quantitative or qualitative (eg., modification or location) of the ligand. Hyper-induction of Fas ligand may allow these cells to kill promiscuously due simply to a concentration effect, whereas physiological levels may require the cooperation of the TCR, coreceptors and other adhesion or accessory molecules for efficient Fas-Fas ligand interactions to occur.

It is significant that an alloreactive, perforin-deficient CTL line killed ConA blasts of the specific MHC haplotype that they were raised against, but spared bystander ConA blasts expressing an irrelevant MHC haplotype in a Fas-dependent manner (Kojima et al.,

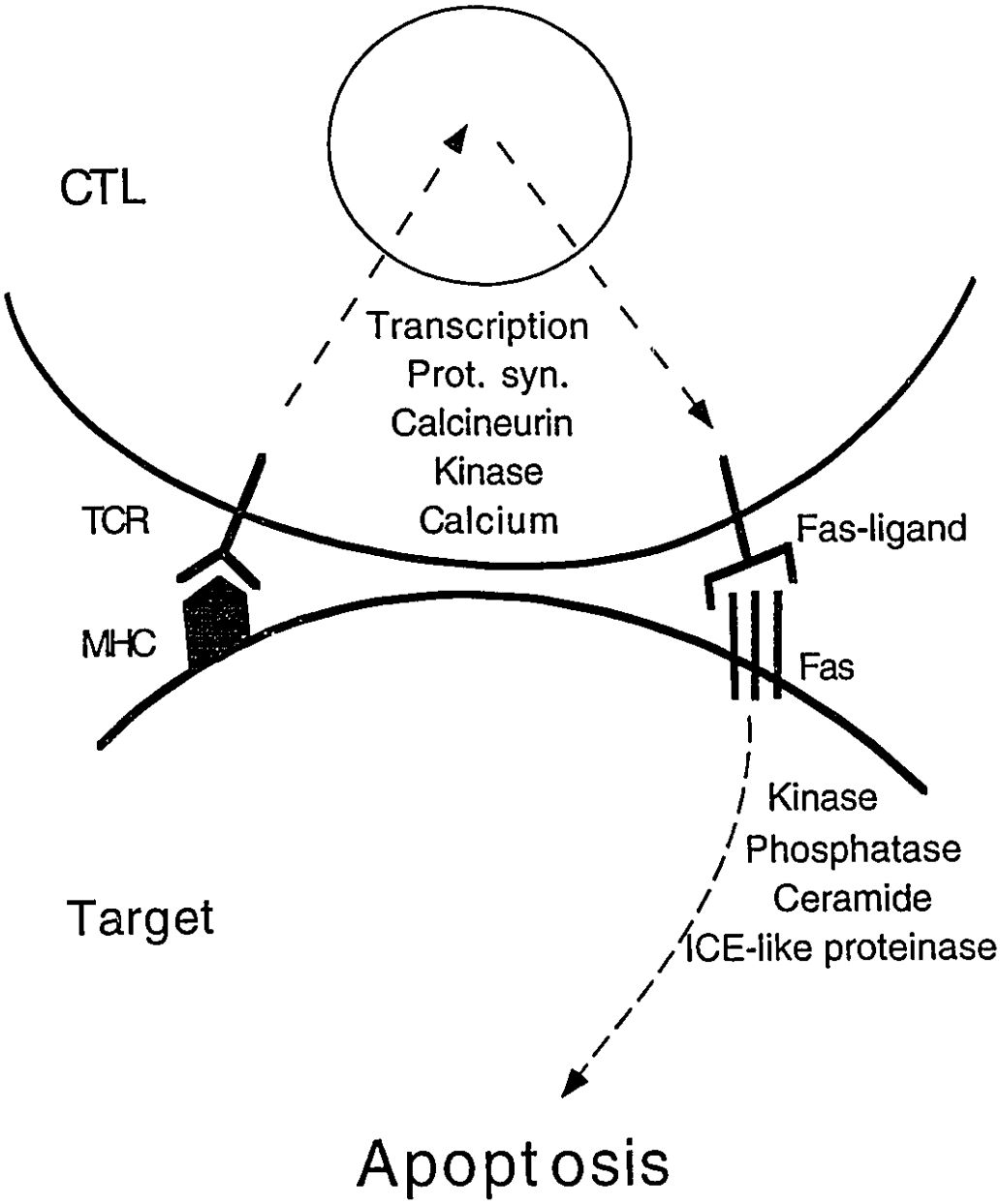
1994). In other experiments with this CTL line, soluble Fas did not block Fas-dependent killing, although it did block Fas-dependent killing of a different killer cell line. Also, Fas-mediated cytolysis was observed to be calcium dependent. While these observations may appear somewhat confusing in light of our overall picture of Fas-based cytotoxicity, they may be explainable by a mechanism involving activation-induced control of Fas ligand expression. The perforin-deficient CTL were not pre-stimulated by antigen or PMA/ionomycin immediately before the cytotoxicity assays. Therefore, their levels of Fas ligand may have been very low or nonexistent, and antigenic stimulation by binding to target cells likely upregulated Fas ligand expression and led to cytolysis. As the induction of Fas ligand appears to be calcium dependent (Garner et al., 1994; Vignaux et al., 1995), it could not be induced by antigen in calcium-free conditions, leading to an apparent calcium-dependency of Fas-mediated killing. Also, if Fas ligand was only induced upon CTL-target cell conjugation, soluble Fas likely could not gain access to the nascently-induced Fas ligands in the area of cell-cell contact. If this is indeed the explanation for these results, it also suggests a mechanism of specificity of Fas-mediated killing. Functional expression of Fas ligand may require recent, or even perhaps continual, signalling through the T cell receptor, as depicted in Figure 1-3. In this way, only antigenically reactive, Fas-bearing cells would be lysed. In this regard, it is interesting that we have observed that after stimulation with PMA/ionomycin, a Fas-dependent cytolytic hybridoma quickly loses its potent cytotoxicity towards Fas-positive targets within a few hours (Atkinson and Bleackley, unpublished). Therefore, rapid up- and down-regulation of Fas ligand on the surface of CTLs likely provides a considerable degree of specificity to normal Fas-dependent cellular cytotoxicity.

III. THE ROLES OF THE PERFORIN- AND FAS-BASED KILLING MECHANISMS

The extent of the involvement of the perforin-dependent and Fas-dependent pathways in cell mediated cytotoxicity, as well as their likely major biological functions

FIGURE 1-3 Activation and Effector Stages of Fas-Mediated Death.

Target cells are recognized by CTL through the antigen receptor (TCR). As a result of this interaction, Fas-ligand is expressed on the surface of the cell in a process that is sensitive to inhibitors of transcription, translation, tyrosine kinases, and to cyclosporin and EGTA. The ligand interacts with Fas antigen on the target, resulting in the delivery of a death signal. Although this does not require target cell protein or RNA synthesis and is insensitive to cyclosporin, it likely involves phosphoproteins, ceramide, and ICE-like enzymes.


CTL
Transcription
Prot. syn.
Calcineurin
Kinase
Calcium
TCR
Fas-ligand
MHC
Fas
Kinase
Phosphatase
Ceramide
ICE-like proteinase
Target

Apoptosis

has recently been better defined. This has become possible with the availability of mice that are deficient in perforin, Fas, and Fas ligand. A number of groups have generated perforin-negative mice (Kagi et al., 1994; Lowin et al., 1994; Walsh et al., 1994) or CTL (Kojima et al., 1994) by gene targeting, while the naturally occurring *lpr* and *gld* mutations in mice have been shown to be the result of Fas and Fas ligand loss of function mutations, respectively (Watanabe-Fukunaga et al., 1992; Adachi et al., 1992; Drappa et al., 1993; Wu et al., 1994; Takahashi et al., 1994; Hanabuchi et al., 1994). These new tools have enabled the clarification of the roles of the calcium-dependent and -independent pathways of cell mediated cytotoxicity.

A. The Role of The Perforin-Dependent Pathway

Perforin deficient mice develop normally, as do their lymphocyte subsets (Kagi et al, 1994a; Walsh et al, 1994b). However, despite apparently normal activation and proliferation responses to viral infection, *in vitro* virus-specific CTL cytotoxic activity is severely impaired (Kagi et al., 1994a; Walsh et al., 1994b). Furthermore, perforin knockout mice cannot clear viral infections and succumb to virus loads that are readily eliminated by their normal counterparts (Kagi et al, 1994a; Walsh et al, 1994b). Likewise, perforin-minus mice fail to reject syngeneic fibrosarcoma cells (Kagi et al, 1994a). In addition, perforin-minus lymphocytes stimulated in an *in vitro* mixed lymphocyte reaction are also severely impaired in their ability to lyse appropriate allogeneic target cells *in vitro* (Kagi et al, 1994a, Walsh et al, 1994b; Lowin et al., 1994a). NK activity is also abolished in these mice (Kagi et al., 1994a; Lowin et al., 1994a). The reduced lytic activity of perforin-deficient cytolytic lymphocytes is not due to a failure to become activated or proliferate or exocytose their granules, as all these responses appear normal (Kagi et al., 1994a; Walsh et al., 1994b, Lowin et al., 1994a).

The *in vitro* lytic activity of perforin-deficient CTL was found to be entirely absent when tested against some target cells. However, when reacted with other targets, mainly of hematopoietic origin, a significant, albeit greatly reduced, level of cytolytic

ability remained (10-30%). By utilizing targets that either expressed or did not express a functional Fas antigen, the entire remaining residual lytic activity could be attributed to the Fas-based pathway, at least in the short-term lytic assays employed (Walsh et al., 1994b ; Kojima et al., 1994; Lowin et al., 1994b; Kagi et al., 1994b). Therefore, all of the lytic activity of CTL as assessed in these assays can be accounted for by either a granule-exocytosis, perforin-dependent pathway or a Fas-dependent pathway.

Protection against the intracellular bacterium *Listeria monocytogenes* was also recently shown to be dependent upon perforin expression (Kagi et al., 1994c). It is known that primary infection by this organism is mainly controlled by granulocytes and activated macrophages, but secondary infection is mainly controlled by $CD8^+$ T cells. The exact mechanism $CD8^+$ T cells utilized to confer resistance was unclear, as lymphokines are known to be involved in anti-*Listeria* responses. Using perforin-deficient mice, it was shown that control of primary infection by *Listeria* remained intact, but that resistance to secondary infection was greatly reduced. Therefore, the immunity imparted by $CD8^+$ T cells against secondary infections by this organism is mainly mediated by a perforin-dependent pathway.

The perforin knockout mice have been highly informative in delineating the role of the granule exocytosis pathway in *in vitro* and *in vivo* cellular cytotoxicity. To date, antigen-specific $CD8^+$ T cell-mediated elimination of all intracellular infectious agents tested has been shown to require the presence of perforin. Interestingly, this is also true for viral infections of the liver, which express abundant levels of functional Fas antigen. Therefore, even elimination of virus from cells that possess an intact Fas-based lytic pathway requires a functional perforin/granule exocytosis mechanism. The Fas death pathway has not been demonstrated to be relevant for elimination of any pathogenic organism as yet. It therefore appears that all *in vivo* cytotoxic activity towards infected cells that is mediated by cytotoxic lymphocytes is achieved via a granule/perforin

dependent pathway. In addition, rejection of tumours, allografts, and syngeneic transplants by NK cells or $CD8^+$ CTL is strictly dependent upon perforin.

B. The Role of the Fas Pathway

What then is the role of the Fas pathway of cytotoxicity? Evaluation of mice with natural mutations affecting either Fas (*lpr*) or Fas ligand (*gld*) would suggest that the Fas death pathway is of vital importance in downregulating immune responses and eliminating activated T cells. Mice harbouring the *lpr* (for lymphoproliferation) or *gld* (for generalized lymphoproliferative disease) mutations exhibit a similar phenotype, characterized by lymphadenopathy and splenomegaly (see Cohen and Eisenberg, 1991 for review). Large numbers of double-negative T cells accumulate in these animals, and are believed to be derived from mature, single positive T cells (Laouar and Ezine, 1994). Both positive and negative thymic selection are apparently normal in these mice, indicating that the Fas apoptosis pathway is not involved in these events (Herron et al., 1993; Sidman et al., 1992; Singer and Abbas, 1994). However, peripheral clonal deletion and elimination of mature, activated T cells is greatly impaired, suggesting a requirement for Fas and its ligand (Singer and Abbas, 1994; Russell et al., 1993; Russel and Wang, 1993). Normally, upon exposure to specific antigen, a T cell will proliferate and generate multiple clones of itself in order to deal with a given infection. Most of these cells must eventually be destroyed in order to avoid increasing numbers of lymphocytes from accumulating with every subsequent infection. In *lpr* and *gld* mice, this does not occur efficiently, and lymphoproliferative disease results. Interestingly, Fas gene mutations were recently discovered to be the cause of a human autoimmune lymphoproliferative syndrome in five unrelated children (Fisher et al., 1995). As in *lpr* and *gld* mice, there is an accumulation of double-negative T cells in people with this syndrome.

As discussed earlier, mature T cells constitutively express low levels of Fas but are not sensitive to Fas ligation (Owen-Schaub et al.). Upon activation, Fas is upregulated and the cell also becomes sensitive to the consequences of Fas cross linking. Also, Fas

ligand expression is restricted to activated T cells (Suda et al., 1993). Therefore, both the ability to kill and be killed by the Fas pathway are attained by T cells upon activation by antigen. Collisions between antigen-specific T lymphocytes within an area of high density of these cells during an immune response is therefore likely to result in Fas/Fas ligand interactions, elimination of large numbers of activated lymphocytes, and down-regulation of the response.

The phenomenon of activation-induced death of T cell hybridomas (Brunner et al., 1995; Ju et al., 1995), preactivated T cells (Dhein et al., 1995), and T cell lines (Alderson et al., 1995; Dhein et al., 1995) has also been shown to be due to the Fas pathway. It has been known for some time that stimulation of these cells with antibodies against the T cell receptor complex, or with antigens or mitogens often resulted in their death via apoptosis (see Green and Scott, 1994 for review). Recently, it was reported that upon activation these cells upregulate Fas and Fas ligand but blocking the ability of these molecules to interact prevents cell death from occurring. Surprisingly, this was also shown in single-cell cultures, indicating that an activated T cell may have the ability to kill itself via this pathway (Brunner et al., 1995; Dhein et al., 1995).

IV. RELATED ISSUES

A. The Significance of DNA Fragmentation in Target Cell Death

The question of whether the nuclear events, and in particular the DNA fragmentation, that occur in dying target cells are fundamentally central to the death process or are simply secondary and dispensable phenomena is still a matter of debate. Based upon the observations that Fas- (Schulze-Osthoff et al., 1994;, Nakajima et al., 1995) and perforin/granzyme (Nakajima et al., 1995) -mediated lysis and induction of apoptotic morphological features of enucleated targets can be achieved with comparable efficiency to targets possessing nuclei, it has been argued that DNA fragmentation in granule- and Fas-based cytotoxicity, and perhaps in other instances of apoptotic death, is merely an epiphenomenon. Other work has also suggested that efficient lysis can occur in

the absence of DNA fragmentation (Ucker et al., 1992). The basic question of whether DNA fragmentation actually contributes to a loss of target cell viability has not really been addressed, however. Since enucleated cells are not themselves viable, even though they can undergo the cytoplasmic events that occur during apoptosis, in a strict sense they cannot be reliably used to determine if genome digestion is necessary for target cell death.

Inhibition of DNA breakdown with the inhibitor ATA results in inhibition of target cell lysis, at least as measured by release of ^{51}Cr (Helgason et al., 1993). In addition, it is interesting that the fragmentins, isolated from lytic granules on the very basis of their ability to mediate DNA fragmentation, have been found to be important in target cell destruction. When these proteins are absent or inhibited, not only is DNA fragmentation impaired, but ^{51}Cr release is also reduced. Although this does not necessarily indicate a cause and effect relationship, it does suggest that molecules that affect the ability of DNA fragmentation to occur can contribute substantially towards the release of cytoplasmic components from a cell. Also, lysis of Fas-bearing cytoplasts by Fas ligand-bearing effectors was shown to require three times more effector cells to achieve the same degree of lysis seen with intact targets (Nakajima et al., 1995). This may be an indication that the nuclear events do indeed contribute to cell lysis. Clearly, then, while cytoplasmic and membrane events in this type of cell death can be separable from nuclear events, they appear to occur in parallel. While the cytoplasmic events may not necessarily always result in loss of cell viability, there can be no recovery from genome digestion. In addition, it may serve a cytolytic lymphocyte well to have the capacity to induce two parallel pathways, one cytoplasmic, and one nuclear, when lysing virus-infected cells. Viral inhibition of one of the pathways may be able to be compensated for by the other pathway. Furthermore, the genome digestion that occurs during apoptosis has the additional benefit of destroying integrated viral DNA that may itself be infectious.

B. Phagocytosis

Recent research in the area of cell mediated cytolytic mechanisms has focused on how the CTL or NK induce destruction of the target. It is striking, however, that *in vivo*, even at sites of intense cytolytic activity, very little evidence of destruction in the form of cellular debris is observed. This is because the cells surrounding an apoptotic cell rapidly recognize and efficiently phagocytose the dying cell. This is an extremely important process as it avoids the release of noxious and inflammatory substances into the surrounding tissue. The ingestion process is accomplished not only by professional phagocytic cells (e.g. macrophages) but also by amateurs, such as fibroblasts and epithelial cells.

Obviously the expression of phagocytic recognition signals on the surface of the dying cell is a key step in this process *in vivo*, yet very little is known about the mechanisms. Most studies have been performed on neutrophils because they have such a limited life span, and thus represent a convenient experimental system to study. A number of mechanisms have been distinguished including the use of the vitronectin receptor and CD36 by the phagocytic cell (Ren et al., 1995; Savill et al., 1990) and the "inappropriate" exposure and subsequent recognition on the cell surface of phosphatidylserine (Fadok, 1992). Future studies on the recognition structures and the regulation of their expression will represent an important area of research. We may be in for some surprises as recent results demonstrate that Bcl-2 inhibits death but not phagocytosis, thus the triggers may be independent (Lagrasse & Weissman, 1994). It will be most interesting to see if the same molecules/mechanisms operate in the phagocytosis of cells under attack by immune effectors.

C. Concluding Remarks on Mechanisms of Lysis

There has been an enormous amount of work done on various aspects of cell mediated cytotoxicity over the last few years. In this review we have tried to focus mainly on the mechanisms of lysis and have dealt only briefly or not at all with many other

important aspects. The reader is referred to two excellent books by Sitkovsky and Henkart (1993) and Griffiths and Tschopp (1995) in which comprehensive review on many other related issues are presented.

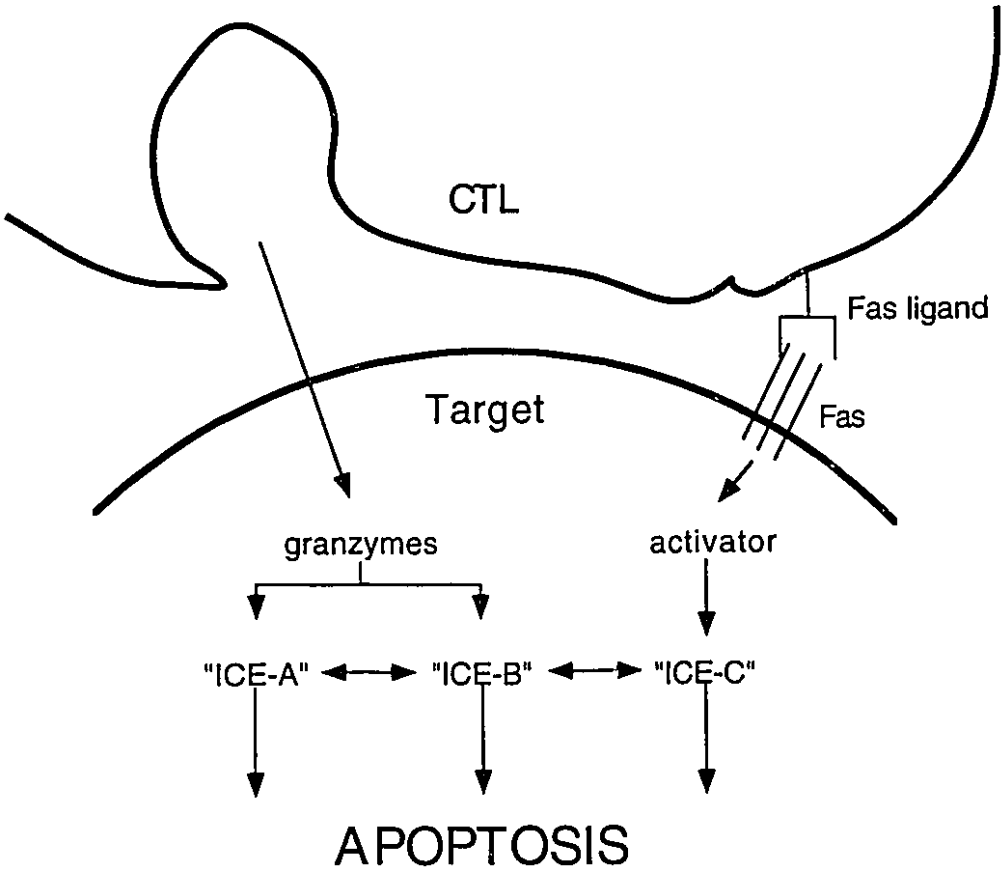
We have described two mechanisms that cytotoxic lymphocytes employ to destroy other cells. Even though they appear quite different, there are some quite remarkable similarities in the way target cell destruction is achieved. ICE-like enzymes have been implicated in both, but the relationships between A,B, and C (Figure 1-4) remain to be elucidated, although our evidence would clearly identify one of these as CPP32. Evidence from granzyme B knockout mice and recently from pox-virus infected cells in which protease inhibitors are expressed (Macen, et al., submitted) would indicate that at least two pathways to apoptosis operate in granzyme-mediated killing. Most likely this relates to the presence of multiple granzymes and possible multiple ICEs. It could also explain why disparate results are found with inhibitors such as Bcl-2, as sensitivity may be a reflection of which pathway is dominant in that cell type.

Both the granule exocytosis perforin/granzyme pathway and the Fas-dependent pathway of cell mediated cytotoxicity result in target cells dying with many of the features of classical programmed cell death. These include morphological changes and cell membrane blebbing, as well as nuclear condensation and DNA fragmentation. Both pathways are unique from many other forms of programmed cell death in that they occur independently from macromolecular synthesis. However, the involvement of ICE-like proteinases, the ability of Bcl-2 or related proteins to at least partially inhibit cell death, as well as the shared physical characteristics of the death process suggest that a common, evolutionarily conserved apoptosis pathway may be followed in target cells lysed by cytotoxic lymphocytes or dying via programmed cell death.

The last few years have witnessed a great leap forward in our understanding of the mechanisms by which cytotoxic lymphocytes kill their target cells. The presence of two separate pathways of cytotoxicity, often existing within the same cell type had previously

FIGURE 1-4 Granule- and Fas-Mediated Killing Occur via Activation of ICE-Like Proteins.

In the granule-mediated exocytosis mechanism, granzymes gain entry into target cells and may activate the apoptotic pathway by cleavage and activation of ICE-like enzymes. Multiple granzymes likely result in activation of multiple pathways. After Fas antigen trimerization, a signal is generated that also results in activation of an ICE-like enzyme. The relationship between ICE-A,-B, and -C is presently unclear, although the identity of at least one in the granzyme pathway is CPP32. During this process the enzymes may act on one another in a cascade mechanism that results in amplification of the original signal.


CTL
Fas ligand
Target
Fas
granzymes
activator
"ICE-A"
"ICE-B"
"ICE-C"
APOPTOSIS

caused a great deal of confusion in this field of research and the exact roles these two pathways played in immunesurveillance was unclear. Now, with the molecular mechanisms of both pathways becoming increasingly defined, and the availability of mice harbouring specific mutations that are central to each pathway, the functional significance of the two mechanisms has been illuminated. CTL and NK cells kill virus-infected and tumour cells via a granule-exocytosis mechanism that is entirely dependent upon the presence of functional perforin. After antigenic activation and clonal expansion, the lymphocytes that are no longer needed once the antigen has been cleared, are eliminated via Fas-Fas ligand interactions. Although much progress has been made at elucidating the various steps involved in the two death signals, there are still many questions concerning the exact molecular requirements for target cell death. The next few years will surely prove to be exciting and enlightening in this field.

V. ACTIVATION OF T CELLS

A. T Cell Signal Transduction and Tyrosine Kinases

1. Antigen Recognition

Mature T lymphocytes express a clonotypic T cell receptor (TCR) associated with a complex of transmembrane signalling proteins collectively termed CD3, and either the CD4 or CD8 coreceptor. All T cells see antigen presented on the surface of cells in the context of self MHC. $CD4^+$ cells see antigen in the context of MHC Class II molecules, whereas $CD8^+$ cells see antigen in the context of MHC Class I. Upon proper presentation of its specific antigen, a T cell generates signals that result in proliferation and the acquisition of certain functional attributes . $CD4^+$ cells become activated helper, or Th cells, and produce a wide-variety of lymphokines necessary for the progression of the immune response. $CD8^+$ cells become activated cytotoxic cells, or CTL, and gain the ability to kill target cells expressing the appropriate antigen. Before stimulation, neither class of T cell is functionally capable of carrying out any of these roles, and acquisition of these abilities requires gene transcription and protein synthesis.

2. T Cell Receptor Structure

The TCR is composed of a clonotypic disulfide-linked α and β chain responsible for antigen/self MHC recogition, noncovalently associated with a cluster of transmembrane proteins collectively called CD3. The α and β chains themselves have very small cytoplasmic domains and are likely not able to transduce signals into the cell interior on their own. Antigen binding by the TCR is relayed into the cell by the chains of the CD3 complex, which have large cytoplasmic domains. The entire CD3 complex consists of an α γ, δ, and ε chain, together with a ζ homodimer or a ζ/η heterodimer. CD3 is expressed on the surface of cells as a complete complex, and all the chains must be present for efficient expression. The importance of the ζ chain in both surface expression of CD3 and signalling from the TCR was demonstrated by using a ζ-deficient T cell line (Schussman et al., 1988). Although mRNA for the remaining chains of CD3 was present, there was greatly reduced CD3 complex surface expression and signalling via the TCR was eliminated. Transfection of this cell line with a plasmid encoding an intact ζ chain rescued both CD3 surface expression and T cell signalling. When plasmids encoding mutant ζ chains having truncations in cytoplasmic regions were used, CD3 surface expression was still recovered, but TCR signalling and IL-2 production were greatly reduced, indicating an important role for the ζ cytoplasmic region in signal transmission from TCR/CD3 (Frank et al., 1990). This was confirmed by transfecting T cell lines with plasmids encoding chimeric receptors consisting of the transmembrane and cytoplasmic domains of ζ fused to the extracellular domains of a variety of different cell surface proteins, including CD4, CD8, and IL-2Rα (Irving and Weiss, 1991; Romeo and Seed, 1991; Letourneur and Klausner, 1991). Ligation of these chimeric receptors resulted in efficient second messenger generation and IL-2 production. In fact, the cellular response to activation via the chimeric receptors was indistinguishable from that achieved by activation of an intact TCR/CD3, indicating an important and substantive role for ζ in delivering signals from the TCR.

ζ is not the only CD3 chain to have signalling capabilities. Other ζ-deficient T cells lines have been found to have some signalling capabilities and could produce IL-2 in response to TCR stimulation (Wegener et al., 1992). This suggests the existence of at least one additional ζ-independent signalling mechanism. Using a similar chimeric receptor approach, it was found that the cytoplasmic domain of the ε chain also has the ability to signal IL-2 production and generate earlier biochemical signals (Letourneur and Klausner, 1992). There is some indication that although the signals generated via ζ and ε may be overlapping, they may not be identical. For instance the pattern of cellular proteins tyrosine phosphorylated in response to ligation of ζ and ε chimeras are overlapping, but also partially distinct (Letourneur and Klausner, 1992). Also, ζ and ε appear to differ in their calcium mobilization activities (Cambier, 1995) It is currently unknown if the γ and δ chains of CD3 are also capable of generating and transmitting TCR signals.

3. TCR Signal Transduction

Signal transduction from the TCR/CD3 complex is an area of intense investigation. It has been known for some time that stimulation of the TCR, like many other receptors, results in activation of the inositol phospholipid specific enzyme phospholipase C (PLC) and the generation of inositol-1,4,5-trisphosphate (IP3) and diacylglycerol (DAG) (Weiss et al., 1986). These second messengers release calcium (Ca^{++}) from intracellular stores and activate protein kinase C (PKC), respectively (Berridge, 1984). Indeed, mimicking the action of these second messengers by the addition of calcium ionophores and phorbol esters results in the activation of T cells that is in many ways indistinguishable from activation in response to appropriately presented antigen. However, it has recently been realized that there are a number of critical events in TCR signal transduction that precede IP3 and DAG production.

One of the earliest events to occur after TCR engagement is the tyrosine phosphorylation of a number of cellular proteins (Baniyash et al., 1988; Hsi et al., 1989).

The importance of tyrosine phosphorylation in TCR signal transduction is illustrated by the observation that protein tyrosine kinase (PTK) inhibitors completely block T cell activation (June et al., 1990a). This inhibition can be by-passed by Ca^{++} ionophores and phorbol esters, suggesting that PTKs are involved before Ca^{++} mobilization and PKC activation take place. Time-course studies have reached similar conclusions regarding the temporal relationships of tyrosine phosphorylation and Ca^{++} mobilization (June et al., 1990b). Only some of the proteins that become tyrosine-phosphorylated upon TCR engagement have been identified. Among the known tyrosine-phosphorylated proteins are vav, valosin containing protein, CD5, the ζ chain of CD3, and the γ isoform of phospholipase C (PLC-γ1) (see Fraser et al., 1993). Tyrosine phosphorylation of PLC-γ1 greatly increases its catalytic activity, and thus provides the link between tyrosine kinase activity and inositol phospholipid breakdown.

4. Tyrosine Kinases in T Cell Signal Transduction

Neither the α or β chains of the TCR nor any of the chains of the CD3 complex have intrinsic tyrosine kinase activity. Therefore, nonreceptor protein tyrosine kinases (PTKs) must be recruited by TCR/CD3 to carry out the requisite phosphorylations that occur upon receptor ligation. To date, 5 PTKs representing 3 different families have been implicated in T cell signalling. These include the src-family PTKs p56lck and p59fyn, the syk family PTKs Zap-70 and syk itself, and the prototype of the csk family of PTKs (see Chan and Weiss, 1994 for review). In addition to these kinases, the tyrosine phosphatase CD45 has also been shown to influence TCR signalling (Trowbridge and Thomas, 1994). Features of src-family PTKs include glycine residue at amino acid position 2 that is the site of myristylation, which allows localization to the inner face of the plasma membrane, a unique N-terminal region of approximately 80 amino acids that is thought to play a role in the interaction of the kinase with specific proteins, a SH3 domain involved in interacting with other proteins containing proline-rich regions, an SH2 domain that is able to bind proteins at phosphorylated tyrosine residues, a C-terminal domain responsible for

the catalytic activity of the enzyme containing an autophosphorylatable tyrosine residue involved in upregulating the kinase activity, and a negative regulatory tyrosine residue C-terminal to the kinase domain. Syk-family PTKs differ from Src-family PTKs in that they contain two SH2 domains and no SH3 domain, they are not myristylated, and they do not possess any known negative regulatory tyrosine residues. Csk PTKs are structurally similar to the src-family, but differ in that they do not have a myristylation site, an autophosphorylation site, or a negative-regulatory C-terminal tyrosine residue (see Figure 1-5)

Effective signal transduction by CD3 ζ and ε is dependent upon the presence of an 18 amino acid signaling motif (Irving and Weiss, 1991; Romeo et al., 1992; Irving et al., 1993). This motif contains two conserved tyr-x-x-leu/ile (YXXL) sequences separated from each other by a conserved aspartate or glutamate residue and has been termed the ITAM, for Immunereceptor Tyrosine Activation Motif (reviewed by Samelson and Klausner, 1992; Abraham et al., 1992; Chan and Weiss, 1994; Cambier, 1995). Interestingly, this motif was first noticed by Reth (1989) who pointed out that all the members of the CD3 complex, as well as the B cell antigen receptor and the Fc-ε receptor on mast cells, possessed this motif in their cytoplasmic regions at least once. ζ is unique, in that it contains three ITAM sequences. Subsequently, additional ITAM-containing signaling molecules have been identified, as listed in Table 1-III. The ITAMs are the core signaling domains of the CD3 chains and are indispensable to T cell signal transduction. The tyrosine residues within these motifs serve as substrates for the src kinases lck and fyn, and upon phosphorylation, recruit the syk kinase ZAP-70 via its double SH2 domains (Chan and Weiss, 1994). Through the ITAMs, the various tyrosine kinases involved in T cell signal transduction can coordinate their activities and initiate the formation of an activation cluster of many different signaling proteins via a cascade of tyrosine phsophorylations and SH2 and SH3 domain interactions (Chan and Weiss, 1994; Cambier, 1995).

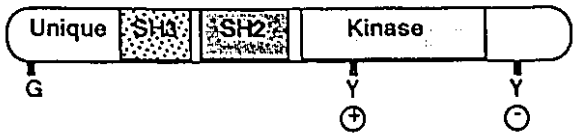
Figure 1-5 Structure of Protein Tyrosine Kinases Involved in T Cell Signal Transduction.

Two src family PTKs, p56lck and p59fynT are important in initiating T cell signaling. Lck interacts strongly with the CD4 and CD8 coreceptors via its unique N terminus and is brought into the vicinity of the CD3 ITAM sequences upon antigen/MHC binding. Abundant evidence supports a model in which lck phosphorylates the tyrosine residues in the ζ and ε chain ITAMs (and perhaps those in other CD3 chains), initiating recruitment and activation of ZAP-70. Fyn weakly interacts with nonphosphorylated ITAM sequences via its unique N terminus and may contribute towards ITAM tyrosine phosphorylation, after which it may interact further with the phosphorylated CD3 chains via its SH2 domain. Fyn and lck are both localized to the cell membrane by virtue of myristilation at an N-terminal glycine (G) residue. Positive and negative regulatory tyrosine residues are indicated by Y. It is thought that phosphorylation of the C-terminal negative regulatory tyrosine allows its interaction with the cis SH2 domain keeping the enzyme in a closed, inactive conformation. Activation of the enzyme then requires both dephosphorylation of the negative regulatory tyrosine residue and phosphorylation (perhaps autophosphorylation) of the positive regulatory tyrosine residue in the kinase domain.

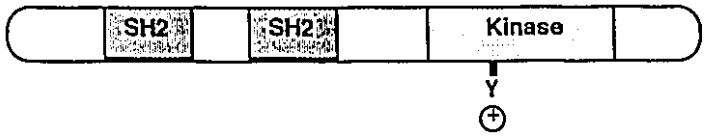
ZAP-70 is not myristilated, nor does it possess the SH3 domain characteristic of the src family members. Instead, its two SH2 domains are spaced such that they interact with the two tyrosine residues of an ITAM motif. Phosphorylation and accurate spacing of the tyrosine residues in the ITAMS has been found to be critical for ZAP-70 binding, as have both SH2 domains of this PTK.

Csk is a src-like PTK in that it possesses an SH3 and an SH2 domain. It is involved in regulating the activity of the src family kinases by phosphorylating their C-terminal negative regulatory tyrosine residue.

Src Family Kinases
-lck
-fyn



Syk Family Kinases
-syk
zap-70

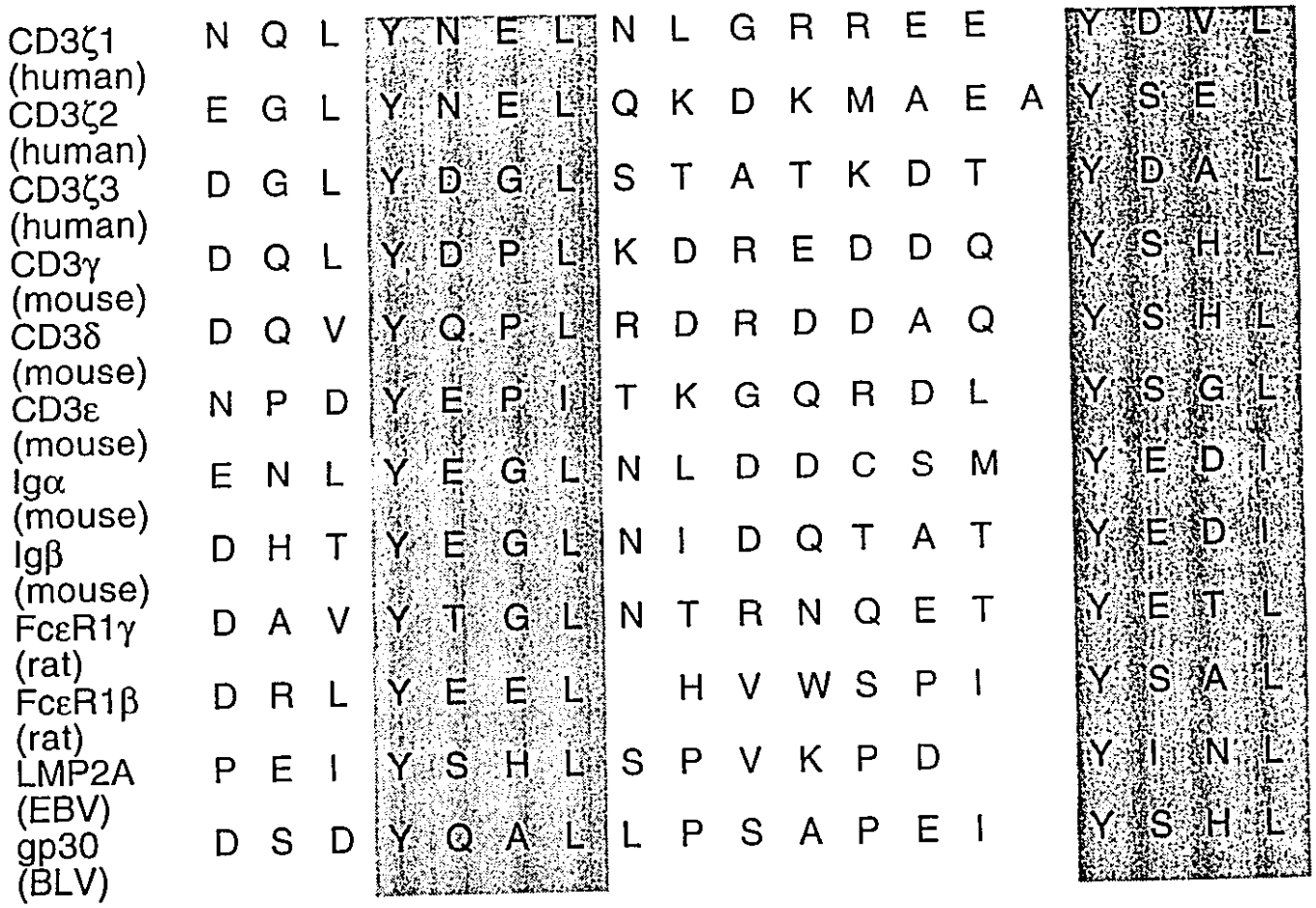


Csk Family Kinases
-csk

Adapted from Chan et al., 1994.

Table 1-III

ITAM Sequences in Various Proteins



B. Acquisition of Lytic Activity by CTL

1. Induction of Lytic Molecules

Cytolytic effector function in CTL can be induced by ligation of the TCR by antibodies, mitogenic lectins, or appropriately presented antigen. The lymphokine IL-2 can also induce cytotoxicity, as can a number of other lymphokines (Liu et al., 1990; Manyak et al., 1989; Smyth et al., 1991; Smyth et al., 1990a and 1990b; Salcedo et al., 1993). Concomitant with the acquisition of cytoxicity in most CTL populations is increased accumulation of messenger RNAs encoding molecules involved in the cytotoxic mechanism, such as perforin and granzyme B (or granzyme 2 in the human system). Perforin and granzyme B can also be induced by calcium ionophores. Interestingly, the protein kinase C-activating phorbol ester PMA synergizes with calcium ionophores in the induction of granzyme B but not in perforin induction (Garcia-Sanz et al., 1992).

Since IL-2 alone can stimulate lytic activity and induce transcription of perforin and granzyme genes, many of the stimulatory effects of TCR ligation on $CD8^+$ cells in mixed cultures may be mediated by IL-2. That is, stimulation of the TCR in heterogeneous mixtures of lymphocytes (peripheral blood lymphocytes [PBL] or splenocytes) may induce CTL effector function by stimulating helper T cells to secrete IL-2 and concomitantly upregulating the IL-2 receptor on pre-CTL. It is, however, very unlikely that this is the only mechanism for the induction of cytotoxicity in CTLs.

A number of pieces of data suggest the existence of IL-2 independent pathways for CTL activation. Using a panel of monoclonal anti-CD3 antibodies recognizing different epitopes, Stohl et al. (1990) have demonstrated the induction of potent cytolytic activity in the absense of detectable IL-2. Also, high levels of IL-2 were not able to induce as much cytolytic activity as some of the anti-CD3 antibodies that resulted in much lower levels of IL-2 being produced, yet exogenous IL-2 could enhance the cytolytic activity induced by the antibodies. Other groups have also provided evidence for

an IL-2 independent pathway of perforin and granzyme induction in $CD8^+$ T cells (Garcia-Sanz et al., 1992; Kaiser et al., 1993). It has also been shown that anti-CD3-induced lytic activity and expression of granzymes B and C in mouse splenocyte cultures was drastically reduced by the immunosuppressant cyclosporin A (CsA) (Kaiser et al., 1993). This effect of CsA was not solely due to its well-documented effect of blocking IL-2 gene transcription, as addition of exogenous IL-2 to Cs-A-treated cultures did not eliminate this suppression. Moreover, neutralization of IL-2 in anti-CD3-stimulated splenocyte cultures had little effect on either granzyme expression or the generation of cytolytic activity. Other groups have also provided data indicating that CsA can suppress perforin and granzyme gene induction in a manner independent of IL-2 production (Garcia-Sanz et al., 1992; Liu et al., 1989).

2. Heterogeneity of TCR-Derived Signals Leading to Differential Responses

The nature of the signals responsible for the IL-2-independent pathway for perforin and granzyme gene induction and acquisition of cytolytic activity is presently unclear. Certainly, the effect of lymphokines other than IL-2 cannot be excluded and could contribute to CTL effector acquisition. The presence of additional lymphokines known to affect cytolytic generation were not tested in the experiments mentioned above. It is also conceivable, however, that distinct signals generated as a consequence of TCR ligation contribute directly or indirectly towards the transcriptional activation of the genes encoding proteins involved in the cytolytic machinery, and consequently, towards the generation of cytolytic potential. It is this possibility that is interesting to explore.

Although not generally appreciated, several lines of evidence have hinted at the possibility that the TCR can transmit distinct and different types of signals. For example, Prendergast et al. (1992) demonstrated that ligation of the TCR in splenocyte cultures by lectin or alloantigen resulted in the induction of almost the entire family of mouse granzyme genes (B through G), whereas only two members of the family (B and C) were induced by TCR ligation by anti-CD3 antibody. Since mitogenic lectin is known to

stimulate T cells in a TCR/CD3-dependent manner (Kanellopoulos et al., 1985; Weiss and Stobo, 1984; Weiss et al., 1984; Weiss et al., 1987; Ohashi et al., 1985) this difference in activation profiles from anti-CD3 antibody is surprising and suggests that the way in which the TCR is stimulated results in different responses, perhaps due to different types of signals being generated from the same complex. This idea is supported by the observation that monoclonal antibodies recognizing different epitopes of the CD3 complex resulted in differential levels of cytolytic activity in PBL cultures that could not be explained on the basis of differences in isotype or avidity. Also, different anti-CD3 antibodies can induce different lymphokine secretion patterns from PBLs (Sherris et al., 1989). Since many of these observations have been made in mixed populations of lymphocytes, alternative explanations for differential cytotoxic responses could be explained by differential responsiveness of different helper T cells to various stimuli. This could result in different cytokine patterns being produced which could influence cytotoxicity and transcriptional activation of perforin and granzymes in variable ways. More compelling, however, are reports of variable responses being elicited from cloned T cells upon stimulating the TCR in distinct ways. It has been demonstrated that superantigens and conventional peptide antigens can activate different biochemical signaling pathways (O'Rourke et al, 1990) and generate different cytokine patterns (Liu et al, 1991) in the same cloned T cell line. Although both types of stimulus are believed to operate through the TCR/CD3, they bind different regions of the TCR and so the different responses elicited may reflect a variablility in the signals generated from the same signaling complex when it is ligated in different ways. This phenomenon is also observed when comparing cloned T cell responses to wild-type or modified peptide ligands. Although presented and recognized in an apparently normal way, the modified peptides could elicit different responses from the T cells that were elicited by the native peptide, such as separating growth of the cells from other responses (Evavold and Allen, 1991; Racioppi et al., 1993; Ruppert et al., 1993). It has been proposed, then, that the TCR is

flexible in the signals it generates, and ligation by different agents may activate different signals by causing different conformational changes in the signaling complex (Janeway and Bottomly, 1994).

Since the CD3 complex is known to be composed of multiple signaling modules (Wegener et al., 1993), it seems possible that differential responses to TCR ligation might be due to selective activation through distinct modules. Some types of stimuli may cause the generation of signals from all the modules, whereas others may activate single modules or different combinations of modules. Each type of signal may be interpreted in different ways, including differential activation of effector function and gene transcription. The feasibility of this notion is supported by recent evidence which indicates that not all ITAM sequences in the cytoplasmic regions of the various immune receptors are functionally redundant. Rather, it appears that various ITAMs may be coupled to different signalling pathways and cellular responses (reviewed in Cambier, 1995). Interestingly, the src-family kinases fyn and lyn have been shown to bind to nonphosphorylated ITAM sequences via their unique N-terminal regions (Clark et al., 1992; Gauen et al., 1994; Pleiman et al., 1994), and the avidity of binding was determined by non-conserved sequences within the ITAMs (Clark et al., 1994). Therefore, one explanation for variable signals conveyed by different CD3 signalling molecules could be due to differential coupling of transduction molecules, including src family kinases.

C. Concluding Remarks on T Cell Activation

Activation of CTL is a complex process resulting from the coordinated cumulative effects of different lymphokines and TCR-derived signals. Each of these signals is transduced by a vast array of different signaling molecules impacting on each other. The initial signals generated then result in gene transcription, which is itself controlled by a complex set of transcriptional regulators. Recent technology involving chimeric protein production and gene targeting of various components in these processes

has begun to unravel the mysteries of T cell function. A more detailed picture of the events that occur between recognition of an antigen-bearing cell and its destruction by a CTL is beginning to emerge. Nevertheless, we have only just begun to scratch the surface, and multiple layers of regulatory circuits likely exist to be discovered. There is still more unknown than known.

VI. AIMS OF THE THESIS

Research on cell mediated cytotoxicity is an extremly competitive and fast-moving field. When I first started these studies, perforin was the only cytolytic effector molecule for which a clear role in the lytic process was defined. It was only beginning to be appreciated that perforin alone could not account for all of the observed target cell responses to CTL attack, most noteably DNA fragmentation. Considerable circumstantial evidence pointed to the granzymes as likely playing an important role in target cell destruction, but the exact contribution these enzymes made was entirely unknown. Also, data were beginning to accumulate that suggested the existence of a non-granule-mediated, calcium-independent lytic pathway that could be utilized by at least some CTL. The nature of this perforin-independent lytic mechanism was a mystery.

In order to investigate the role that the most abundant granule serine proteinase, granzyme B, played in cytolysis, I set out to produce granzyme B-deficient mice via homologous recombination in mouse embryonic stem cells. Gene targeting technology was quite new at the time and had never been attempted at the University of Alberta. However, an increasing number of labs worldwide were successfully knocking out a variety of genes, so I decided to take the risk and attempt this project. The resulting mice would surely prove to be an extremely valuable research tool and would allow new insight into the function of granzyme B. Unfortunately, our competitors thought similarly, and we lost the race to produce granzyme B knockouts to Timothy Ley's lab in St. Louis. Jurg Tschopp's lab in Lausanne was similarly "scooped" and they, like us, decided to abandon the project midway, before producing any mutant mice.

In any rapidly advancing field, adaptation to new realities is a must and projects must quickly evolve as new information and tools become available. When mice deficient for the T cell tyrosine kinase p59fyn^T were offered to us, I decided to investigate if this enzyme played a role in the T cell signals that lead to activation of CTL effector functions. During the course of these studies, I noticed a definite increased longevity of fyn-deficient lymphocyte cultures. Fas had not yet been implicated in CTL function, and its role in regulating T lymphocyte lifespans was only just being speculated at. When I noticed that the Fas cytoplasmic domain had a tyrosine residue within a YXXL motif similar to that found in the CD3 chains, I hypothesized that fyn might interact with this sequence as it does with a similar sequence in CD3 ζ, and if Fas was indeed important in downregulation of immune responses, this might explain the sustained viability of activated fyn-deficient lymphocytes. My research then began to focus on investigating this intriguing possibility.

The specific aims of this thesis, then, were as follows:

i) To knockout the granzyme B gene in mouse ES cells via homologous recombination.

ii) To investigate the role p59fyn plays in the generation of CTL effector function.

iii) To examine the possibility that p59fyn may be involved in Fas signal transduction and in regulating lymphocyte lifespans.

CHAPTER TWO: MATERIALS AND METHODS

MATERIALS

Antibodies

Hamster anti-mouse Fas antibody Jo2 (with and without sodium azide) and control hamster IgG were purchased from Pharmingen. Anti-human Fas antibody was obtained from Upstate Biotech, Inc. Anti-mouse Fas peptide Fab_2 fragment was a generous gift of Dr. K. Elkon. Anti-fyn antibodies were purchased from Santa Cruz Biotech, Inc. HRP-conjugated donkey anti-rabbit antibody was from Amersham, and HRP-conjugated goat anti-rabbit was from BioRad. FITC-conjugated anti-hamster was purchased from Pharmingen. Hamster anti-mouse CD3ε–producing hybridoma 145-2C11 was obtained from Dr. J. Bluestone. It was used as partially purified culture supernatant as described (Leo et al., 1987). Anti-mouse CD45 antibody was obtained from Dr. H. Ostergaard (University of Alberta).

Animals

p59fyn-deficient mice were a generous gift of Drs. M. Appleby and R. Perlmutter, University of Washington, Seattle. BABL/c and C57Bl/6 mice were obtained from the University of Alberta Laboratory Animal Services or from Jackson Laboratories.

Radiochemicals

$\alpha[^{32}P]$-dCTP [deoxycytidine 5'-triphosphate tetra-(triethylammonium) salt], ^{3}H-thymidine, ^{51}Cr, ^{3}H-thymidine and $\gamma[^{32}P]$-ATP [deoxyadenosine 5'-triphosphate tetra-(triethylammonium) salt] were obtained from New England Nuclear.

Tissue Culture Materials

RPMI-1640, Dulbecco's Modified Eagle's Medium (DMEM), Ca^{2+}/Mg^{2+}-free phosphate buffered saline (PBS), trypsin, penicillin/streptomycin, leukemia inhibitory factor (LIF) and geneticin G-418 sulfate (G418) were obtained from Gibco/BRL. Fetal Calf Serum (FCS) was obtained from either Gibco/BRL or Hyclone. Gancyclovir (GANC) was purchased from Syntex. LIF/ESGRO was from Gibco/BRL.

Molecular Biological Reagents

Restriction endonucleases, DNA modifying enzymes proteinase K, and Taq DNA polymerase were obtained from Boehringer Mannheim, New England Biolabs, and/or Gibco/BRL Life Technologies. All enzymes were used in buffers provided by the manufacturer. Primers for PCR and in vitro mutagenesis were obtained from the DNA Synthesis Facility, Dept. of Biochemistry, University of Alberta.

METHODS

Cell Culture

All cell lines were grown in a 5% CO_2 humidified incubator at 37° C. Tumour target cells YAC-1, P815, EL-4, L1210, L1210-Fas, and Jurkat were all grown in RPMI-1640 supplemented with 10% FCS, 20 mM HEPES, 100 uM 2-mercaptoethanol, 100 ug/ml streptomycin, and 100 U/ml penicillin (RHFM). SNL feeder layer cells (STO mouse embryonic fibroblasts transfected with a neo/LIF-expression construct) were obtained from Dr. J. Stone (University of Alberta) and grown as monolayers in (DMEM), 10% FCS. They were removed by brief treatment with 0.05% trypsin, 0.53 mM EDTA (trypsin/EDTA) and washed in FCS-containg media, before being mitotically inactivated by γ-irradiation with 5000 rads from a cesium source. ES cell lines AB-1, JS-1, and D3J8 were obtained from Drs. Alan Bradley (Baylor College), Jim Stone (University of Alberta) and Mark Appleby (University of Washington), respectively. They were maintained on feeder layers of irradiated SNL cells in DMEM supplemented with 15% FCS. Before electroporation or DNA isolation, ES cells were removed with trypsin/EDTA treatment and split at least 1:5 in order to dilute out the fibroblasts feeders. When grown in the absence of feeders, 1000 units/ml of leukemia inhibitory factor (LIF/ESGRO) was added to the ES cells to maintain an undifferentiated state.

Splenocyte Culture and Activation

Splenocytes were obtained from mutant or normal mice by disruption through wire mesh under pseudo-sterile conditions in RHFM. Stimulator splenocytes were

irradiated with 2500 rads γ radiation before use. For alloantigen stimulation (MLR), $1x10^6$ $H2^b$ responder cells (C57Bl/6 fyn+/+ or 129-C57Bl/6 fyn-/-) were mixed with $1x10^6$ $H2^d$ stimulator cells (BALB/c) per ml RHFM containing 60 U/ml rIL-2 for the indicated time periods. For anti-CD3 stimulation, responder splenocytes were initially cultured at 5x105 cells per ml RHFM containing 60 U/ml rIL-2 and anti-CD3 antibody diluted to a final concentration of 1:500. In both activation conditions, dead cells were removed by centrifugation over ficol after stimulation for 3 days.

Genomic DNA Library Screening and Isolation of Genomic Granzyme B Clone

A genomic library made from 129/J mouse liver in λ/DASH phage (Stratagene) was obtained from Dr. J. Stone, University of Alberta. The library was titred and found to be approximately $1.5x10^9$ pfu/ml. It was diluted 1000 fold in SM buffer. 34.4 µl of the diluted phage ($1.5x10^6$ pfu/ml) was added to 0.3 ml of LE392 host cells (OD_{600}=0.425) in 23 separate tubes and incubated at 37° C for 30 minutes, at which time 8 ml of molten 0.7% NZY top agar at 49° C was added. The solution was quickly mixed and poured onto 150x15mm NZY agar plates, allowed to solidify, and then grown inverted at 37° C overnight. The plates were chilled at 4° C before pulling duplicate Hybond N nylon filters and treating them in denaturing (pH 14) and neutralizing (pH 8) solutions. The DNA was fixed to the filters using a Stratagene Stratalinker on the autocrosslink setting.

Duplicate filters were hybridized with either a 1128 bp DraI fragment from the 5' region of genomic granzyme B, or a 925 bp XhoI/HindIII fragment from the 3' end of granzyme B (the HindIII site comes from the plasmid multiple cloning site) in 50% formamide-containing hybridization buffer at 42 C overnight, washed with 0.1X SSC, 0.1% SDS at 60° C and autoradiographed. Plaques hybridizing with both probes were plugged and replated and reprobed to obtain isolated clones.

Phage was isolated from liquid lysate cultures grown with agitation overnight at 37° C and treated with a few drops of chloroform. Phage was precipitated with PEG and

DNA was obtained by phenol:chloroform extraction. The Not I inserts were subcloned into pBS KS$^+$ and analyzed by restriction enzyme mapping.

Electroporation of ES Cells

Log phase ES cells were harvested and single cell suspensions obtained by brief treatment with trypsin/EDTA and two washes with room temperature DMEM/15% FCS. Two additional washes in Ca^{2+}/Mg^{2+}-free PBS were performed before the cells were resuspended in room temperature PBS at a concentration of $1.25x10^7$ cells/ml. 18-20 µg of Not-1 linearized, EtOH sterilized targeting vector DNA in about 25 µl PBS was mixed with 10^7 cells (0.8 ml) in a 0.4 cm electroporation cuvette and held at room temperature for 5 minutes. The cells were then subjected to a single pulse from a BioRad Gene Pulser electroporator equipped with a Capacitance Extender unit, set to 230 volts and 500 µF. Time constants were all within 7.5-10 useconds. After electroporation, the cells were left undisturbed in the cuvettes for 10 minutes, before equally distributing them between 3 or 4 10 cm plates, each containing a feeder layer of irradiated SNL fibroblasts and 15 ml DMEM/FCS. After two days growth, the old media was removed and replaced with fresh DMEM/FCS containing 1 or 2 µM GANC and/or 0.25 mg/ml G418. Half the drug-containing media was changed every day for the next 4 or 5 days, and then every other day after that. After 6 or 7 days, the media was supplemented with 1000 U/ml LIF. Colonies were counted and picked under a dissecting microscope after about 10 days growth, dispersed in trypsin/EDTA and plated onto 6-well plates.

Isolation of ES Cell Genomic DNA and Southern Blotting

After a few days growth in the 6-well plates, 1/4 of the cells were frozen, and DNA was isolated from the remainder by resuspending in 250 µl of lysis solution (100 mM Tris HCl, pH 8.5; 5 mM EDTA; 0.2% SDS; 200 mM NaCl; 100 µg/ml proteinase K). Samples were agitated at 55° C for 24-48 hours and then precipitated with 0.5 volumes of 7.5 M NH_4Ac and 2 volumes of 95% EtOH. The DNA was then washed with

70% EtOH, resuspended in TE buffer, and subjected to the appropriate restriction enzymes.

Restriction enzyme digestion was generally carried out overnight to ensure complete cutting. Samples were then electrophoresed overnight through 0.8% agarose gels at a rate of 1 volt/cm in TBE buffer. The gels were stained with EtBr and visualized on a UV light box before being soaked in 0.2 N HCl for 20 minutes, followed by 45 minutes each in pH 14 denaturing solution (0.5M NaOH, 1.5M NaCl) and pH 8 neutralizing solution (1.5M NaCl, 1M Tris HCl). The DNA was then transferred onto Hybond N nylon filters (Amersham) by blotting the gel under weight overnight. The DNA was cross-linked to the filter using the autocrosslink setting of a Stratagene Stratalinker apparatus.

Hybridization of the filters with granzyme B randomly primed probe was carried out in hybridization buffer contain 50% formamide overnight at 42° C. The filters were washed in 0.1X SSC, 0.1% SDS for 20 minutes before being exposed to X-ray film at -70° C.

Assessment of Lytic Activity (^{51}Cr-Release Assays)

Tumour target cells in log phase of growth were pelleted at 800-1000 RPM in a Sorval centrifuge. After removal of the supernatant, the cells were resuspended in 100 µCi ^{51}Cr per $2x10^6$ cells and incubated at 37° C for 60-90 minutes. The cells were washed once in about 10 ml room temperature RHFM, resuspended in 20 ml RHFM and incubated at least 30 minutes longer at 37° C. The cells were then washed two more times in RHFM, counted, and resuspended at $1\text{-}2x10^5$ cells per ml. Effector cells were added to wells of V or U-bottomed 96-well plates in growth medium at various concentrations in a volume of 100 µl. 50 or 100 µl (10^4) labelled target cells were then added to each well containing the effectors, as well as to additional wells containing only 100 µl of media for assessment of total labelling and background release of ^{51}Cr. When inhibitors or DMSO were added, they were in a volume of 50 µl. The final volume of all assays was 200 µl

per well and each condition was assessed in triplicate. The plates were lightly centrifuged to ensure immediate cell-cell contact and then incubated in a 37° C humidified incubator for 4 hours. After incubation, the plates were centrifuged at 500 RPM to ensure the pellets were resistant to slight perturbation, and the top half (100 µl) of the supernatant from each well was removed for counting in a Rack-Gamma gamma counter. Lytic activity was determined using the standard formula

% Specific Lysis = 100 x (sample - spontaneous release)/ (total - spontaneous release). Spontaneous release was the amount of ^{51}Cr released into the supernatant in the absence of effector cells. Totals were obtained by counting 50 µl-equivalents of the original target cell suspension.

One lytic unit is the number of effector cells necessary to achieve 30% Specific Lysis. Lytic units were calculated by plotting the specific lysis data from a number of effector to target ratios on semi-log graph paper and determining the number of cells required to obtain 30% Specific Lysis.

Thymidine Uptake Proliferation Assays

Splenocytes were removed from stimulated bulk cultures on a daily basis and added to flat-bottomed 96-well plates at a final concentration of $5x10^5$ cells per well in RHF. 1 µCi ^3H-thymidine/well was added and the cells were cultured for 16 hours at 37 C, 5% CO_2. All cultures were set up in double triplicates, harvested onto glass fiber filter paper using a cell harvester (Skatron) and incorporated radioactivity was counted in a Beckman LS scintillation counter.

Assessment of DNA Fragmentation

Quantitation of DNA fragmentation was performed using the method of Duke (1993) with slight modifications. Briefly, target cell DNA was labelled by overnight incubation in RHFM containing 1.5 µCi/ml ^3H-thymidine. The next day, the cells were washed twice in label-free RHFM and incubated in fresh media for at least 1 hour, after which they were washed two more times, counted, and resuspended to a concentration of

$2x10^5$ cells per ml. 50 or 100 µl of cell suspension were added to 1.5 ml Eppendorf tubes and mixed with target cells at various concentrations in the presence or absence of inhibitors, in a final volume of 200 µl. Each condition was assayed in duplicate or triplicate. The tubes were briefly centrifuged to initiate cell-cell contact and then incubated at 37° C for 2-4 hours, depending on the experiment. After incubation, an equal volume of 0.5% Triton X-100 in PBS was added to all tubes except those designated as Totals, which had an equal volume of 1% SDS in PBS added instead. The tubes were vortexed well and then centrifuged at 14000 RPM in an Eppendorf centrifuge at 4° C for 10 minutes. Half the supernatant from each tube was removed and placed into plastic scintillation vials containing 3 ml ACS scintillant (Amersham), mixed well, and counted in a Beckman scintillation counter. Percent DNA fragmentation was calculated using the formula % Specific Fragmentation = 100 x (experimental released DNA - spontaneous released DNA) / (Total labelled DNA - spontaneous released DNA).

Qualitative visualization of DNA fragmentation was done using a modification of the method described by Smith et al. (1989). 10^6 unlabelled target cells were mixed in Eppendorf tubes with various concentrations of effector cells in the presence or absence of inhibitors in a final volume of 1 ml RHFM. After incubation at 37° C for 2-4 hours, depening on the experiment, the cells were pelleted in an Eppendorf centrifuge for 10 seconds at maximum speed. The pellets were resuspended in 35 µl of lysis buffer composed of 10 mM EDTA, 50 mM Tris-HCl (pH 8.0), 0.5% sodium lauryl sarkosinate, and 0.5 mg/ml proteinase K. The proteinase K was always added just prior to use. The tubes were then incubated at 50° C for one hour, at which time DNase-free RNase A was added to a final concentration of 0.5 mg/ml and incubation was continued for an additional hour. The samples were electrophoresed through 1.0% agarose gels in Tris-Borate EDTA running buffer. DNA was visualized by staining the gels in ethidium bromide staining buffer, destaining extensively in water, and illumination on a UV light box.

Quantitation of Apoptosis by Flow Cytometry

Splenocyte DNA was labelled by the *in situ* nick translation method of Meyaard et al.,(1992). Briefly, 1 million cells were fixed on ice in 70% ethanol and 1% glutaraldehyde, washed 2 times with PBS and then incubated in a total volume of 10 µl with 55 µM biotin dUTP, a mixture of 19 µM dATP, dGTP and dCTP and *E. coli* DNA polymerase I as normally done using a Boehringer Mannheim nick translation kit for 90 minutes at 15° C. The samples were then washed in PBS plus 0.1% Triton X100 and resuspended in 2.5 µg/ml avidin-fluorescein isothiocyanate, 20µg/ml RNase, 4X SSC, 0.1% Triton X-100, and 5% skim milk powder. Finally, the samples were washed and resuspended in PBS plus 0.1% Triton X-100 and 5 µg/ml propidium iodide and analyzed on a Becton Dickinson FacsScan Flow Cytometer using Lysis II software.

This assay was performed by R.S. Garner and M.J. Pinkoski.

Isolation and Northern Blot Analysis of Total RNA

RNA was isolated by solubilizing cells in guanidinium thiocyanate containing 2-mercaptoethanol and either centrifuging through cesium chloride following the method of Chirgwin et al. (1979) or extracting with phenol chloroform, as per Chomski et al. (1985). RNA pellets were resuspended in a solution of EDTA, SDS and measured by EtBr fluoresence at pH 8.0 in a fluorometer calibrated with standards consisting of 0.5 µg calf thymus DNA. 10 or 1 µg of RNA was loaded onto full-sized or mini northern gels, respectively. The gels were 1% agarose containing 0.7% formaldehyde and 0.33 µg/ml EtBr. Before loading, the RNA was mixed with two volumes of FFMOPS [50% formamide, 6.5% formaldehyde, 1X MOPS buffer (ie, 20 mM MOPS, 5 mM sodium acetate, 1 mM EDTA, pH 7.0)] and denatured by heating at 60° C for 15 minutes. Electrophoresis was done in circulating 1 X MOPS buffer. After size separation, the ribosomal RNA bands were viewed under UV illumination to ensure the integrity of the RNA samples. The RNA was then transferred onto Hybond N nylon filters (Amersham) by weighted overnight contact in a 10x SSC solution, and then cross-linked to the

membranes via the autocrosslink setting in a UV Stratalinker 1800 apparatus (Stratagene).

Northern blots were incubated overnight at 42° C with various ^{32}P-labelled cDNA probes produced using the BRL random primers kit in hybridization buffer containing 50% formamide, 5X SSC, 5X Denhardt's, 50 mM $NaPO_4$, pH 6.5, 1 mM $NaPyPO_4$, 0.1% SDS, 100 µM ATP, 2.5 mM EDTA. The filters were then serially washed in solutions of diminishing salt concentrations at increasing temperatures, up to a stringency of 0.1x SSC, 0.1% SDS at 55° C. Hybridizing bands were visualized by placing the filters in contact with Kodak X-OMAT AR X-ray film for various lengths of time at -70° C and developing on a Kodak X-OMAT film processor.

Immunoprecipitations, Western Blotting, and *In Vitro* Kinase Assays

5 x 10^6 YAC-1 cells and an equivalent number of activated PMM-1 cells were incubated together for 10 minutes at 37° C in serum-free medium (RPMI:AIM-V, 1:1, Gibco) and lysed in 1 ml lysis buffer (0.5% Triton X-100, 150 mM NaCl, 50 mM Tris-Cl, pH 7.5, 1 mM EDTA, 5mM sodium fluoride, 1 mM sodium ortho vanadate, 0.2M PMSF, and multi-protease inhibitor cocktail) for 30 minutes on ice. Post-nuclear supernatants were pre-cleared with 20 µl protein A/G agarose (Pierce) and subjected to immunoprecipitration using the indicated antibodies. Hamster anti-mouse Fas (Jo2), hamster control Ig, and rat anti-mouse CD45 (I3/2) were used at 5 µg/ml and immunoprecipitated with 20 µl protein A/G agarose. 5 µl of agarose-conjugated anti-fyn antibody (FYN15, Santa Cruz Biotech.) per ml was used to immunoprecipitate p59fyn. Immunoprecipitates were washed extensively in lysis buffer without EDTA and either dissociated in SDS sample buffer, separated by SDS-PAGE (8%), and electroblotted onto nitrocellulose, or subjected to *in vitro* kinase assays. Western blotting was performed using either rabbit-anti fyn antibody (FYN3, Santa Cruz Biotech) or rabbit anti-Fas peptide Fab_2 fragment (kindly provided by Dr. K. Elkon) and HRP-conjugated donkey anti-rabbit (Amersham) and visualized using the ECL method (Amersham).

Kinase assays were performed exactly as described by Samelson et al., 1990. Unstimulated samples contained exactly the same amount of protein from YAC-1 and PMM-1 cells but the cells were lysed separately and then mixed together just before removal of nuclei. For the reprecipitation of p59fyn from the anti-Fas immunoprecipitates, the washed pellets from the kinase reactions were resuspended in 80 µl SDS sample buffer containing B-mercaptoethanol, boiled 5 minutes, centrifuged, and 40 µl of the supernatant removed and diluted in 500 µl RIPA buffer containing the same inhibitors as the lysis buffer, then subjected to immunoprecipitation with anti-fyn, as above. Kinase reactions were also separated via 8% SDS-PAGE and visualized via autoradiography.

Site-Directed Mutagenesis of Fas

This was carried out by Dr. A. Caputo (University of Alberta) using the Amersham Sculptor *in vitro* mutagenesis kit according to the manufacturers instructions. Briefly, a 650 bp Xba1-BamH1 fragment from mouse Fas in expression vector pHBAPrI-neo/Δ Eco RI (Rouvier et al., 1993) containing most of the cytoplasmic domain, including the tyrosine residues of interest, was subcloned into M13mp18. Single stranded DNA template was prepared, and mutagenic primers of the following sequences were annealed and extended.

Primer 272: CTGTGCTGGTTCCCAATCTCATG

Primer 281: AGTGATGCATTTCAAGATTTAATC

In both cases, the underlined T residue replaces an A in the wildtype sequence and converts a tyr codon to a phe codon.

The resultant mutated DNA from a number of phage plaques was sequenced to confirm the presence of the dersired mutation, and then reinserted into the XbaI-BamH1 site of the Fas expression vector.

Electroporation of L1210 Cells

Expression vectors encoding wildtype and mutant Fas were linearized with ScaI. 40 μg of DNA was mixed with $1x10^7$ washed L1210 cells in PBS in 0.4 cm electroporation cuvettes in a final volume of 0.5 ml. The cells and DNA were allowed to sit at room temperature together for 10 minutes before a single pulse of 250V, 250 μF was delivered by a BioRad Gene Pulser. Time constants were all between 5.8 and 6.1 μsec. After resting for 10 minutes at room temperature, transfected cells were distributed between four 10 cm plates containg 12.5 ml RHFM. After two days, 1.5 mg/ml G418 was added and the cells were selected over a period of about two weeks in drug.

CHAPTER THREE: GENE TARGETING OF GRANZYME B

INTRODUCTION

Gene targeting via homologous recombination in mouse embryonic stem (ES) cells has become an extremely powerful and valuable tool for assessing the function of a variety of genes. By knocking out specific genes at will in these pluripotent, cultured cells, and then introducing them into a host blastocyst, mice can be created that are deficient for the protein encoded by that gene. According to a recent compendium, more than 325 independently-derived mutant mice have been generated, representing knockouts of over 260 different genes (Brandon et al, 1995). A wealth of information regarding the function of these genes that was previously impossible to obtain has now been collected by analyzing the phenotypes of these mice. A thorough discussion of this relatively new technology of gene targeting will not be given here, but the reader is refered to a number of important reviews and methodology papers (Lin et al., 1985; Smithies et al., 1985; Thomas et al., 1986; Song et al., 1987; Doetschman et al, 1987; Thomas and Capecchi, 1987; Mansour et al, 1988; von Melchner et al, 1992).

At the time the project which this chapter describes was initiated, the granzyme B gene was an excellent candidate for a gene knockout attempt. Very little was known about its actual function, although as previously discussed, several lines of circumstantial evidence suggested a potential role for this and/or other cytotoxic granule-localized serine proteases in the lytic mechanism utilized by cytotoxic T lymphocytes (CTL). A genomic DNA granzyme B clone had previously been isolated in our lab, and the entire coding region had been sequenced. Since gene knockout technology was beginning to be successfully applied by an increasing number of labs, we decided to embark on a project to disrupt the granzyme B gene in mouse ES cells. It was our hope that this would allow us to create a granzyme B-deficient mouse and provide us with an extremely useful tool with which to study the contribution this enzyme makes towards CTL-mediated killing. We were partially successful in this endeavour, in that a number of independently derived ES colonies were found to contain correctly-targeted granzyme B alleles. However,

before we could complete this project, another lab reported that they had generated granzyme B-deficient mice and published their findings on the effect of this mutation on target cell lysis by CTLs derived from these mice. This chapter describes the construction of a granzyme B targeting vector and its application in disrupting one allele of the granzyme B gene in mouse ES cells.

RESULTS AND DISCUSSION

Basics of Gene Targeting

In designing a gene targeting vector, a number of factors must be considered. The vector must contain a certain amount of sequence that is homologous to the genetic region one wishes to ablate in order for the targeting process to take place. Also, the coding sequence of the gene must be disrupted, so that replacement of the endogenous DNA with the vector DNA results in a null mutation. A mechanism for positive selection must be used in order to identify the ES cells which have been transfected with the vector, and a negative selection protocol to select against cells which randomly integrate the vector is also useful. Finally, a strategy for screening the selected cells and identifying correctly targeted ES cell colonies must be decided upon.

We utilized the positive/negative selection protocol first described by Mansour et al. (1988) in constructing a granzyme B gene targeting vector. In this system, the coding sequence of the gene is disrupted via the insertion of a DNA cassette which consists of a cDNA encoding neomycin phosphotransferase (neo) under the control of a strong promoter. Not only does the neo cassette act to interrupt the coding sequence of the target gene, but it also serves to provide a means of positive selection of cells integrating the vector, since the neomycin phosphotransferase enzyme confers G418-resistance to mammalian cells. Negative selection is provided by the addition of a herpes simplex virus thymidine kinase gene (HSV-tk) (under the same promoter as the neo gene) immediately at the end of the DNA sequence homologous to the genomic locus being targeted. If correct targeting occurs, the HSV-tk gene is eliminated and the neo gene is integrated, via

recombination between the homologous sequences of the vector and the genomic locus (Figure 3-1A). If the vector DNA is randomly integrated into the ES cell genome, however, the HSV-tk gene will also often be retained, since integration of linearized exogenous DNA occurs most often via the free ends (Folger et al., 1982; Roth et al., 1985; Thomas et al., 1986) (Figure 3-1B). The anti-HSV drug gancyclovir (GANC) can then be used to select against HSV-tk$^+$ cells. This drug is a nucleoside analogue that targets the HSV-tk enzyme, which has a less stringent substrate specificity than the mammalian thymidine kinase (See Mansour et al., 1988 and references therein). Phosphorylation of GANC by the HSV-tk enzyme allows its integration into replicating DNA strands, where it acts as a chain terminator and therefore kills the cell. Therefore, by growing ES cells transfected with a targeting vector employing this design in the presence of G418 and GANC, one can select for cells integrating the homologous DNA containing the neo cassette and against cells which have randomly integrated the entire DNA including the HSV-tk gene. This positive/negative selection method is not absolute though, in that it only enriches for targeted clones but does not ensure that double-resistant cells have in fact integrated the exogenous DNA via homologous recombination. The drug resistant colonies must still be screened to identify which ones are truly targeted.

Two main methods exist for screening transfected ES colonies for homologous recombinants. Both rely on the fact that proper insertion of the neo cassette into the target locus results in the introduction of a unique DNA sequence of known composition, thereby altering the entire locus in a defined manner. The first method makes use of the fact that the restriction enzyme map of the locus will be altered in a predictable way. Probing Southern blots of genomic DNA digested with appropriate enzymes with DNA probes derived from the targeted locus will result in a defined shift of the hybridizing band if the locus has been altered via homologous recombination. Failure to observe the

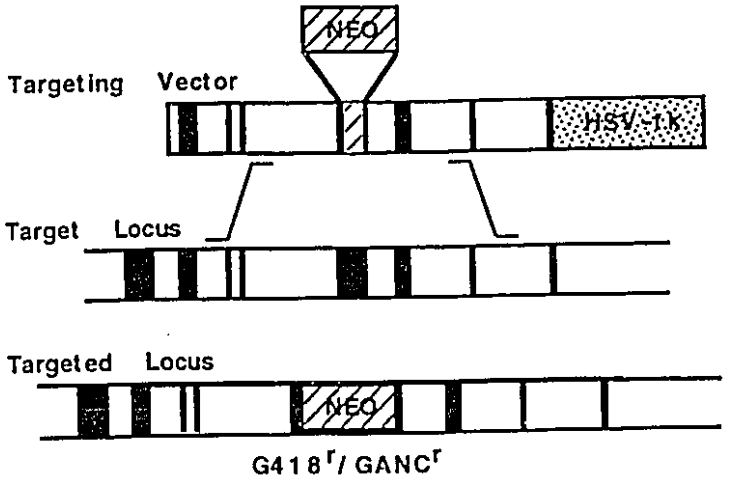
Figure 3-1 Homologous Recombination vs Random Integration of Targeting Vector.

A. Homologous Recombination. The linearized sequence replacement targeting vector is introduced into an ES cell via electroporation. The coding sequence of the gene of interest is disrupted by the insertion of a neo cassette, encoding a neomycin phosphotranspherase which confers resistance to the drug G418. Identical sequences between the vector and the target locus align themselves and homologous recombination occurs, accurately replacing the chromosomal DNA with the vector DNA and incorporating the neo cassette into the genome. The HSV-tk enzyme is not integrated and so the cells remain GANC-resistant.

B. Random Integration. The free ends of the linearized targeting vector drive integration of the vector by single strand invasion at a random chromosomal locus. The entire vector, including both the neo cassette and the HSV-tk gene, is thereby integrated into the genome. The cell is therefore resistant to G418 (by virtue of the neo cassette) and GANC-sensitive (by virtue of the HST-tk gene).

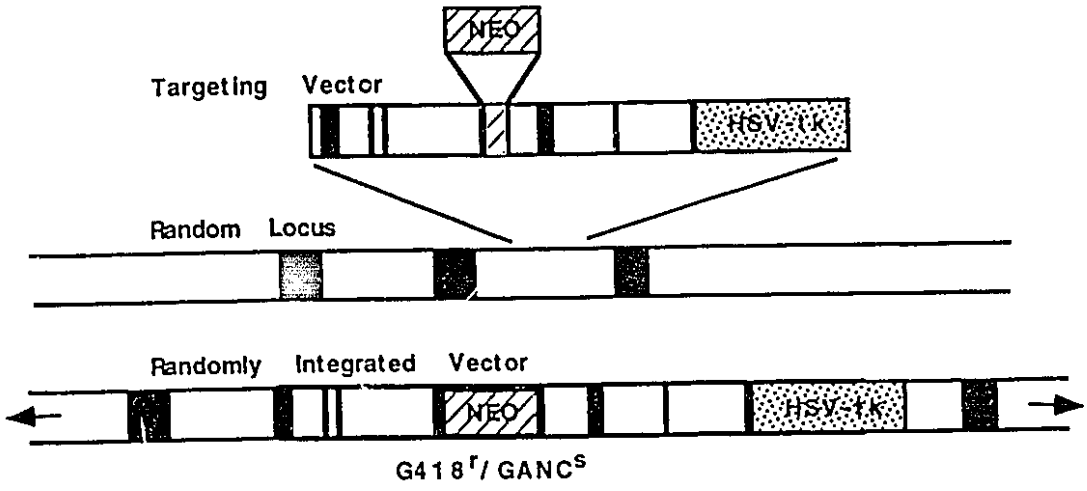
A

Gene Targeting
via
Homologous Recombination



B

Random Integration
via
Free Ends



expected sized restriction fragment indicates that random integration has taken place and the gene of interest has not been modified.

The second screening method utilizes the polymerase chain reaction (PCR). By selecting one amplification primer within the neo cassette and the second primer within the genomic sequence of the target locus but outside of the region of homology utilized in the targeting vector, a PCR product of a predictable size will only be obtained if the two primer sites are brought into correct juxtaposition with each other via homologous recombination (Kim and Smithies, 1988). This screening method has some drawbacks, in that it is susceptible to both false positive and false negative results. In addition, it requires the placement of the neo cassette fairly close to one end of the targeting vector, since PCR efficiency declines as the product size increases. However, it has a tremendous advantage over the restriction map method, since far fewer cells are required and each drug-resistant colony does not have to be independently expanded. This makes the screening process much faster and more efficient.

First Generation Granzyme B Targeting Vector

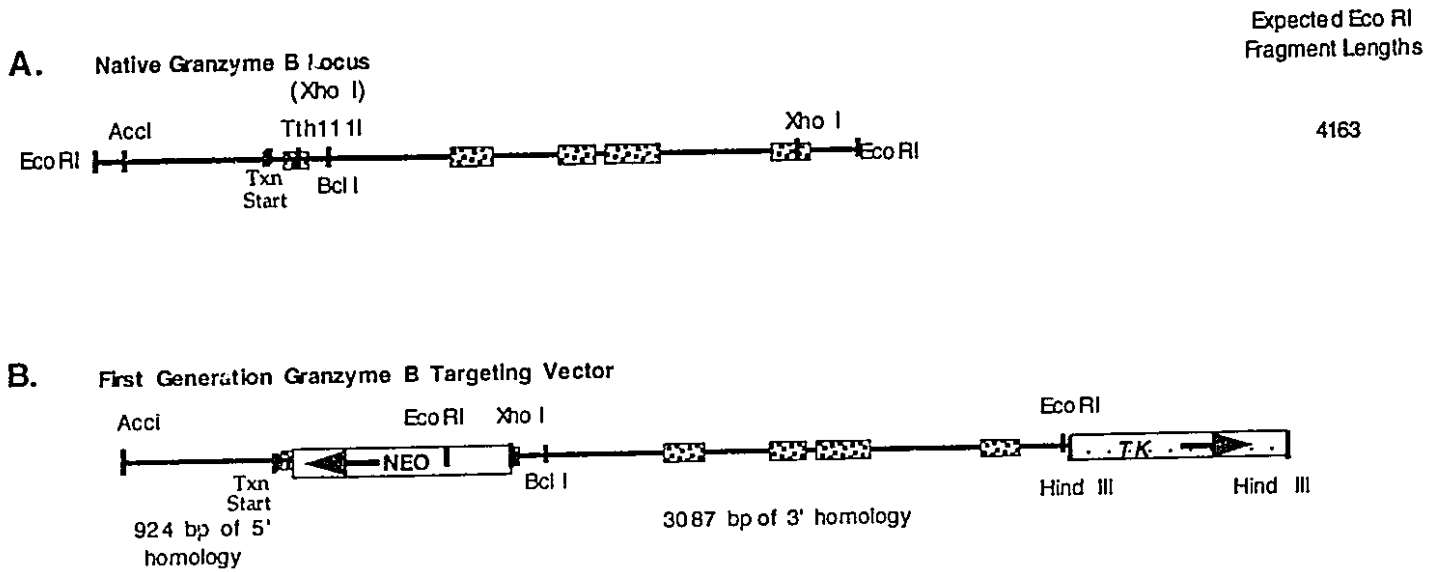
In an initial attempt at targeting the granzyme B gene, we constructed the vector shown in Figure 3-2B. A genomic 4163 bp Eco RI fragment encompassing the entire coding region of the granzyme B gene had been previously sequenced in our laboratory and was used as the starting material for the vector (Figure 3-2A). A pMC1-neo cassette (Thomas and Capecchi, 1987) was inserted into the first exon at a unique Tth111 I site, which we modified to become a Xho I site in order to accept the Xho I/Sal I ends of the neo cassette. The neo gene inserted in the opposite transcriptional orientation to the granzyme B gene. A 153 bp Eco RI-Acc I fragment was removed from the 5' end of the genomic fragment used in order to allow the sequences found in this region to be utilized as a PCR primer site. This resulted in a targeting vector with 4011 bp of total homology with the granzyme B locus, 924 bp of this homology being located on the short (5') arm and 3087 bp on the long (3') arm. PCR primer sites were picked within the neo cassette

Figure 3-2 Design of The First Granzyme B Targeting Vector.

A. Granzyme B Genomic Locus. 4.1 Kb of the granzyme B locus within an Eco RI fragment had been previously sequenced and isolated. Exons are indicated by the stippled boxes and the location of the transcription start site is shown.

B. Granzyme B Targeting Vector. A PMC1-neo cassette was introduced into a modified Tth 111 I site in the first exon of granzyme B, thereby disrupting the coding sequence of the gene. The neo cassette inserted in the optional transcriptional orientation from the granzyme B gene. A small Eco RI-Acc I fragment was removed from the 5' end of the genomic DNA clone. 924 bp of homologous DNA sequence was retained on the short (5') arm of the vector and the long arm was 3087 bp. A HSV-tk gene was added to the 3' end of the vector just outside the region of homology.

C. Expected Structure of Granzyme B Locus After Gene Targeting. Homologous recombination of the targeting vector with the granzyme B gene would result in the introduction of a new Eco RI site found within the neo cassette. This would cause conversion of a wildtype 4163 bp Eco RI fragment to two fragments of 2047 bp and 3262 bp. PCR primer sites within the neo cassette and just outside the region of homology used in the targeting vector are indicated by facing arrows. Homologous recombination between the vector and the granzyme B locus would allow a 1140 bp PCR fragment to be amplified with these two primers.


A.
Native Granzyme B Locus
(Xho I)
Expected Eco RI
Fragment Lengths
AccI
Tth111I
Xho I
Eco RI
Eco RI
4163
Txn
Start
Bcl I
B.
First Generation Granzyme B Targeting Vector
AccI
Eco RI
Xho I
Eco RI
NEO
TK
Txn
Start
Bcl I
Hind III
Hind III
924 bp of 5'
homology
3087 bp of 3' homology
C.
Expected Structure of Locus After Homologous Recombination
AccI
Eco RI
Xho
Eco RI
Eco RI
NEO
Txn
Start
Bcl I
2047 and 3262
1140 bp PCR fragment

and the Eco RI-Acc I fragment found just outside of the 5' end of the targeting vector, such that homologous recombination between the targeting vector and the chromosomal granzyme B locus would allow the amplification of a 1140 bp PCR product (Figure 3-2C). An HSV-tk gene was placed just outside the 3' end of homologous granzyme B sequence to provide negative selection. Since the neo cassette introduces 1146 bp of additional DNA and a new Eco RI site, digestion of genomic DNA from correctly targeted cells would result in the conversion of the wild type 4163 bp Eco RI fragment into two fragments of 2047 and 3262 bp (Figure 3-2C). The diagnostic 2047 bp fragment could then be detected on Southern blots using the 5' EcoR I-Acc I fragment as a probe.

A number of unsuccessful attempts to target the granzyme B gene in mouse ES cells with this vector were made. These attempts will not be documented here. Description of the construction of this first generation targeting vector was given in order to provide background information necessary to understand the design of a more efficient, second generation targeting vector.

Redesign of the Granzyme B Targeting Vector

Many factors were considered in the redesign of the granzyme B targeting vector. First, newly published data indicated that the efficiency of homologous recombination could be significantly enhanced if the targeting vector was made with DNA from the same strain of mouse as the recipient ES cells. Vectors made with isogenic DNA were shown to be anywhere from four or five (Deng and Capecchi, 1992) to twenty (Riele et al., 1992) times more efficient than comparable vectors made with nonisogenic DNA. Second, the relationship between targeting frequency and the length of homologous DNA sequences used in the targeting vector has been controversial. One report had suggested a strong correlation between length of homology and targeting efficiency (Thomas and Capecchi, 1987), whereas another report showed that this effect of length saturated at about 6 kb (Hasty et al., 1991). New data indicated that there was an exponential increase in targeting efficiency with length of homology up to 14-15 kb (Deng and Capecchi,

1992). Furthermore, while one report indicated that vectors having short arms of homology less than 500 bp were as efficient as those with short arms in excess of 1 kb (Hasty et al., 1991), another group reported a reduction in targeting frequency and a propensity for unpredictable rearrangements at the target locus when the short arm contained less than 1 kb of homologous DNA (Thomas et al., 1992). Indeed, short arms of at least 2 kb in length were advised (M. Capecchi, personal communication). Finally, the nature of the neo cassette used for gene disruption and positive selection was considered. We had routinely obtained a far lower number of G418-resistant ES cell colonies when we transfected ES cells with our original granzyme B targeting vector than what we obtained using a granzyme C targeting vector constructed in a similar way with the pMC1-neo cassette (data not shown). This appeared to be consistent with a context-dependency of the promoter used in this construct which other investigators had reported anecdotally. We therefore decided that our new granzyme B targeting vector would utilize a neo disruption/selection cassette with a stronger promoter, flanked by isogenic DNA sequences of as great a length as was feasible, and with the short arm being no less than 2 kb.

Most of the available ES cell lines are of 129 mouse strain origin. We therefore obtained a genomic library constructed in the λ/DASH vector with DNA from a 129/J mouse in order to obtain an isogenic DNA clone. Duplicate filters were pulled from plates containing in excess of 1.5 million plaques, and independently probed with 5' and 3' genomic granzyme B probes. Ten plaques were found to hybridize to both of these probes. Phage from these positive plaques was isolated and replated using an infectivity ratio that resulted in isolated plaques. Secondary screening with both probes resulted in four strongly positive clones being identified. Phage from isolated plaques of each of these clones was generated from liquid lysates and DNA isolated. Digestion with the restriction enzyme Eco RI resulted in the expected 4164 base pair fragment of genomic granzyme B from all four samples (data not shown). Erroneous isolation of a granzyme C

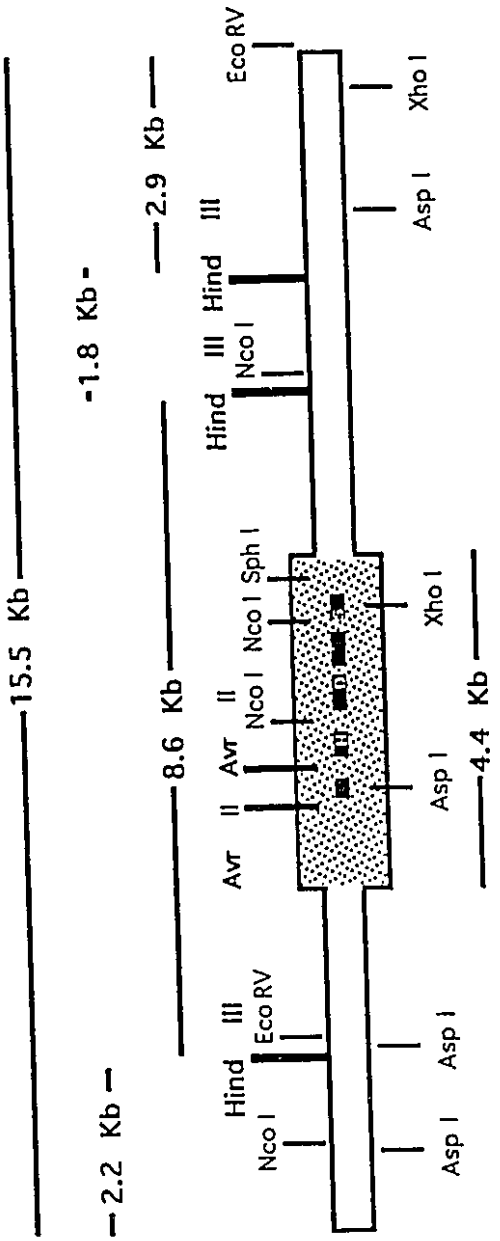
clone by cross-hybridization was therefore ruled out, as an Eco RI digest would have resulted in a smaller fragment of 3893 base pairs being obtained. Also, probing Southern blots of DNA from these four phage isolates with a granzyme C cDNA probe even at low stringency did not detect additional granzyme sequences (data not shown). Of the four plaques isolated, two gave identical fragment patterns with Eco RI and the other two gave distinct, but overlapping patterns. Therefore, three unique inserts were represented, each containing the entire granzyme B gene (data not shown). The approximately 15.5 kb Not I insert of one of these genomic clones, designated gC11-DASH2, was subcloned into the plasmid vector pBluescript II KS$^{(+)}$ (hereafter called pBS). Extensive restriction enzyme mapping, making use of known sites in pBS and the 4.1 kb sequenced region of genomic granzyme B, allowed the generation of the restriction map shown in Figure 3-3.

Second Generation Granzyme B Targeting Vector

The 8.6 kb Hind III fragment containing the granzyme B gene was utilized to provide a large amount of homologous sequence for the new targeting vector. A 350 bp AvrII-AvrII fragment containing all of exon 1, including the initiating ATG codon, and part of the first intron, was removed and replaced with a PGK-neo cassette to provide gene disruption and positive selection with G418 (McBurney et al., 1991). PGK-neo has the advantage over pMC1-neo in that it is driven by the phosphoglycerol kinase promoter, which is reportedly less sensitive to positional effects, and also codes for a neomycin phosphotransferase enzyme that has much higher specific activity, due to the correction of a point mutation present in many neo genes including pMC1-neo (Yenofsky et al, 1990). PGK-neo was inserted in the same transcriptional orientation as the granzyme B gene. PGK-tk, a herpes simplex thymidine kinase gene driven by the PGK promoter, was added at the end of the region of homology to provide negative selection of ES cells ramdomly integrating the targeting vector. Two separate complete targeting vectors were generated, differing only in which end of the homologous DNA PGK-tk was added to. pGB-TV2 had PGK-TK attached to the 3' end of the genomic granzyme B

Figure 3-3 Restriction Enzyme Map of 15.5 Kb Granzyme B Genomic DNA Clone.

The Not I genomic clone obtained from a library made from 129/J mouse strain DNA contained an 8.6 Kb Hind III fragment containing the entire granzyme B gene. The stibbled box indicates the 4.4 Kb region for which sequence information was available and which was used in the construction of the previous targeting vector. The location of the exons are showed by the smaller black boxes and the codons for the His, Asp, and Ser residues of the serine proteinase catalytic triad are indicated in white by H, D, and S, respectively. The extreme 3' Eco RV site is outside the genomic cone obtained and was mapped using isolated genomic DNA.


15.5 Kb
2.2 Kb
1.8 Kb
8.6 Kb
2.9 Kb
4.4 Kb
Hind III
Nco I
Eco RV
Asp I
Avr II
Nco I
Sph I
Xho I
1 cm = 1 Kb

DNA whereas pGB-TV14 had PGK-tk attached to the 5' end. Both of these vectors were utilized in case the position of the negative selection cassette influenced its efficiency (Figure 3-4A).

As shown in Figure 3-4A, our new isogenic granzyme B targeting vectors contained approximately 8.3 kb of total homology with the target locus, with the short arm being about 3.4 kb in length. We decided against utilizing a PCR-based screen method with this vector, since it would be necessary to generate a large amplification product of about 3.5 kb and we were concerned about the efficiency of the PCR reaction on crude genomic DNA preparations with a product of this size. Also, we did not have any sequence information for the locus outside the 4.3 kb central area (designated by the shaded box in Figure 3-3), so an outside PCR primer could not be designed. Instead, we opted to screen drug-resistant colonies solely by Southern blot analysis of restriction enzyme fragments, utilizing a rapid genomic DNA preparation protocol supplied to us by Dr. Mark Appleby (University of Washington). The fragment sizes expected from wild-type and targeted DNA cut with a variety of enzymes are indicated in Figure 3-4.

Analysis of Enrichment Factor by Negative Selection

Experiments were carried out in which the two targeting vectors were electroporated into three different ES cell lines: JS-1, AB-1, and D3J8. 20 μg of vector DNA linearized at the unique Not I site in the pBS multiple cloning site was electroporated into 10^7 ES cells. The surviving cells from each transfection were plated onto SNL-feeder cell layers in three (for JS-1 and AB-1) or four (for D3J8) 10 cm plates and drug selection was applied after two days. All but one of the plates received both G418 and GANC while the remaining plate received only G418 in order to assess the efficiency of negative selection imparted by the PGK-TK gene. After ten days growth in drug-containing medium, surviving ES cell colonies were counted. As seen in Table 3-I, the position of the PGK-tk gene had no impact on the enrichment factor provided by negative selection. In all cases, regardless of the vector or the ES cell line used, addition

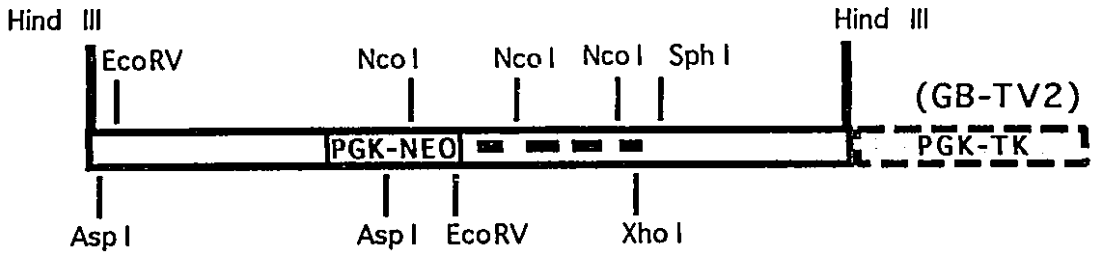
Figure 3-4 Isogenic Granzyme B Targeting Vector and Structure of Wildtype and Modified Locus.

A. New Granzyme B Targeting Vector. An 8.6 Kb Hind III fragment containing the entire granzyme B gene was used to construct an isogenic targeting vector. A PGK-neo cassette replaced an Avr II fragment containg the entire first exon of granzyme B. The targeting vector GB-TV2 is shown, in which a PGK-tk cassette was added to the 3' end of the vector, just outside the region of homology. A second vector, GB-TV14 was also constructed, and differed from GB-TV2 only in that the PGK-tk cassette was added to the 5' end.

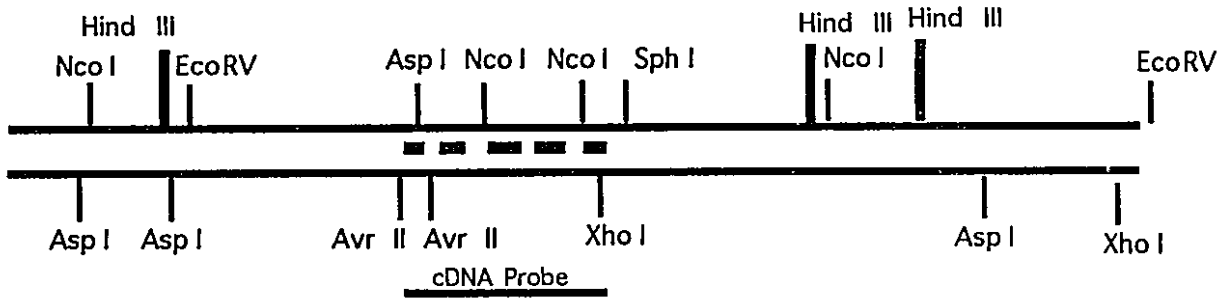
B. Native Granzyme B Chromosomal Locus. The mapped 15.5 Kb granzyme B locus is shown, as are the sizes of a number of restriction enzyme fragments that would be detected with a granzyme B cDNA probe.Location of the exons encoding granzyme B are indicated by small black boxes.

C. Structure of the Granzyme B Locus After Gene Targeting. Homologous recombination between the vector and chromosomal sequences results in the insertion of the PGK-neo cassette, replacing the wildtype Avr II fragment and introducing new Nco I, Asp I and Eco RV sites. The altered restriction enzyme fragment lengths that would be detected with a cDNA probe are indicated at the right.

A Granzyme B Targeting Vector



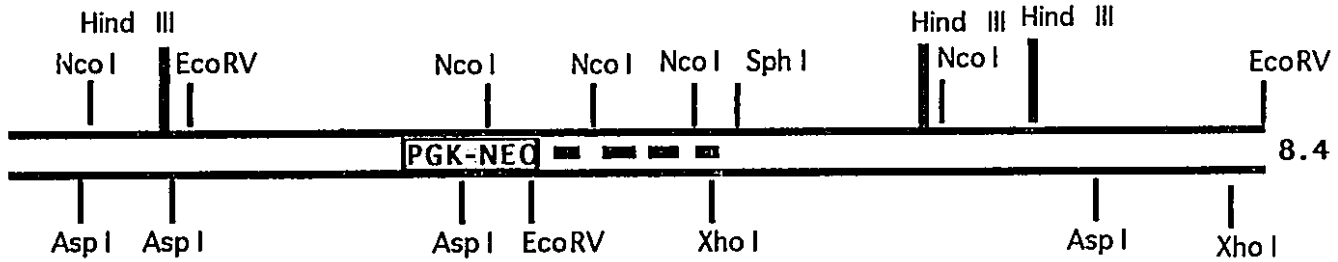
B Native Granzyme B Locus



Restriction Enzyme Fragment Sizes (Kb)

Asp I	Eco RV	Nco I	Hind III
7.5	13	5.5 1.3	8.6

C Targeted Granzyme B Locus



8.4	9.5	5.5 1.3	10.2

Table 3-I

Positive/Negative Selection of ES Cells

Electroporated With Granzyme B Targeting Vectors

	Number of Cells Per Plate				
	G418 + GANC			G418 ONLY	Enrichment Factor
Plate Number:	**1**	**2**	**3**	**4**	
(ES Cell)/ **Vector**					
(AB-1)					
GB-TV2	206	218		430	2.03
GB-TV14	121	134		249	1.95
(JS-1)					
GB-TV2	33	35		65	1.91
GB-TV14	27	31		62	2.1
(D3J8)					
GB-TV2	94	94	119	169	1.7
GB-TV14	102	110	116	178	1.6

of GANC resulted in a 1.6 to 2-fold enrichment. This is surprisingly low, but is similar to the enrichment factor obtained with our first generation granzyme B targeting vector (data not shown). Surprisingly, our granzyme C targeting vectors gave an enrichment factor of between 4 and 8 fold (data not shown). The reason for the low efficiency of negative selection with our granzyme B vectors is unknown. Even when fresh GANC was used and the concentration added to the cultures was doubled from 1 μM to 2 μM (as was the case for the D3J8 cells), the enrichment factor was not increased.

The JS-1 ES cell line gave several fold fewer colonies after transfection and drug selection than the AB-1 and D3J8 cell lines. The reason for this is unknown, but could be due to a number of factors, including differential sensitivity to the electroporation conditions employed (which had been optimized for AB-1 cells) or a reduced capacity for integrating exogenous DNA. Both targeting vectors resulted in almost identical numbers of drug-resistant colonies when JS-1 and D3J8 cells were used. However, when AB-1 cells were transfected, 1.7 times more G418- and G418/GANC-resistant colonies were obtained with pGB-TV2 compared with pBG-TV14. The reason for this difference is not known.

Gene Targeting of Granzyme B in ES Cells

We obtained an aliquot of D3J8 cells from a passage that Dr. Mark Appleby (University of Washington) had confirmed was germline-competent. These cells were electroporated with pGB-TV2 and grown in medium containing G418 and GANC for 10 days. At this time, 200 individual colonies were picked and expanded. A portion of each of the cloned cells were frozen at -70° C, and genomic DNA was isolated from the remainders. An aliquot of each sample containing approximately 10 μg of DNA was digested with the restriction enzyme Asp I. The digests were electrophoresed on 0.8% agarose gels and transferred to nylon membranes in preparation for Southern blotting.

Ideally, screening of Southern blots from transfected ES cells should be done with a probe coming from outside the region used in the construction of the targeting vector. If

a shift in the hybridizing band of the anticipated size is observed with such a probe, it is almost certain that this is due to modification of the locus by homologous recombination of the vector DNA. Unfortunately, every piece of DNA from regions flanking the 8.6 kb Hind III fragment used to construct our targeting vector was not suitable as a probe due to the presence of repetitive DNA elements. After a number of attempts to suppress the hybridization of the repetitive elements in these probes with random sequences on the Southern blots had failed, we were forced to use an internal probe. Probes originating from sequences found within the DNA used in the targeting vector have the disadvantage of hybridizing not only with the endogenous loci, but also with the targeting vector itself, no matter where it integrates. It is therefore possible that false positives may be obtained if a hybridizing band of the anticipated size is fortuitously generated by a random integration event an appropriate distance from a distinct recognition sequence for the restriction enzyme employed. Therefore, a number of different restriction enzymes must be utilized to ensure the clones scored as positive with the initial enzyme continue to give the predicted sized fragments.

The Southern blots of Asp I digests of 192 different clones were hybridized with a granzyme B cDNA probe. Homologous recombinants should show a wildtype band of 7500 bp and a band of 8400 bp representing a targeted allele (see Fig. 3-4). Seven different clones gave a pattern entirely consistent with this prediction and ten additional clones gave a non-wildtype band that was only slightly larger than 8400 bp. Since it was difficult to convincingly determine the exact size of the bands, all seventeen of these clones were designated as potential positives and analyzed further.

DNA samples from each of the the seventeen potentially targeted ES cell clones was separately digested with the restriction enzymes Eco RV, Hind III, and Nco I. As seen in Figure 3-4, correctly targeted DNA digested with Eco RV and probed with granzyme B cDNA should give a 13 kb wildtype band and a reduced band of about 9.5 kb due to the presence of an Eco RV site in the PGK-neo cassette. Hind III should give

the 8.6 kb wildtype fragment and a larger fragment of about 10.2 kb due to the addition of the PGK-neo sequences. Correctly targeted DNA digested with Nco I should look identical to wildtype DNA, since a 5.5 kb fragment would be obtained from both wildtype and targeted loci, due to the removal of the Avr II fragment that PGK-neo replaced and the presence of an Nco I site in the neo cassette at the precise location to generate a band of this size. Wildtype and targeted DNA, as well as DNA with a randomly integrated vector would give an Nco I band of 1.3 kb, due to two additional sites found within the region used to construct the targeting vector. Importantly, no additional bands should be present in correctly targeted DNA that are not present in wildtype DNA, and observation of unexplained fragments would eliminate a clone as being considered a true positive.

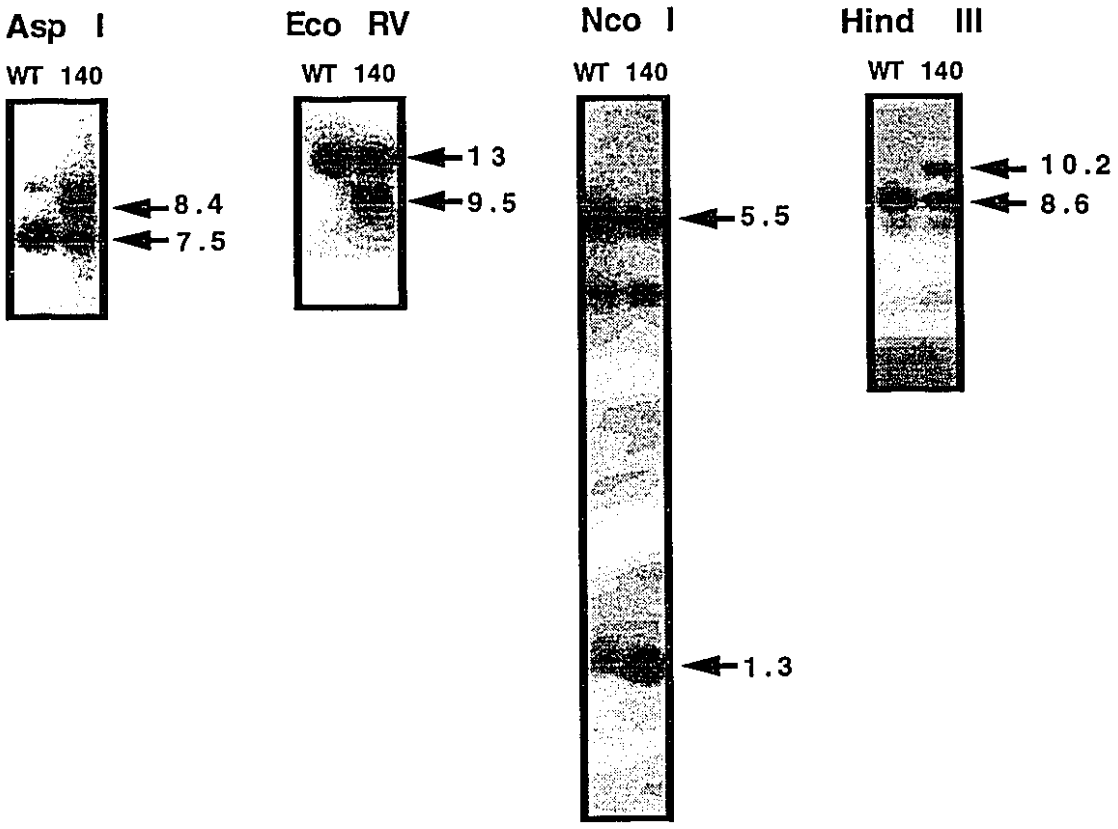
Of the seventeen samples analyzed with the three additional restriction enzymes, five gave results entirely consistent with those predicted for a homologous recombination event. The results of the Southern blot analysis of one of these clones, designated number 140, is shown in Figure 3-5. Reprobing of the Southern blots containing Nco I-digested DNA with a NEO probe confirmed that three of these five samples had the pattern of hybridizing fragments diagnostic of a single targeting vector integrating at the granzyme B locus. However, two of the samples gave additional bands, indicating that more than one vector molecule had integrated into a single genome (data not shown). Therefore, of 192 G418/GANC-resistant colonies tested, three were appropriately targeted, giving a final targeting frequency of 1 in 64.

Granzyme B-Deficient CTL

Before we had determined that we had indeed knocked out the granzyme B gene, Heusel et al. (1994) reported that they had generated granzyme B-deficient mice via gene targeting of ES cells. In light of this development, we decided to discontinue our efforts to produce our own knockout mice, due to the complexity, time, and cost involved in continuing this project. Interestingly, the targeting vector utilized by this group was very

Figure 3-5 Southern Blots of ES Cells Showing Targeted Granzyme B Locus

Genomic DNA was isolated from wildtype (WT) ES cells and GANC/G418 double-selected ES colonies and digested with the indicated restriction enzymes. Southern blots were probed with a granzyme B cDNA probe. Clone number 140 (shown) is representative of a targeted colony, giving the expected bands for each enzyme as shown in Figure 3-4. Both wild-type and targeted bands are obtained from clone 140 since only one granzyme B allele is knocked out.


Asp I
WT 140
8.4
7.5
Eco RV
WT 140
13
9.5
Nco I
WT 140
5.5
1.3
Hind III
WT 140
10.2
8.6

similar to our first vector, in that the 4.1 kb Eco RI fragment containing the granzyme B gene was used as the region of homology. However, isogenic DNA was employed. The same Avr II fragment was removed and PGK-neo inserted, similar to our second generation vector. A targeting frequency of about 1 in 19 was achieved with this vector, which is surprisingly three times more efficient than our own, larger vector. The reasons for our observed low efficiency of targeting are unknown, but may be due to the occurrence of repetitive DNA elements at the ends of our homologous sequences (Gruss et al., 1991). Indeed, a number of our clones gave restriction enzyme patterns that were consistent with the granzyme B locus being disrupted, but with one side of the vector recombining inaccurately (data not shown). These clones were not used in our calculation of targeting frequency.

The granzyme B-deficient mice have provided a significant amount of information regarding the contribution of this protease to CTL-mediated killing. These mice develop normally, as do their immune systems, ruling out a central role in T cell development and the various occurrences of programmed cell death involved in this process. When used as effector cells, alloantigen-stimulated splenocytes lacking granzyme B show a significant but partial reduction in their ability to cause lysis of appropriate target cells in a standard 4 hour ^{51}Cr-release assay. This decrease in cytolysis can be compensated for with extended assay times, reaching normal values by 18 hours. A much greater defect is seen in the ability of granzyme B-deficient CTL to induce target cell DNA fragmentation, a hallmark of cell-mediated killing. Virtually no DNA fragmentation is detected upon exposure of appropriate target cells to granzyme B$^{-/-}$ CTL for four hours, even at high effector to target ratios that result in normal control CTLs fragmenting 40% of the targets' DNA. Extending the assay period to 18 hours reveals a delayed onset of DNA fragmentation which does not completely reach control levels. Therefore, granzyme B is intimately involved with the fragmentation of the target cell

genome observed upon CTL attack, and this DNA digestion appears to contribute towards cell lysis, at least at early time points.

The fact that cell lysis is only partially affected and increased incubation times allows normal levels of cytolysis to be achieved, indicates that while granzyme B may be centrally involved in target cell destruction, it is not indispensable. Presumably, membrane attack by the CTL pore-forming protein perforin is responsible for the significant levels of lysis achieved, even at early time points. The delayed onset of DNA fragmentation observed may be mediated by other serine proteases of the granzyme family found in the cytotoxic granules. Interestingly, work by Greenberg and colleagues (Shi et al., 1992a; Shi et al., 1992b) has recently identified granzyme B as being a potent mediator of a DNA fragmenting activity found in CTL lytic granules. Two other, less potent granule "fragmentins" were also found to be granzymes, and could be involved in the slow target cell DNA fragmentation induced by granzyme B-deficient CTL.

A role for granzyme B in target cell DNA fragmentation is also confirmed by work from Henkart's group. These investigators expressed granzyme A or B in mast cell granules along with perforin. Although perforin alone was cytolytic, it killed target cells in the absence of DNA breakdown (Shiver et al., 1991). Coexpression of a granzyme was necessary for target cell DNA fragmentation to occur and also contributed towards the extent of lysis achieved (Shiver et al., 1992; Nakajima et al., 1994; Nakajima et al., 1995). A role for granzyme B in target cell apoptosis has therefore now been convincingly demonstrated.

How granzyme B effects genome digestion is not known at this time. Recent attention has focused on its ability to cleave and activate the ICE-family protease CPP-32 (Darmon et al., 1995). This Asp-ase has recently been found to play a central point in apoptosis and is responsible for the cleavage of the DNA repair enzyme poly(ADP-robose) polymerase (PARP), an early event in programmed cell death (Nicholson et al., 1995; Tewari et al., 1995). The relationship between CPP-32 activation, PARP cleavage,

and DNA fragmentation is unclear. New evidence from our laboratory indicates that attack by granzyme B-deficient CTL does not lead to target cell CPP32 cleavage and activation as it does in response to normal CTLs, thus confirming the *in vitro* identification of CPP-32 as a granzyme B substrate (A.J. Darmon and R.C. Bleackley, manuscript in preparation). Intriguingly, CPP-32 activation remains undetectable even at late time points, when significant levels of DNA fragmentation begin to occur. This suggests that if other granzymes are involved in the late onset DNA fragmentation seen in target cells exposed to granzyme B-deficient CTL, they accomplish this in a CCP-32-independent manner.

The granzyme B knockout mice have established an important role for this protease in the cytotoxic process. As the biochemical events involved in target cell destruction become increasingly defined, CTL derived from these mice will continue to be valuable tools in the elucidation of these events. We are currently establishing perforin$^{-/-}$ and granzyme B$^{-/-}$ CTL lines in order to test the hypothesis that granzyme B-mediated DNA fragmentation requires perforin in order to gain access into the target cell interior. An alternate explanation for the granzyme dependency on perforin in both Greenberg's *in vitro* fragmentin assay and Henkart's mast cell transfectants is that perforin delivers a second signal to the target cell which synergizes with a granzyme signal initiated at the cell membrane. These two possibilities can be directly tested by individual and co-attack of target cells by CTLs lacking either perforin or granzyme B. We expect both the perforin and granzyme B knockouts to continue to provide us with a wealth of information about the mechanism of CTL-mediated cytolysis for years to come.

CHAPTER FOUR: ANALYSIS OF FYN-DEFICIENT CTL

INTRODUCTION

In general, resting $CD8^+$ T lymphocytes do not possess cytotoxic activity. Acquisition of this effector function requires cellular activation, which often involves proliferation, blast formation, and new synthesis of the proteins that make up the cytolytic machinery. Unlike T helper cell activation and differentiation, very little is known about the signals required for a resting $CD8^+$ T cell to become a fully functional CTL. Although several aspects of T cell activation are surely similar in both $CD4^+$ and $CD8^+$ subsets, there are likely unique features as well. Elucidation of the cell activation process is hindered by its complexity. Activation results from the combined signals derived from a number of different soluble lymphokines, cell-associated accessory and costimulatory molecules, and stimulation of the antigen-specific T cell receptor which is itself composed of multiple signaling modules. How this array of signals is integrated and deciphered by the pre-CTL is not understood. It is also not known if certain of the various activation signals are redundant or if they each affect different aspects of CTL maturation.

Many of the activation signals, such as those delivered by soluble lymphokines like interleukin-2, are antigen non-specific. *In vivo*, it is likely that they are delivered to other cells within a microenvironment and over very short distances, perhaps even across a cell-cell synapse (see Paul and Seder, 1994 for a recent discussion). Another more important aspect of the specificity of CTL activation are the signals generated by the clonotypic, antigen-specific T cell receptor (TCR). Not only does antigen-specific TCR ligation likely deliver important signals that are directly involved in cellular activation, it also causes the T cell to become more responsive to certain lymphokines. For instance, T cell stimulation results in the generation and presentation of the high-affinity IL-2 receptor chain, which greatly increases the sensitivity of the T cell to the effects of this lymphokine. It is therefore often difficult to separate the various signals for CTL activation delivered via the TCR/CD3 complex from those delivered by lymphokines and

accessory molecules on the target cell. Normally, all these signals cooperate during T cell activation. Analysis of T cells that are devoid of specific signal transducing molecules implicated in the various activation pathways may allow dissection of the heterogeneous signals delivered upon T cell stimulation and more closely define the basic requirements for generation of cytotoxic ability.

Signal transduction via the TCR is carried out by the CD3 complex which is composed of at least two autonomous transduction modules (Wegener et al., 1992). CD3 consists of a number of different transmembrane proteins, designated δ, ε γ, and ζ. An alternatively-spliced form of ζ, termed η, can also be present. Both the ζ and ε chains of CD3 have been shown to be capable of transducing signals, and this ability is dependent upon the tandem YXXL Immune Receptor Tyrosine Activation Motif (ITAM) sequences in their cytoplasmic domains (Letourneur and Klausner, 1992; Chan and Weiss, 1994). Since the CD3 γ and δ chains also contain ITAMs, it is possible that they too can relay signals independently, although this has not yet been determined. Initiation of the signalling process is thought to occur by recruitment and activation of src-family tyrosine kinases, including lck and fyn, followed by the syk-family kinase ZAP-70. A current model involves phosphorylation of the tyrosine residues in the CD3 ITAM sequences by lck and/or fyn followed by binding of the dual ZAP-70 SH2 domains to the two phosphorylated tyrosines of an ITAM.

The tyrosine kinase p59fyn exists as two mutually exclusive isoforms as a result of alternative splicing (Cooke and Perlmutter; 1989). Fyn^B (most highly expressed in the brain) differs from fyn^T (found mainly in T lymphocytes) within a short region between the SH2 domain and the beginning of the kinase domain. Both physical and genetic evidence suggests a role for fyn in T cell signalling although its exact role is still unclear. Fyn has been shown to associate with CD3 chains, although at a relatively low stoichiometry (Samelson et al., 1990; Sarosi et al., 1992; Gauen et al., 1992). Even when fyn is overexpressed with a chimeric protein containing the CD3 ζ cytoplasmic domain,

only 1-5% of the fyn can be co-immunoprecipitated with the ζ chimera (Gauen et al., 1992). Overexpression of fyn in thymocytes of transgenic mice resulted in augmented responses signalled via the TCR, including increased tyrosine phosphorylation of cellular proteins, increased magnitude and kinetics of Ca^{2+} mobilization, and increased levels of proliferation (Cooke et al., 1991). Expression of a catalytically-inactive form of fyn functions as a dominant negative in thymocytes, resulting in diminished responses (Cooke et al., 1991). In many of these responses both fyn^B and fyn^T seem to function equally well. However, although overexpression of either isoform of activated fyn in a T cell hybridoma resulted in an enhancement of tyrosine phosphorylation of cellular proteins and IL-2 production in response to anti-TCR antibodies, only fyn^T resulted in enhanced IL-2 production in response to appropriately presented specific antigen (Davidson et al., 1992). The different catalytic domain of fyn^T and not the altered SH2 domain was later shown to be responsible for its enhanced function in T cells, as compared to fyn^B (Davidson et al., 1994). Fyn^T was also shown to be more adept than fyn^B at mobilizing intracellular Ca^{2+} in these studies (Davidson et al., 1994). Therefore, a role for fyn in TCR-mediated Ca^{2+} fluxes and IL-2 production has been suggested.

Recently, mice devoid of both isoforms of fyn (Stein et al., 1992) or the T isoform specifically (Appleby et al., 1992) have been generated by gene targeting technology. It was observed that while some aspects of T cell activation are impaired in thymocytes of fyn-deficient mice, mature splenocytes seem to reacquire significant signaling ability. Although overall tyrosine phosphorylation of cellular proteins is still significantly reduced and Ca^{2+} fluxes are blunted in fyn^- splenocytes stimulated by anti-CD3 antibody, they are still able to proliferate relatively normally. Interestingly, Appleby et al. (1992) observed a less-complete reacquisition of proliferative capabilities in fyn-negative splenocytes than did Stein et al. (1992). The reason for this discrepency is unknown. Also, Stein et al. (1992) observed a significant reduction in IL-2 production in fyn-

deficient splenocytes stimulated with anti-CD3. This supports the suggestion of Davidson et al. (1992) that fyn is involved in Ca^{2+} mobilization and IL-2 production.

It appears, therefore, that fyn may not play a unique or indispensable role in some aspects of mature T cell activation since many responses to TCR ligation that are severely affected in fyn-deficient thymocytes are reacquired by splenocytes. However, although fyn-deficient splenocytes responded better than thymocytes, the fact that responses were still often reduced compared to wild type controls does suggest that fyn may contribute to TCR signaling.

Only some aspects of T cell signaling and activation have been studied in fyn-deficient mice. Neither of the groups who generated fyn^{null} mice investigated the generation or function of CTL responses. Since the activation requirements for generation of cytolytic potential in CTLs has not been well defined, we decided to investigate if p59fyn plays a role in this process. Utilizing the fyn^{T} knockout mice created by Appleby et al. (1992), we studied both cellular and molecular aspects of the CTL response to anti-CD3 antibody or alloantigen. Little difference in CTL generation or function was observed in fyn-negative splenocytes, although a surprising finding was that fyn appears to influence the lifespan of the activated lymphocytes in culture.

RESULTS

Generation of Cytotoxic Ability in $fyn^{-/-}$ vs $fyn^{+/+}$ Splenocyte Cultures

Splenocytes from $fyn^{-/-}$ or $fyn^{+/+}$ ($H2^{b}$) mice were isolated and stimulated *in vitro* with either anti-CD3 antibody or in a mixed lymphocyte reaction (MLR) with alloantigen (irradiated $H2^{d}$ splenocytes from Balb/c mice). Over a week-long time course, the cytotoxic activity of the CTL's generated by both stimulation regimens was determined daily against the P815 ($H2^{d}$) tumour target cell line that had been loaded with ^{51}Cr. Lysis of the target cells was determined by specific release of ^{51}Cr into the medium, indicating loss of membrane integrity and leakage of cytoplasmic components. For each day, a range of effector to target ratios was employed, and lytic units were

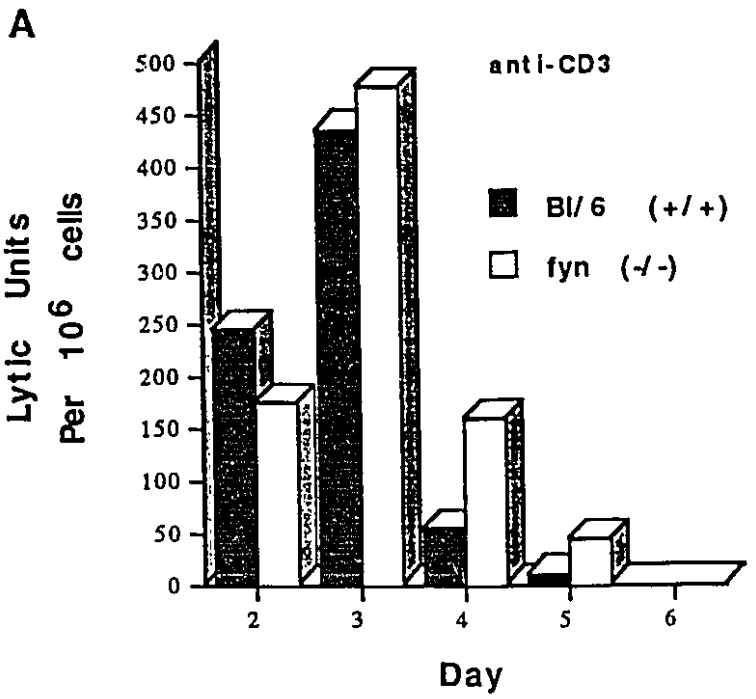
calculated from the per cent specific lysis data obtained. This allows a more accurate representation of cytotoxic potential in the cultures than achieved by simply reporting per cent specific lysis.

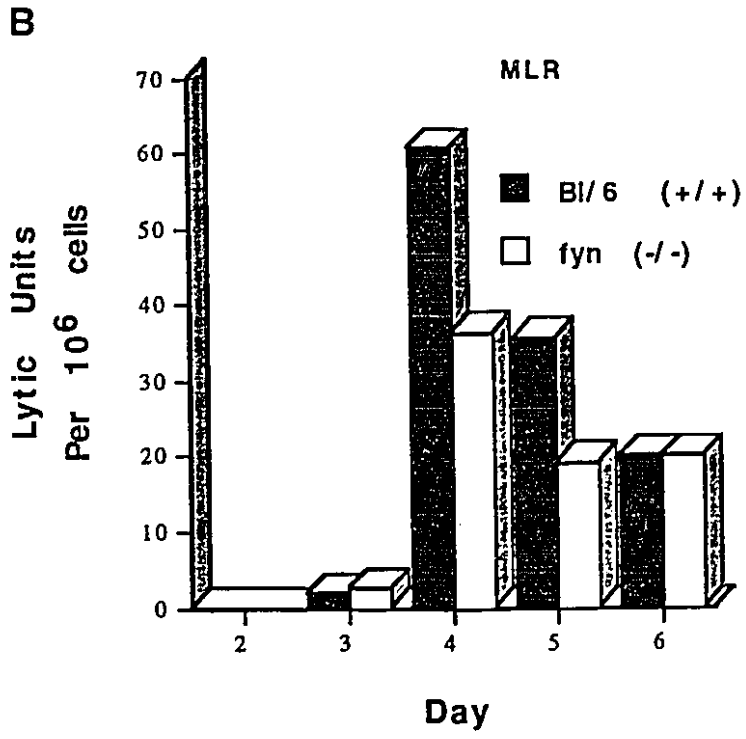
Acquisition of lytic activity followed similar kinetics in both wild-type and fyn-deficient splenocyte cultures stimulated with either anti-CD3 or alloantigen (Figures 4-1A and B). The time after stimulation that detectable cytolytic activity was obtained, peaked, and began to decline was identical for both normal and mutant cultures. Therefore, the kinetics of induction of lytic activity were not affected by the fyn-null mutation. Since anti-CD3 antibody strongly activates virtually all the T cells in the culture, responses to this type of stimulation are generally faster and more intense than those achieved by alloantigenic stimulation, which stimulates only those T cells possessing TCRs capable of recognizing allo-specific epitopes. Therefore, while significant cytotoxic activity against P815 targets was seen by day two and peaked at day 3 after anti-CD3 stimulation, lytic activity stimulated by alloantigen first became detectable on day 3 and peaked on day 4.

Early in the time course, the control cells appeared to have higher levels of killing activity compared to the $fyn^{-/-}$ cells. On day 2 after anti-CD3 stimulation, $fyn^{-/-}$ cultures exhibited about 70% of the lytic activity against P815 compared to wildtype controls, and day 4 alloantigen-stimulated fyn-deficient cultures exhibited approximately 60% of wildtype lytic activity. However, once the peak of lytic activity had been achieved and began to decline, the $fyn^{-/-}$ CTL appeared to lose lytic activity considerably slower than control cells. Therefore, on day 4 and 5 after anti-CD3 stimulation, the more rapid decline of the wildtype lytic activity resulted in the fyn-deficient cultures exhibiting greater cytolytic potential. In alloantigen-stimulated cultures, the faster decline of the wildtype lytic activity resulted in the fyn-deficient cultures displaying comparable levels of activity against P815 on day 6. These results suggest that fyn is not absolutely required for resting CTL to gain cytotoxic function, but it may contribute in this process, since fyn-deficient

Figure 4-1 Lytic Activity of Normal and Fyn-Deficient CTL.

Splenocytes from normal (black bars) or fyn-knockout (white bars) were stimulated in culture with either anti-CD3 antibody (A) or alloantigen (B). Their ability to lyse the allogeneic target cell P815 was tested daily over a week-long timecourse in a ^{51}Cr-release assay. Lytic Units were calculated by plotting the specific lysis data obtained from a number of different E:T ratios on a semi-log graph and deducing the degree of lysis achieved by a million cells. The data shown are typical of those obtained from four separate experiments.


A
anti-CD3
Bl/6 (+/+)
fyn (-/-)
Lytic Units
Per 10^6 cells
500
450
400
350
300
250
200
150
100
50
0
2
3
4
5
6
Day


B
MLR
Bl/6 (+/+)
fyn (-/-)
Lytic Units
Per 10^6 cells
70
60
50
40
30
20
10
0
2
3
4
5
6
Day

cultures had slighty less cytotoxic ability in the early days after stimulation. They also suggest that fyn may play some role in the downregulation of the cytotoxic response, since loss of effector function appeared to occur more slowly in the absence of this tyrosine kinase.

Induction of Granzyme B and Perforin Message in $fyn^{-/-}$ vs $fyn^{+/+}$ CTL

In order to further evaluate the generation of CTL effector function in $fyn^{-/-}$ splenocyte cultures, we decided to investigate if genes encoding cytolytic effector molecules were induced normally in response to TCR stimulation. RNA was isolated from anti-CD3- or alloantigen-stimulated $fyn^{-/-}$ and $fyn^{+/+}$ splenocytes over a time course. Northern blot analysis was performed using granzyme B and perforin cDNA as probes. As discussed previously, these genes encode proteins that are important in the cytotoxic effector function of CTL, and they are normally induced upon TCR stimulation of $CD8^+$ lymphocytes. The amount of RNA loaded per lane was shown to be equivalent by probing the filters with a ribosomal protein cDNA probe.

As with cytotoxicity, the kinetics of granzyme B and perforin message induction was similar in $fyn^{-/-}$ and $fyn^{+/+}$ cells stimulated by either regimen (Figure 4-2 and data not shown). Therefore, the ability to signal transcription from the cytotoxicity-related perforin or granzyme B genes does not appear to require the presence of p59fyn kinase activitiy.

Comparison of T Cell Subsets

One explanation for any differences in cytotoxicity observed between the $fyn^{-/-}$ and the $fyn^{+/+}$ cultures could be that different numbers of $CD8^+$ CTLs were induced upon stimulation, so actual numbers of effector cells assayed may have been different. In order to test this, cells from the anti-CD3 and alloantigen-stimulated cultures were removed daily and analyzed for surface expression of CD4 and CD8 subset markers by flow cytometry (Table 4-I). After anti-CD3-stimulation, very little difference was seen in the percentage of cells staining for CD8, although $fyn^{-/-}$ cultures appeared to maintain

Figure 4-2 Induction of Granzyme B and Perforin RNA in Anti-CD3 Stimulated Splenocytes from Normal and Fyn-Deficient Mice.

Total RNA was purified from resting (day 0) and anti-CD3 stimulated splenocytes from normal (+) and fyn knockout (-) mice. RNA was electrophoretically separated on a formaldehyde-agarose gel, blotted onto nylon filters and hybridized overnight with randomly-primed cDNA encoding the entire granzyme B and perforin genes at 42 C in hybridization buffer containing 50% formamide. The filter was then washed up to a stringency of 0.1 x SSC, 0.1% SDS at 55 C for 15 minutes and exposed to X-ray film for 24 hours at -70 C. After stripping, the filter was reprobed under the same conditions with randomly primed rpl32 cDNA (from Dr. R. Perry, Fox Chase Cancer Center, Philadelphia) encoding a ribosomal protein to indicate the degree of RNA loading per lane. RNA from the mouse CTL line MTL was included as a positive control.

These results are representative of 4 different experiments. Also, RNA from alloantigen-stimulated splenocytes was analyzed in the same way and gave similar results, differing only in that strong induction of both granzyme B and perforin lagged by one day and first occured on day 3 after stimulation.

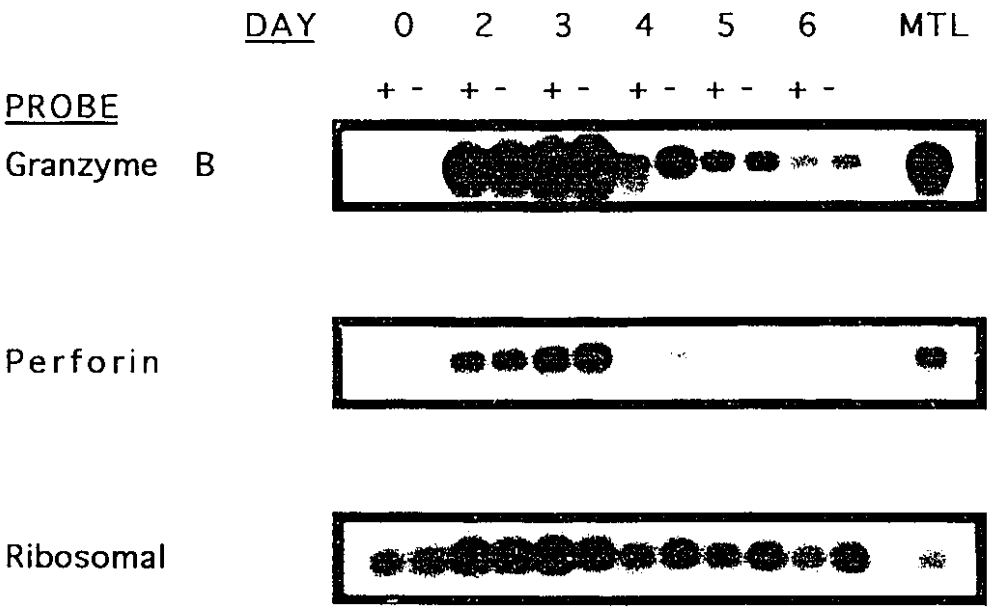

DAY 0 2 3 4 5 6 MTL
+ - + - + - + - + - + -
PROBE
Granzyme B
Perforin
Ribosomal

Table 4-I

Comparison of CD4+ and CD8+ Cell Levels in Stimulated Splenocytes from Normal and Fyn-Deficient Mice

Alloantigen Stimulation (MLR)

	% CD4+		% CD8+	
DAY	fyn +/+	fyn -/-	fyn +/+	fyn -/-
0	29.4	31.0	19.4	14.4
2	19.2	24.5	31.7	32.8
3	21.0	27.0	35.0	35.2
4	15.9	26.0	61.0	49.0
5	9.5	22.5	77.2	65.7
6	9.2	20.9	81.1	67.0

Anti-CD3 Stimulation

	% CD4+		% CD8+	
DAY	fyn +/+	fyn -/-	fyn +/+	fyn -/-
0	29.4	31.0	19.4	14.4
2	20.8	26.0	39.0	39.9
3	17.7	24.9	75.4	66.4
4	13.6	20.1	87.9	82.7
5	11.3	19.7	87.6	87.4

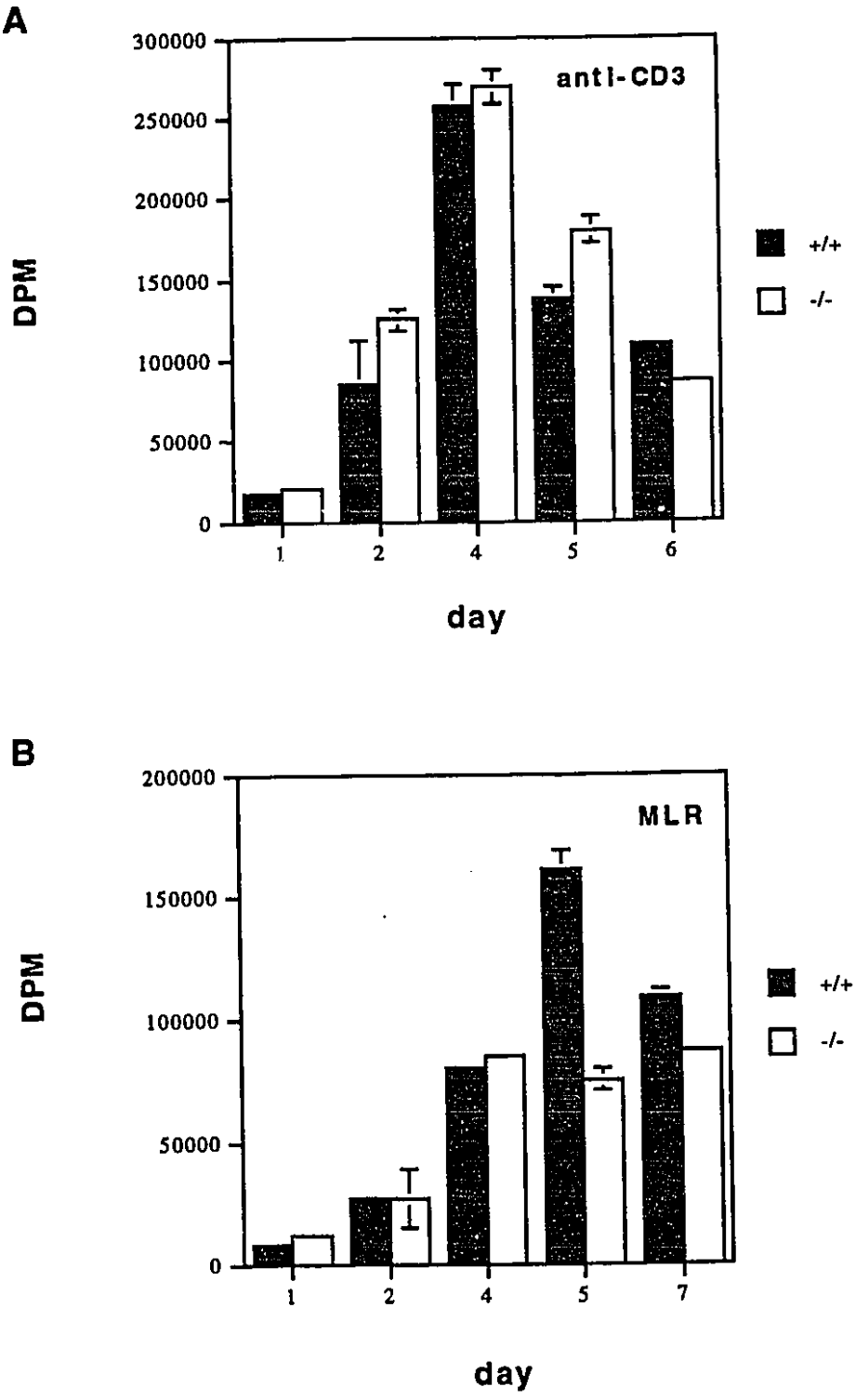
more $CD4^+$ cells over the time course than $fyn^{+/+}$. In alloantigen-stimulated cultures, $CD8^+$ staining levels seemed comparable between $fyn^{+/+}$ and $fyn^{-/-}$ cells for the first 3 days in culture. After that, however, the $fyn^{+/+}$ showed slightly more CD8 staining than $fyn^{-/-}$. As with anti-CD3 stimulation, the alloantigen stimulated $fyn^{-/-}$ splenocytes exhibited less of a decline in the $CD4^+$ cells over the time of the experiment. Therefore, although lower numbers of $CD8^+$ CTL effectors may partially account for the decreased cytotoxicity in day 4 and 5 $fyn^{-/-}$ MLRs, it cannot explain why day 6 MLR cytotoxic levels were equivalent in wild-type and fyn-deficient cultures. It also does not explain the elevated levels of cytotoxicity in day 4 and 5 anti-CD3-stimulated fyn-deficient cultures since the proportion of $CD8^+$ cells was equivalent between the two cells types.

Proliferation and Viability of $fyn^{-/-}$ and $fyn^{+/+}$ Splenocytes

The proliferation of wild-type and fyn-deficient splenocytes in response to stimulation with anti-CD3 or alloantigen was compared by performing thymidine-uptake assays (Figures 4-3A and B). Over a week-long time course, splenocytes were removed from the stimulated bulk cultures and assayed for overnight incorporation of ^3H-thymidine. The proliferation of anti-CD3 stimulated cells appeared relatively normal throughout the timecourse (Figure 4-3A). If anything, fyn-deficient cultures exhibited slightly increased thymidine uptake compared to controls. Interestingly, this was also seen by Stein et al. (1992) but is not in agreement with Appleby et al. (1992) who observed somewhat diminished proliferative responses to anti-CD3 in $fyn^{-/-}$ splenocytes. Alloantigen-stimulated fyn splenocytes exhibited normal proliferative responses up to and including day 4 after stimulation (Figure 4-3B). On day 5, however, wildtype cells appeared to proliferate more than fyn-deficient cells, although this difference was beginning to equalize by day 7. In agreement with Appleby et al. (1992) then, fyn-deficient splenocytes proliferated relatively normally although perhaps slightly less, in response to alloantigen. On the whole, loss of p59fyn activity did not seem to greatly affect the ability of the splenocytes to proliferate in response to either type of stimulation.

Figure 4-3 Proliferation of Activated Splenocytes from Normal and Fyn-Deficient Mice.

Splenocytes from normal (black bars) or fyn knockout (white bars) mice stimulated with anti-CD3 antibody (A) or alloantigen (B) were cultured in 96-well plates overnight in medium containing ^{3}H-thymidine and then harvested onto glass filters with extensive washing using a cell harvester. The amount of thymidine incorporated into the cells' DNA was measured by scintillation counting. Each data point is the average of at least 5 replicate samples, and the data shown are representative of 3 separate experiments.


A
300000
250000
200000
150000
100000
50000
0
DPM
anti-CD3
+/+
-/-
1
2
4
5
6
day
B
200000
150000
100000
50000
0
DPM
MLR
+/+
-/-
1
2
4
5
7
day

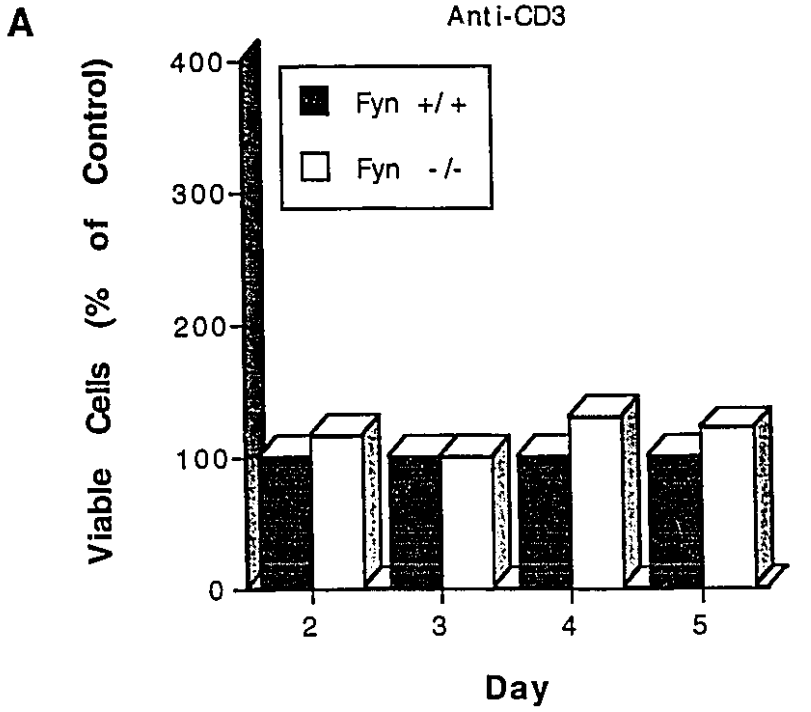
At the same time, daily counts of viable cells in the cultures were made. Cell number and viability was assessed on the basis of exclusion of the vital dye eosin and counting live cells under a light microscope (Figure 4-4A and B). For the first week after stimulation, fyn-deficient splenocytes stimulated with either anti-CD3 or in an MLR exhibited essentially normal growth characteristics. After 6 days of anti-CD3 stimulation, both cell types died off, a common feature of splenocytes stimulated in this way. The alloantigen cultures were allowed to grow untouched for 17 days, when the number of live cells was again assessed. Remarkably, there was an almost 3-fold greater number of live cells in the $fyn^{-/-}$ MLRs than in the wild-type MLRs (Figure 4-4B). The cells in the fyn-deficient cultures also looked healthier than those in the $fyn^{+/+}$ cultures, being larger, rounder, smoother, and brighter under the light microscope. At this point, both cultures were equilibrated to equal concentrations of viable cells and restimulated with irradiated allogeneic spleen cells. Over the next ten days after the secondary stimulation, the fyn-deficient cells continued to remain more viable than their normal counterparts.

Cloning Efficiency of $fyn^{-/-}$ vs $fyn^{+/+}$ Splenocytes

The ^{3}H-thymidine proliferation assays suggested that MLR-stimulated fyn-deficient splenocytes proliferated at a rate comparable to control splenocytes. This was reflected in equivalent viable cell numbers being assessed in the first week of MLR culture. It was therefore difficult to explain the large difference in the number of viable cells at later time points on the basis of differential growth rates. One explanation for the elevated numbers of live cells in the fyn-deficient cultures, however, was that loss of fyn activity somehow reduced the rate of death in alloantigen-stimulated T cells. This possibility was tested by determining the cloning efficiency of the T cells from $fyn^{+/+}$ and $fyn^{-/-}$ MLRs (Figure 4-5). After 7 days of primary stimulation, splenocytes were removed from the cultures and transferred into 96-well plates at densities of 5 or 10 cells per well, where they were restimulated with irradiated Balb/c allogeneic splenocytes. Most stimulated primary T cells die in culture, a phenomenom which likely reflects the

Figure 4-4 Growth Record of Stimulated Splenocytes from Normal and Fyn-Deficient Mice.

Splenocytes from normal (black bars) or fyn knockout (white bars) mice were stimulated with either anti-CD3 antibody (A) or alloantigen (B). The number of viable cells in the cultures were counted on the indicated days using eosin staining and a light microscope. On day 17, the alloantigen-stimulated cultures were equilibrated and restimulated with alloantigen (arrow).


A
Anti-CD3
Viable Cells (% of Control)
400
300
200
100
0
Fyn +/+
Fyn -/-
2
3
4
5
Day

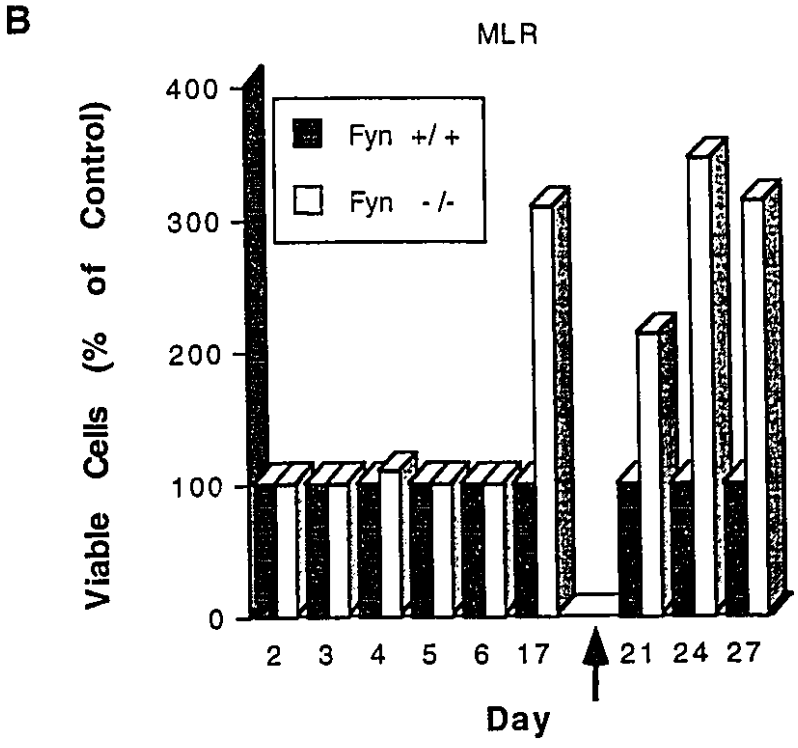
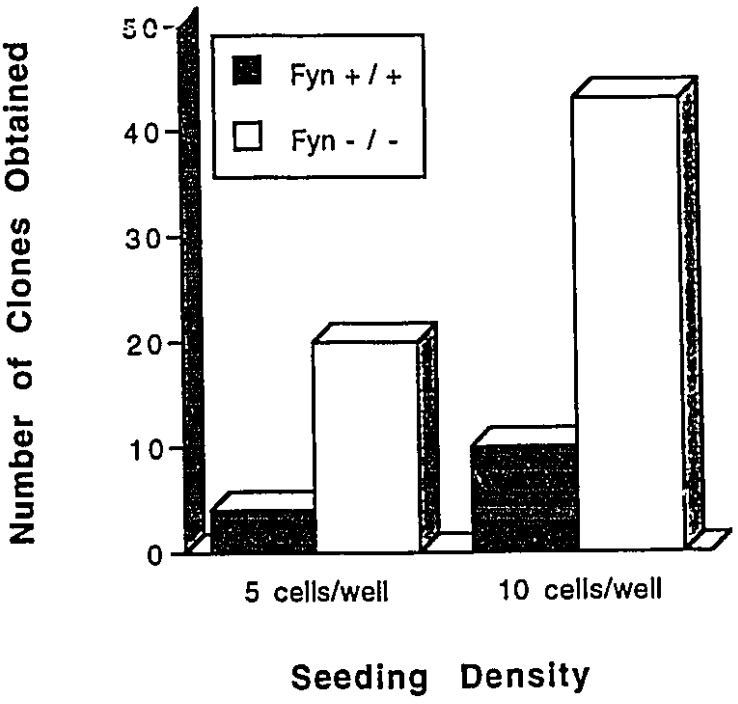

B
MLR
Viable Cells (% of Control)
400
300
200
100
0
Fyn +/+
Fyn -/-
2 3 4 5 6 17 21 24 27
Day

Figure 4-5 Cloning Efficiency of Alloantigen Stimulated Splenocytes from Normal and Fyn-Deficient Mice.

Cells were removed from the primary MLRs on day 7, set up at the indicated densities in 96-well plates, and stimulated with 5 x 10^5 irradiated Balb/c splenocytes per well. Plates were scored for growth 7 days after plating. The results are typical of 4 separate cloning experiments from independent MLRs.


Number of Clones Obtained
50
40
30
20
10
0
Fyn + / +
Fyn - / -
5 cells/well
10 cells/well
Seeding Density

body's normal requirement to eliminate expanded clones of activated T cells once the immune system has dealt with an infection. Therefore, obtaining T cell clones with the ability for prolonged survival *in vitro* is a rare event and generally requires many cells to be stimulated to recover a small number of viable clones. Four separate cloning experiments were carried out, and in each case, although actual numbers varied, an increased cloning efficiency of $fyn^{-/-}$ T cells of approximately 5:1 was obtained. Therefore, these results strongly implicate a role for fyn in the regulation of T cell lifespans.

Since T cell death is generally accomplished via apoptosis, it was possible that the increased lifespans of fyn-deficient splenocytes was due to decreased levels of apoptosis. Death by apoptosis is usually accompanied by fragmentation of a cell's DNA into an oligonucleosomal ladder. Therefore, as a measure of apoptosis the extent of DNA fragmentation occuring in $fyn^{-/-}$ and $fyn^{+/+}$ alloantigen-stimulated splenocytes was assessed over a time course (Figure 4-6). Genomic DNA isolated from an equal number of fyn-deficient and control cells was electrophoresed on an agarose gel and then stained by ethidium bromide and visualized on a UV light box. At all time points, the extent of DNA degradation into a nucleosomal ladder was far greater in control cells than in $fyn^{-/-}$ cells. This suggests that loss of fyn activity coincides with a reduction in apoptosis.

Although the DNA fragmentation gel gave a strong indication that apoptosis was reduced in fyn-deficient MLR cultures, it is not a quantitative method. We therefore attempted to assess the differences in apoptosis in a more quantitative manner. The extent of DNA fragmentation was determined by labelling nicked DNA with biotin-dUTP followed by avidin-fluorescein isothiocyanate *in situ* from day 14 MLR cultures and analyzing the degree of fluorescence via flow cytometry (Figure 4-7). Cells that exhibited fluorescence above background were determined to be apoptotic, and the intensity of fluorescence indicated the extent of DNA fragmentation in each cell. There was a 1.5 fold decrease in the number of apoptotic $fyn^{-/-}$ splenocytes in culture, compared to controls.

Figure 4-6 Alloantigen-Stimulated $fyn^{-/-}$ Splenocytes Exhibit Reduced Apoptosis in Culture.

Pictured is a typical DNA fragmentation gel of ficol-purified normal (+/+) and fyn-deficient (-/-) splentocytes from various days of mixed lymphocyte culture. Each lane contains the DNA from 1 million cells. DNA was visualized by ethidium bromide staining and UV illumination. The results shown are typical of three separate experiments.

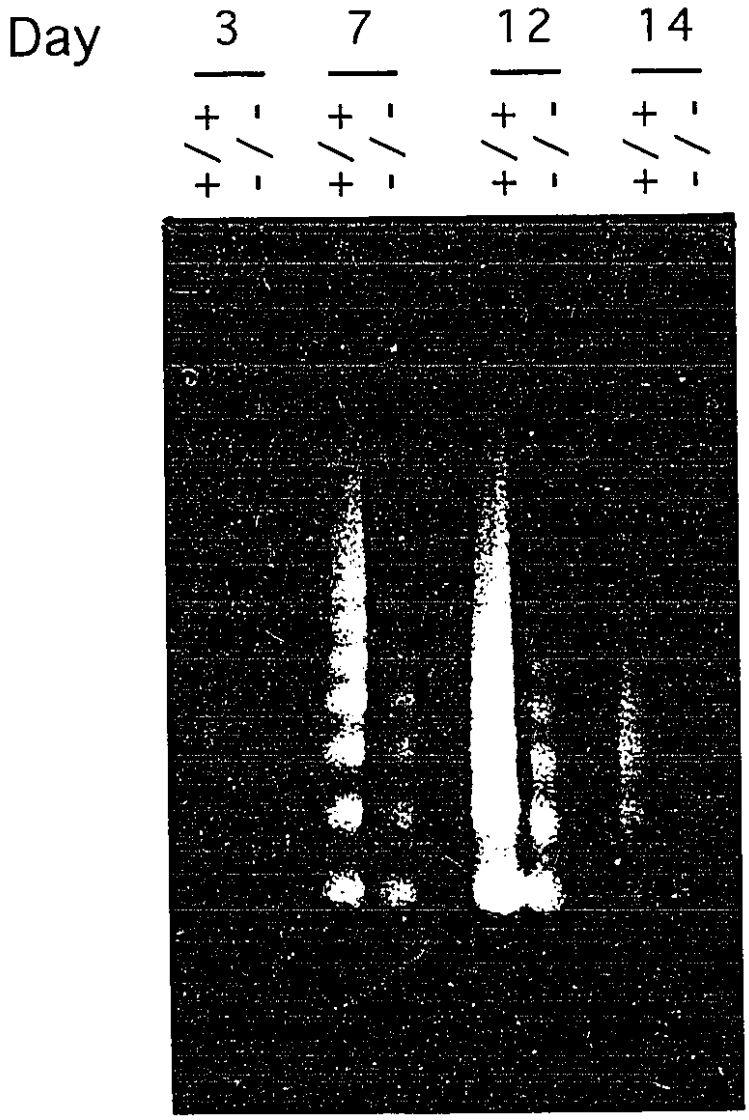
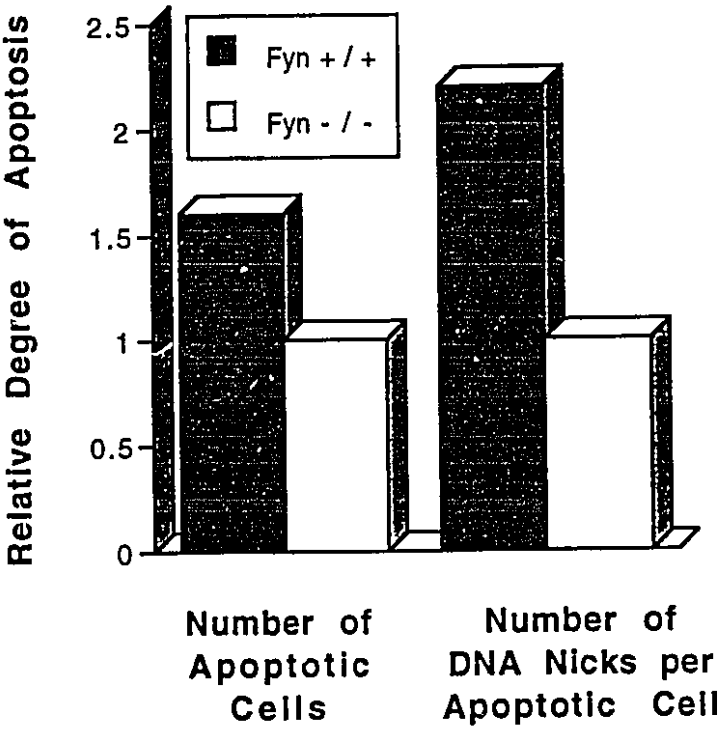

Day
3
7
12
14
+
+
+
+
+
+
+
+

Figure 4-7 Quantitation of Apoptosis in Normal and Fyn-Deficient Mixed Lymphocyte Cultures.

Splenocytes were obtained from day 14 MLCs and their nicked DNA was fluorescently labelled via *in situ* nick translation with biotin-dUTP followed by avidin-fluorescein isothiocyanate. The number of cells with labelled DNA nicks and the number of DNA nicks per cell was quantitated via flow cytometry. This experiment was performed once.


Relative Degree of Apoptosis
2.5
2
1.5
1
0.5
0
Fyn + / +
Fyn - / -
Number of Apoptotic Cells
Number of DNA Nicks per Apoptotic Cell

In addition, in those cells that were determined to be apoptotic, there was a greater than 2 fold reduction in fluorescence in $fyn^{-/-}$ cells, indicating that there were less than half the number of DNA nicks per apoptotic fyn-negative lymphocyte as compared to apoptotic control cells. Although the difference between the mutant and normal cells was relatively modest using this method, the DNA fragmentation gels reveal this time point to show a less dramatic disparity in DNA fragmentation. Nonetheless, these results confirm the observation of a reduction in fyn-deficient T cell apoptosis.

DISCUSSION

Activation of T cell effector function is a complex process resulting from the combined effect of multiple signals. Unique contributions of certain signaling molecules towards various aspects of cellular activation may be revealed by analyzing a variety of activation responses in their absence. The experiments described in this chapter were aimed at investigating if the tyrosine kinase p59fyn had a unique role in the generation of cytolytic effector function. Stimulation of splenocytes with alloantigen versus anti-CD3 antibodies has been shown to result in differences in the activation of granzyme gene transcription (Prendergast et al., 1992), and also revealed a functional difference between fyn^T and fyn^B (Davidson et al., 1992). Therefore, we utilized both types of stimulation regimens in case unique contributions of fyn might be masked or revealed by one or the other.

Fyn-deficient splenocytes acquired cytolytic capabilities with similar kinetics after either mode of stimulation. Although both mutant and wild-type cultures exhibited their peak of cytotoxicity at the same time, the fyn-deficient cells were not able to kill allogeneic target cells quite as efficiently as wild-type splenocytes. Therefore, while not strictly required for the generation and delivery of cytotoxicity, the fyn kinase may contribute towards this process.

The exact contribution of fyn towards the generation of cytolytic ability of CTLs was not determined. It does not appear to involve the signals required for transcription of

the genes that encode either perforin or granzyme B, two molecules known to be important members of the cytotoxic machinery. Both these genes were activated with normal kinetics and to an extent comparable to wild type. Nor does fyn appear to be involved in the expansion of the $CD8^+$ subset of T cells, as this too was relatively normal in fyn-deficient mice. It is possible that the actual delivery of cytotoxic granules to the target cell is impaired in $fyn^{-/-}$ CTL, and this possibility was not addressed. However, preliminary data from experiments recently performed in our laboratory suggest that degranulation of fyn-deficient splenocytes triggered by immobilized anti-CD3 is essentially normal (data not shown)

It is possible that fyn normally does play a role in one or more aspects of CTL activation, but that it is compensated for by other src family kinases in its absence. This appears to be a possibility in other responses, such as proliferation and Ca^{2+} mobilization, which are blunted in fyn-deficient thymocytes, but are significantly reacquired upon maturation into splenocytes. Since the splenic responses are still sensitive to tyrosine kinase inhibitors (Appleby et al., 1992), compensation by a functionally-redundant kinase seems likely.

A compensating kinase may not be quite as efficient at delivering the appropriate signals as fyn. Alternatively, fyn may normally function to simply enhance and contribute towards the phosphorylations of the same cellular substrates as other kinases. This may actually be the case, since lack or overexpression of fyn does not alter the pattern of phosphorylated proteins, only the degree to which they are phosphorylated (Cooke et al., 1991; Stein et al., 1992). In either case, the observed similarity in the kinetics of cytotoxic acquisition would be expected. Furthermore, a slight reduction in the amount of cytolytic potential, as observed, would not be surprising. Therefore, due to the possibility of a functionally redundant kinase compensating for the lack of fyn, a normal role for fyn in the generation of cytotoxicity could be masked. Compensation by other src-family members for each other has recently been demonstrated by producing mice

with null mutations in two family members. Alone, a single src-kinase knockout often gives an imperceptable phenotype, but when expressed within the context of a null mutation in another member, severe abnormalities in a number of processes are revealed. Therefore, determination of the role of fyn in a complex process such as the generation of cytotoxicity may require the utilization of a double knockout mouse in which another src kinase gene is also ablated.

An interesting phenotypic alteration in fyn-deficient MLR cultures was the increased longevity of the lymphocytes. The higher cell numbers in long-term cultures and the elevated cloning efficiency cannot be explained by a concomitant increase in proliferation. However, the reduction in the amount of apoptosis, as assessed by DNA fragmentation, in $fyn^{-/-}$ cultures was striking. These observations suggest that a unique role of fyn may be to control T cell lifespans by affecting their ability to undergo apoptotic death.

In light of these findings, it is interesting to note that in a recent investigation of the neural effects due to ablation of both fyn isoforms, an increased number of granule cells in the dentate gyrus and of pyramidal cells in the CA3 region of the brains of $fyn^{-/-}$ mice was found (Grant et al., 1992). This effect was not observed in mice with other src-family kinase genes knocked out, including the genes coding for src and yes. Since it is believed that the final number of pyramidal cells in the CA3 region of the brain is determined by cell death (Boss et al., 1987), it was suggested that one possible mechanism that could account for this increase in neuron number could be a failure of cell death. Therefore, it appears that fyn activity in the brain and in T lymphocytes, the two sites that express fyn in greatest abundance, is involved in regulating the number of mature cells allowed to persist.

Elimination of activated T cells is important in maintaining homeostasis. It is now believed that co-expression of the cell surface death protein Fas, along with Fas ligand, on activated T cells is responsible for destroying most of the T cells generated in an

immune response. Ligation of Fas with Fas ligand delivers a signal to the T cell to initiate apoptosis. The nature of this death signal is currently unknown. Our findings that fyn seems to be involved in regulating the lifespans of activated T lymphocytes suggests that fyn may be a normal mediator of Fas signal transduction. Experiments aimed at investigating this possibility are described in the following chapter.

CHAPTER FIVE: p59FYN IN FAS SIGNAL TRANSDUCTION

A version of this chapter has been submitted for publication. E.A. Atkinson and R.C. Bleackley, 1996. J. Biol. Chem.

INTRODUCTION

The immune system is capable of recognizing a vast array of potential antigens. As with B cells that rearrange their immunoglobulin genes, T cell development involves T cell receptor (TCR) gene rearrangement and recombination. This results in each mature T cell expressing a unique TCR, capable of recognizing a unique antigen. Upon interacting with its appropriate antigen presented by self MHC molecules, a T cell proliferates in order to generate multiple clones, each expressing an identical TCR. In this way, the number of cells capable of recognizing the particular antigenic threat are expanded and the infection is efficiently eliminated. Once the antigen is cleared from the body, the multitude of new lymphocytes generated must obviously be reduced. If this was not the case, each subsequent infection would result in the accumulation of ever increasing numbers of lymphocytes. Not surprisingly, then, the immune system has a built-in mechanism for the elimination of activated lymphocytes once they are no longer needed.

Although deletion of activated T cells *in vivo* and death of stimulated lymphocytes *in vitro* has been a widely recognized phenomenon for some time, it is only within the last two years that a molecular basis for this has been defined. The identification of the defect in *lpr* and *gld* mice, which develop a late-onset lymphoproliferative disease characterized by the accumulation of mature T cells, as being a mutation in the genes encoding Fas and Fas ligand, respectively, provided a clue as to the mechanism of activated T cell elimination (Watanabe-Fukanaga et al., 1992; Adachi et al., 1993; Takahashi et al., 1994). Subsequently, it was shown that peripheral deletion of activated T cells was severely impaired in *lpr* and *gld* mice (Singer and Abbas, 1994; Russell and Wang, 1993). Therefore, it is now believed that Fas and Fas ligand are involved in signalling the death of normal activated lymphocytes (See Nagata and Golstein, 1995 for recent review).

The nature of the Fas death signal is not known. However, a cytoplasmic region in Fas that is essential for killing has been identified and termed the "death domain" (Itoh and Nagata, 1993). A similar motif exists in the cytotoxic TNF receptor (TNFR1), a member of the NGF receptor family, to which Fas also belongs (Tartaglia et al., 1993). This death domain also shares some homology with part of the Drosophila protein reaper, which is an important mediator in apoptosis (Golstein et al., 1995). Both Fas and TNFR appear to be coupled to a sphingomyelinase-dependent, ceramide-producing pathway, which could explain, at least in part, the apoptotic outcome of ligating these receptors (Cifone et al., 1993; Dressler et al., 1992; Kim et al,. 1991; Schutze et al., 1992). Whether this coupling is direct or not is unknown.

Fas contains no previously known signaling motifs or catalytic activities. In an effort to identify molecules that might interact with Fas and link it to signal transduction pathways, a number of groups have employed the yeast 2-hybrid system, with the Fas cytoplasmic region as "bait". This approach has identified three different proteins that can interact with Fas: FADD (MORT1) (Boldin et al., 1995; Chinnaiyan et al., 1995), RIP (Stanger et al., 1995), and FAP-1 (PTP-BAS) (Sato et al., 1995), although evidence that these proteins interact in a physiologically-relevant system is lacking. FADD and RIP both contain death domain-like motifs, and interaction with Fas may occur via dimerization of this region. The functions of FADD and RIP are not known, although RIP may be a protein kinase. FAP-1 is a tyrosine phosphatase, and interestingly, interacts with the Fas carboxyl terminus, which has been shown to be involved in negatively regulating Fas killing (Itoh and Nagata, 1993). The level of endogenous FAP-1 seems to correlate with the Fas-sensitivity of a cell, and transfection of cells with FAP-1 encoding DNA also leads to a reduction in Fas sensitivity (Sato et al., 1995). This suggests that one or more tyrosine kinases may play a role in Fas killing. Indeed, it has recently been demonstrated that Fas activity is inhibited by tyrosine kinase inhibitors and ligation of Fas leads to the tyrosine phosphorylation of a number of cellular proteins (Eischen et al., 1994).

Therefore, there is now strong evidence from two different sources that tyrosine kinase activity is involved in Fas signal transduction.

Our previous experiments with fyn-knockout mice suggested that the fyn tyrosine kinase may play a role in regulating the lifespan of activated lymphocytes. Since Fas was beginning to emerge as an important mediator in lymphocyte death, we hypothesized that fyn may be involved in the signal transduction pathway utilized by Fas. The experiments described in this chapter were performed in order to investigate this possibility.

RESULTS

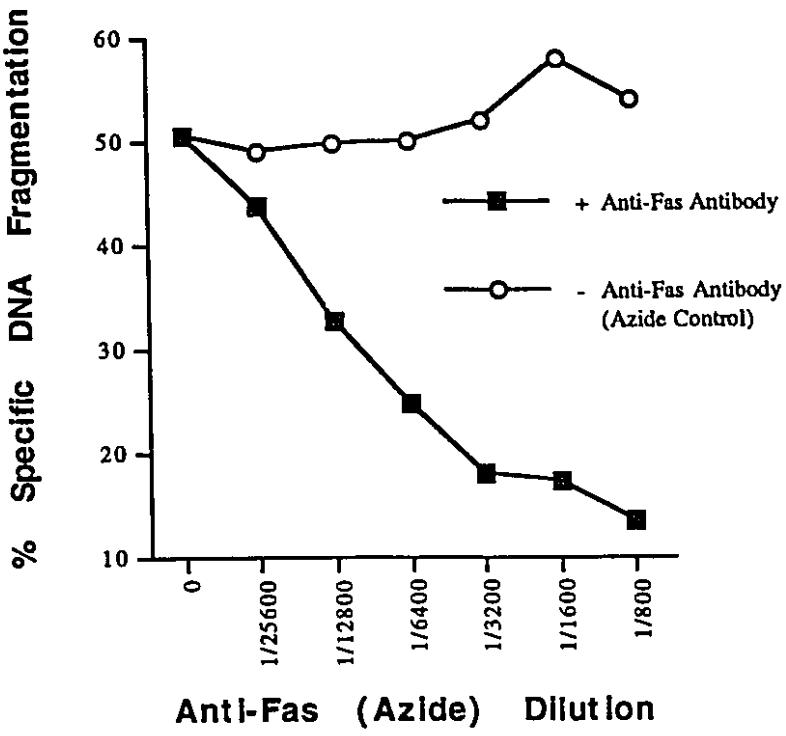
PMM1 Kills Targets Exclusively via the Fas Pathway

As a model system to investigate biochemical aspects of Fas-dependent apoptosis, we utilized PMM-1 cells, a CTL line that is known to kill entirely via a Ca^{+2}-independent, Fas-dependent pathway (Garner et al., 1994), and target cells expressing low (L1210) or elevated (L1210-Fas) levels of Fas antigen. L1210-Fas was created by Golstein and colleagues by stably transfecting L1210 cells with a Fas expression vector (Rouvier et al., 1993). This cell line is many times more sensitive to death via the Fas pathway than the parental line. PMM1 requires cellular activation in order to kill Fas-bearing target cells, presumably for the upregulation of Fas-ligand. Therefore, PMM1 were routinely pre-activated with PMA and ionomycin for 3 hours before use in the assays described below. In order to verify that activated PMM1 cells killed target cells in a strictly Fas-dependent manner, we used an anti-Fas IgG to block Fas recognition on L1210-Fas cells by Fas-ligand on PMM1. The Jo2 anti-Fas antibody itself will kill Fas-bearing target cells, but with much slower kinetics than activated PMM1. We therefore employed a short-term killing assay of two hours. Since Fas ligation results in apoptotic death of cells, and DNA fragmentation is an early consequence of apoptosis, we utilized an assay which measures the release of soluble (fragmented) DNA from the target cell nuclei as a readout of apoptosis. The L1210-Fas cells were incubated in ^{3}H-thymidine-containing medium overnight in order to specifically label their DNA. After incubation with PMM1 effectors, the cells were solubilized with 0.5% Triton X-100. This allows dissolution of the plasma membrane, while keeping the nucleus intact. By pelleting the nuclei and counting the amount of radioactivity in the supernatant, a quantitative measurement of cytoplasmic, fragmented DNA can be obtained. As shown in Figure 5-1, increasing the concentration of anti-Fas antibody effectively blocked DNA fragmentation in L1210-Fas cells exposed to PMM1 killer cells. Therefore, we were confident that

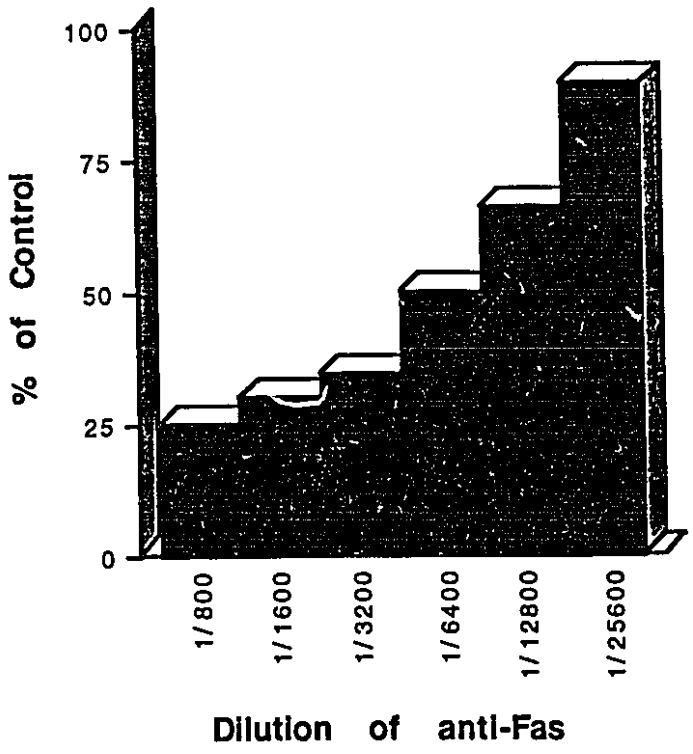
Figure 5-1 PMM1 Cause Apoptosis in Target Cells in a Strictly Fas-Dependent Manner.

L1210-Fas cells were grown overnight in medium containing ^{3}H-thymidine, and then incubated with activated PMM1 cells (E:T=0.5:1) for 2 hours with different dilutions of anti-Fas antibody or an equivalent amount of sodium azide. Data are shown either as % specific fragmentation values obtained (A) or as the % of fragmentation achieved in antibody-containing samples compared to the equivalent azide controls (100%) (B). The data shown have been verified in 2 additional experiments.

A


% Specific DNA Fragmentation
60
50
40
30
20
10
+ Anti-Fas Antibody
- Anti-Fas Antibody
(Azide Control)
0
1/25600
1/12800
1/6400
1/3200
1/1600
1/800
Anti-Fas (Azide) Dilution

B


% of Control
100
75
50
25
0
1/800
1/1600
1/3200
1/6400
1/12800
1/25600
Dilution of anti-Fas

PMM1 cells could be used to investigate death caused solely by a Fas-dependent pathway.

Fas-Dependent Apoptosis is Inhibited by the Tyrosine Kinase Inhibitor Genistein

If p59fyn tyrosine kinase activity was involved in the transduction of the Fas death signal, we reasoned that Fas-dependent apoptosis should be sensitive to tyrosine kinase inhibitors. We therefore studied the effects of the tyrosine kinase inhibitor genistein on PMM1-induced DNA fragmentation of L1210 and L1210-Fas target cells. Utilizing a DNA fragmentation gel, we observed an apparent dose-dependent inhibition of DNA fragmentation by genistein (Figure 5-2). This observation was followed-up using genistein in our quantitative DNA fragmentation assay. As shown in Figure 5-3A and B, genistein inhibited DNA fragmentation in both L1210 and L1210-Fas targets in a dose-dependent fashion. Consistent with the elevated levels of Fas on the surface of L1210-Fas, these cells were much more sensitive to PMM1 than the parental L1210 line (approximately 60% specific DNA fragmentation vs. approximately 10%). Interestingly, when expressed in terms of % fragmentation of that observed in control samples treated with an equivalent amount of DMSO vehicle, the degree of inhibition by genistein was somewhat greater for L1210 targets versus L1210-Fas (Figure 5-3C). 90μg/ml genistein resulted in L1210-Fas targets fragmenting only 25% of the DNA that was measured in control cells. However, this concentration of genistein resulted in L1210 targets fragmenting less than 7% of the amount in control cells. This may indicate that this inhibitor is more effective at inhibiting the Fas death pathway when the initial death signal is less strong (in this case, due to less surface Fas being engaged).

This differential inhibition effect of genistein was also seen when the strength of the death signal was modulated by varying the effector to target ratio (E:T) used in the killing assay (Figure 5-4). In a standard ^{51}Cr-release lytic assay, 60 μg/ml genistein had a much greater inhibitory effect at a lower E:T than at higher ratios. At an E:T of 0.5:1, this concentration of drug inhibited ^{51}Cr release from L1210-Fas targets to just over 50% of

Figure 5-2 PMM1-Induced DNA Fragmentation is Sensitive to the PTK Inhibitor Genistein.

Shown is a DNA fragmentation gel of DNA isolated from 1 million L1210 or L1210-Fas target cells exposed to PMM1 killer cells (E:T=0.5:1) for 2 hours in the presence or absence of genistein. The control lanes contain the DNA from the indicated cells that were incubated alone, and in the absence of drug, but in the presence of an equivalent amount of DMSO. This experiment was performed twice with comparable results.

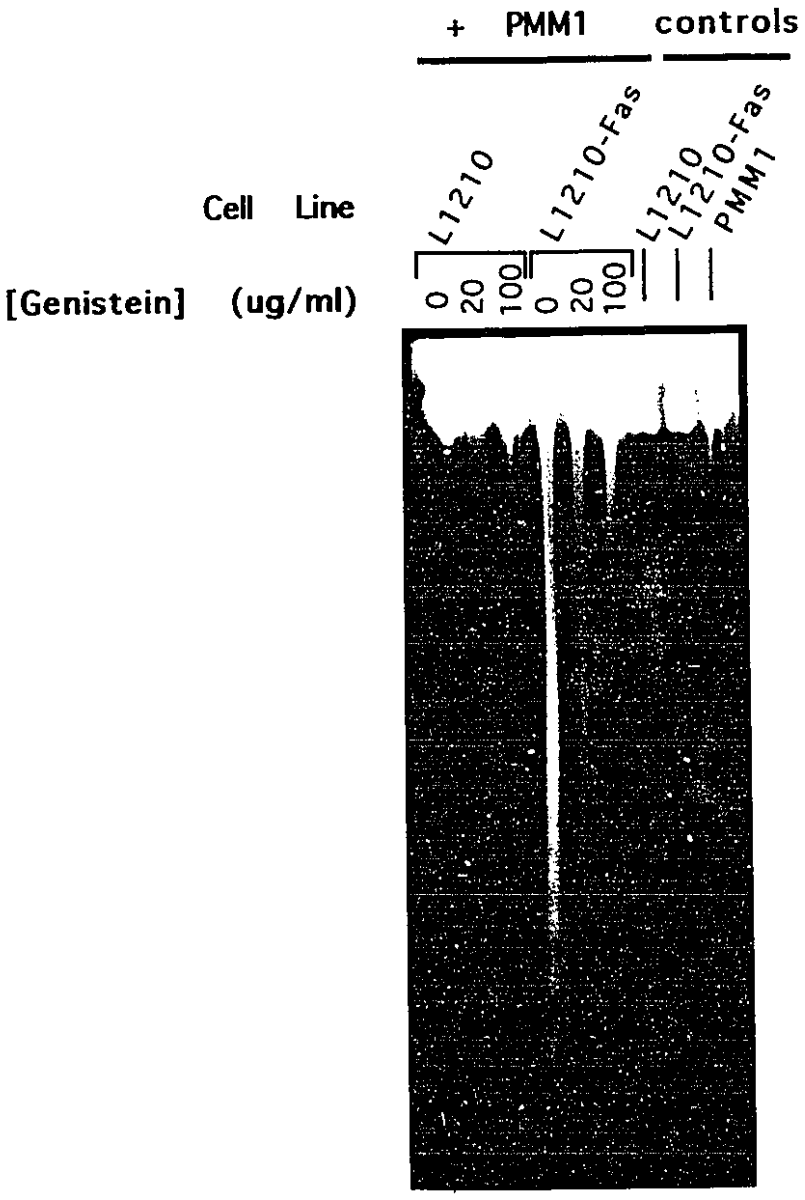

+ PMM1
controls
Cell Line
L1210
L1210-Fas
L1210
L1210-Fas
PMM1
[Genistein] (ug/ml)
0
20
100
0
20
100

Figure 5-3 Genistein Inhibits Fas-Mediated DNA Fragmentation in a Dose-Dependent Manner.

^{3}H-labelled L1210 (A) or L1210-Fas (B) target cells were incubated with PMM1 cells (E:T=0.5:1) for 2 hours in the presence of a range of concentrations of genistein (black columns) or an equivalent amount of DMSO (white columns). The data are represented both as the % of DNA fragmentation obtained (A and B) or as the % fragmentation obtained in genistein-treated samples compared to DMSO controls (designated as 100%) (C). The data shown are typical of six separate experiments.

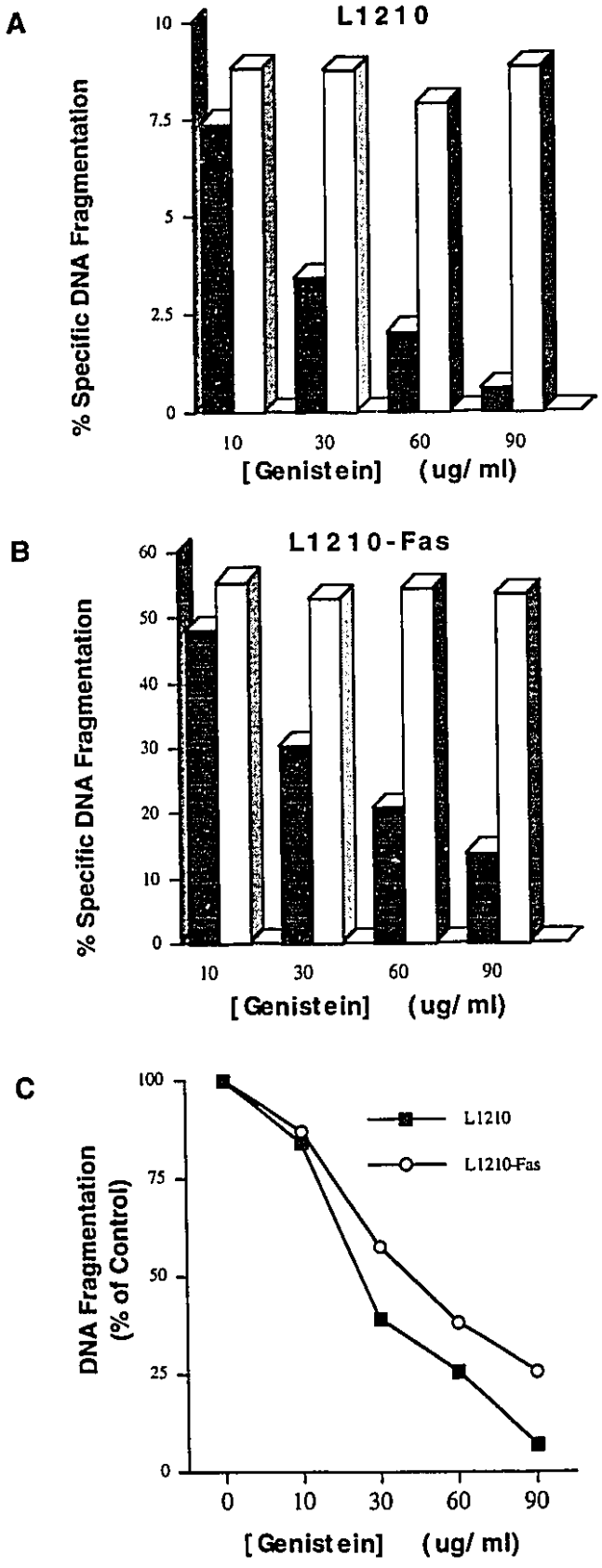
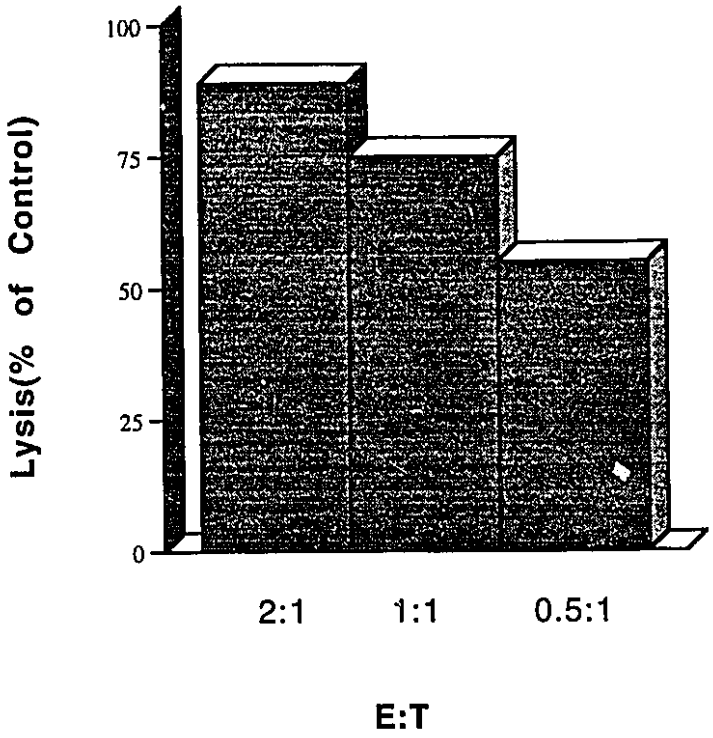

A
L1210
% Specific DNA Fragmentation
10
7.5
5
2.5
0
10
30
60
90
[Genistein] (ug/ml)
B
L1210-Fas
% Specific DNA Fragmentation
60
50
40
30
20
10
0
10
30
60
90
[Genistein] (ug/ml)
C
DNA Fragmentation (% of Control)
100
75
50
25
0
L1210
L1210-Fas
0
10
30
60
90
[Genistein] (ug/ml)

Figure 5-4 The Degree of Genistein Inhibition of Fas-Dependent Death Depends on the Strength of the Death Signal.

^{51}Cr-labelled L1210-Fas target cells were incubated with PMM1 cells at a range of effector to target ratios (E:T) in the presence of 60 μg/ml genistein or an equivalent amount of DMSO. The data shown represent the % of lysis achieved in genistein-treated samples compared to the DMSO controls (designated as 100%).


Lysis(% of Control)
100
75
50
25
0
2:1
1:1
0.5:1
E:T

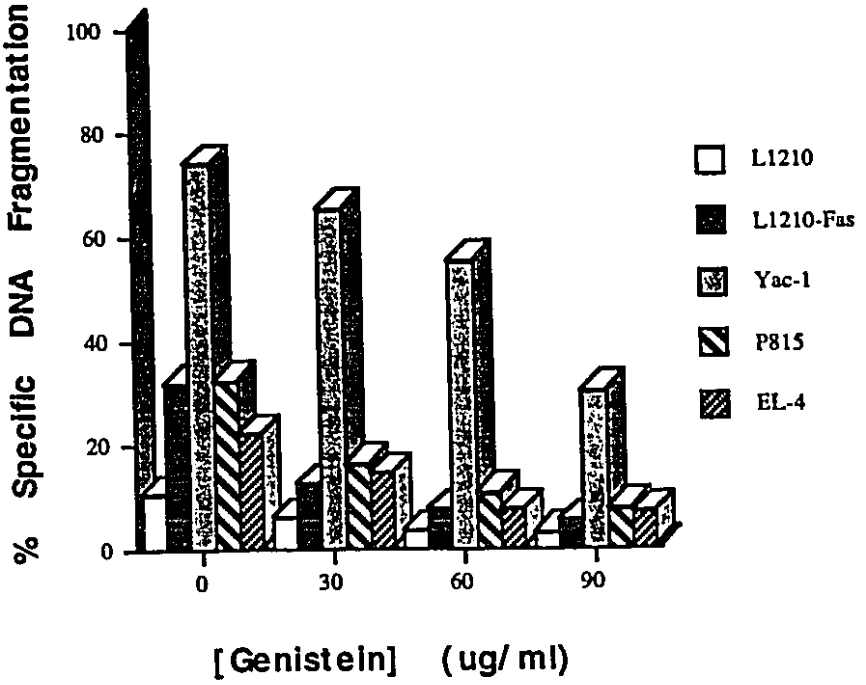
control values, whereas at a 2:1 E:T, about 90% of control levels of ^{51}Cr were still released in the presence of the drug. The amount of inhibition seen at a 1:1 E:T was intermediate between the two, resulting in about 75% of the ^{51}Cr released from control samples. A similar differential inhibition at different E:T ratios by genistein was seen when a DNA fragmentation assay was used as a readout of apoptosis (data not shown). Therefore, the tyrosine kinase inhibitor genistein can indeed inhibit Fas-mediated cell death, but the degree of inhibition depends upon the strength of the death signal. This may indicate that tyrosine kinase activity contributes towards signals generated by Fas ligation but may not be absolutely essential for a death signal to be delivered. Other components of the death signal may be able to counteract the effects of the tyrosine kinase inhibitor if the stimulus is sufficiently strong.

Since genistein is a reversible inhibitor, its presence was required throughout the DNA fragmentation and lytic assays. It was possible, then, that it was actually affecting the PMM1 effector cells and not the target cells. An anti-mouse Fas antibody with the ability to induce cell death of Fas-positive murine cells became commercially available as these inhibitor experiments were being performed. This antibody, Jo2, kills cells with much slower kinetics than PMM1 effector cells. The effects of genistein on DNA fragmentation induced by either anti-Fas or PMM1 were compared using five different target cells (Figures 5-5A and B). For all cell lines, genistein caused a dose-dependent inhibition of PMM1-induced DNA fragmentation. Curiously, however, genistein enhanced the DNA fragmentation induced by anti-Fas antibody. This enhancement effect did not show strict concentration dependence, however, and the level of killing plateaued at either 30 μg/ml or 60 μg/ml of the drug. It is possible that because the DNA fragmentation assay was carried out for 8 hours when anti-Fas was used and only 2.5 hours when PMM1 was used, genistein may be affecting different aspects of death. Tyrosine kinases may be involved in the death process farther downstream from the initial Fas signal and genistein may be influencing these events. Alternatively, genistein

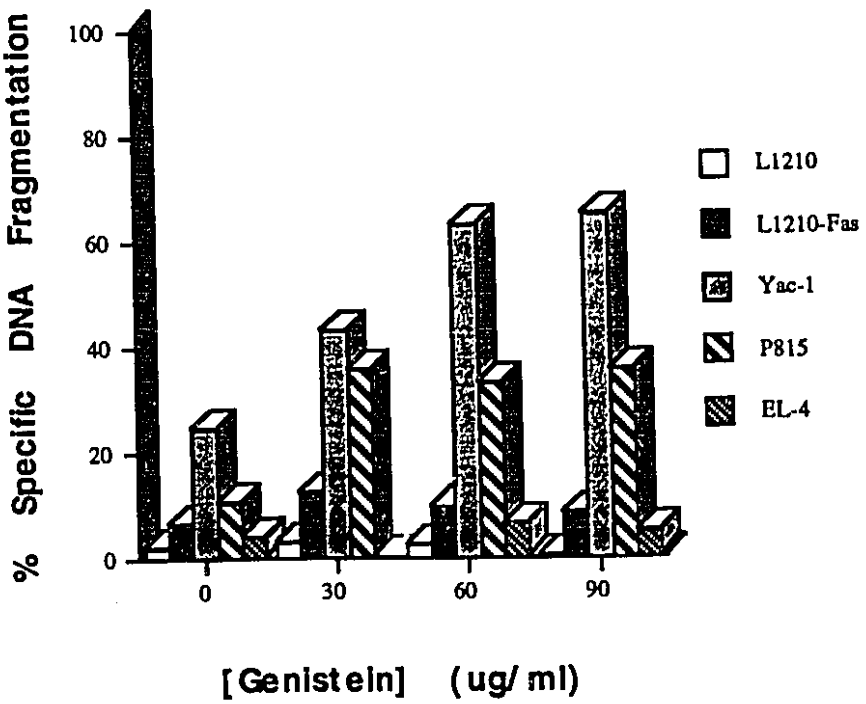
Figure 5-5 Effect of Genistein on DNA Fragmentation Induced by PMM1 or Anti-Fas Antibody in 5 Different Cell Lines

DNA fragmentation assays were performed on five different cell lines exposed to either PMM1 for 2.5 hours (A) or anti-Fas antibody for 8 hours (B) in the presence of the indicated concentrations of genistein. For all samples, the DMSO concentration was kept constant, as was the sodium azide concentration when anti-Fas was used. PMM1 was used at an E:T of 0.5:1 and anti-Fas was used at a 1:1000 dilution (1 μg/ml final concentration). Three separate experiments gave comparable results.

A


% Specific DNA Fragmentation
100
80
60
40
20
0
0
30
60
90
[Genistein] (ug/ml)
L1210
L1210-Fas
Yac-1
P815
EL-4

B


% Specific DNA Fragmentation
100
80
60
40
20
0
0
30
60
90
[Genistein] (ug/ml)
L1210
L1210-Fas
Yac-1
P815
EL-4

may have an effect on death that is not related to its tyrosine kinase inhibitory activity, and this may explain the lack of dose-responsiveness in its enhancement of anti-Fas mediated DNA fragmentation.

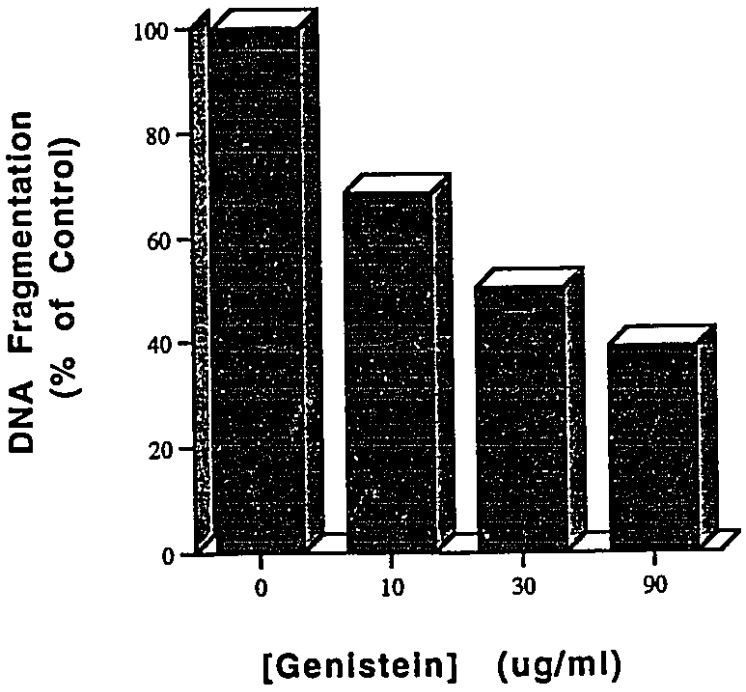
Whatever the reason, the opposite effects of genistein on PMM1 versus anti-Fas mediated DNA fragmentation was disturbing. Since the Jo2 antibody was not ideal in that it induces Fas-based cell death with such slow kinetics, we decided to investigate the effects of an anti-human Fas antibody on Fas-bearing human MOLT4 cells. Anti-human Fas is an IgM, which is pentavalent, and so has the capacity to crosslink surface Fas to a much higher degree than anti-mouse Fas, which is an IgG and only bivalent. We have found MOLT4 cells to be moderately sensitive to anti-Fas in a concentration-dependent fashion and to be killed with faster kinetics than mouse cells exposed to Jo2 (data not shown). As seen in Figure 5-6, genistein had a dose-dependent inhibitory effect of anti-Fas induced DNA fragmentation of MOLT4 cells. As with PMM1-mediated DNA fragmentation of target cells, inhibition was not absolute. 90 μg/ml of genistein was able to inhibit DNA fragmentation to approximately 40% of control values. Therefore, in the human system, and in the absence of possible effects on effector cells, the tyrosine kinase inhibitor genistein was also shown to inhibit Fas-mediated DNA fragmentation.

p59fyn Physically Associates with Fas

If p59fyn is involved in Fas signal transduction, it seemed possible that it might physically associate with Fas. In order to test this, co-immunoprecipitation experiments were performed. We chose to study YAC-1 cells, which are very sensitive to Fas-mediated death and express relatively high levels of both Fas and p59fyn (data not shown). In case Fas ligation was required for fyn association, the YAC-1 cells were exposed to an equal number of activated PMM1 cells for 10 minutes before being lysed with a 0.5% Triton X-100 lysis buffer. Post-nuclear supernatants were pre-cleared with protein A/G agarose beads, and then immunoprecipitated with either anti-Fas or anti-fyn antibodies. After extensive washing, the immunoprecipitates were separated via SDS-

Figure 5-6 Genistein Inhibition of anti-Fas induced DNA Fragmentation in Human MOLT-4 Cells.

A DNA fragmentation assay was performed on ^{3}H-labelled MOLT-4 cells exposed to anti-human Fas IgM for 4 hours in the presence of the indicated concentrations of genistein. For each condition, a DMSO control was performed, and the results are expressed as the % of fragmentation obtained from genistein-treated samples compared to controls (which were designated as 100%). This experiment was performed three times with similar results.


DNA Fragmentation
(% of Control)
100
80
60
40
20
0
0
10
30
90
[Genistein] (ug/ml)

PAGE and transferred to nitrocellulose. Western blots of anti-Fas immunoprecipitates were probed with anti-fyn antibody and anti-fyn immunoprecipitates were probed with anti-Fas antibody. As seen in Figure 5-7A, p59fyn was co-immunoprecipitated with anti-Fas antibody, and vice versa. The ability to co-immunoprecipitate the two proteins with the reciprocal antibodies clearly establishes their association. In order to further demonstrate the specificity of this interaction, similar cell lysates were immunoprecipitated with antibodies specific for Fas, p59fyn, or CD45, a transmembrane phosphatase. These immunoprecipitates were then subjected to western blotting with anti-fyn. Unlike anti-Fas and anti-fyn, anti-CD45 failed to co-immunoprecipitate p59fyn (Figure 5-7B). Therefore, p59fyn specifically associates with the Fas death protein.

To further confirm the presence of a protein kinase associated with Fas, sensitive *in vitro* kinase assays were performed. Lysates of YAC-1 cells that had been stimulated with PMM1 cells or left unstimulated were immunoprecipitated with either anti-Fas or anti-fyn and kinase assays performed on the resulting pellets. A strikingly similar pattern of phosphorylated proteins was observed from anti-Fas and anti-fyn immunoprecipitate kinase reactions, suggesting the presence of a common kinase (Figure 5-8). The presence of p59fyn in the anti-Fas immunoprecipitated kinase reactions was further demonstrated by dissociation of the immune complex and re-immunoprecipitation with anti-fyn antibody. It is interesting to note that stimulation of YAC-1 cells with PMM1 does not appear to be required for p59fyn to associate with Fas (Figure 5-8 and our unpublished observations). Also, our *in vitro* kinase assays have not revealed an increase in p59fyn autophosphorylation upon YAC-1 stimulation with PMM1. This may be due to the fact that this type of cell-cell stimulation results in only a fraction of the available Fas molecules being engaged. Alternatively, we may also be immunoprecipitating the FAP-1 tyrosine phosphatase believed to associate with the Fas carboxyl terminus (Sato et al., 1995), and its activity may not be entirely inhibited by the phosphatase inhibitors

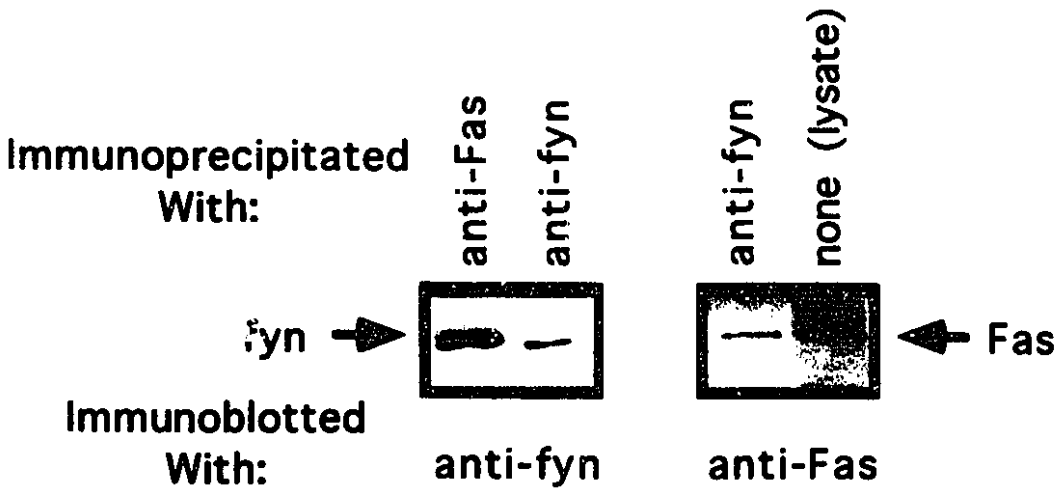
Figure 5-7 Association of p59fyn Tyrosine Kinase with Fas.

A. Western blot showing co-immunoprecitation of Fas and fyn from PMM-1 stimulated YAC-1 cell lysates. The right lane of each panel is present solely as a positive control for the blotting antibodies and to show the mobilities of fyn and Fas, but is not meant to be quantitative. In the left panel, the positive control was a small portion of an anti-fyn immunoprecipitate from a RIPA buffer lysate of YAC-1 cells previously determined to contain specifically immunoprecipitated p59fyn using two separate anti-fyn antibodies. In the right panel, the positive control is 20 µl of a YAC-1 post-nuclear cell lysate.

Due to lack of anti-Fas probe antibody, this experiment was only performed once.

B. Western blot showing that p59fyn specifically associates with Fas. Anti-Fas and anti-fyn antibodies immunoprecipitate detectable levels of p59fyn from cell lysates, but anti-CD45 antibodies do not. Even extreme over-exposure of the filter did not reveal any fyn in the CD45 immunoprecipitates.

The data shown are typical of three independent experiments.


Immunoprecipitated With:
anti-Fas
anti-fyn
anti-fyn
none (lysate)
Fyn
Fas
Immunoblotted With:
anti-fyn
anti-Fas

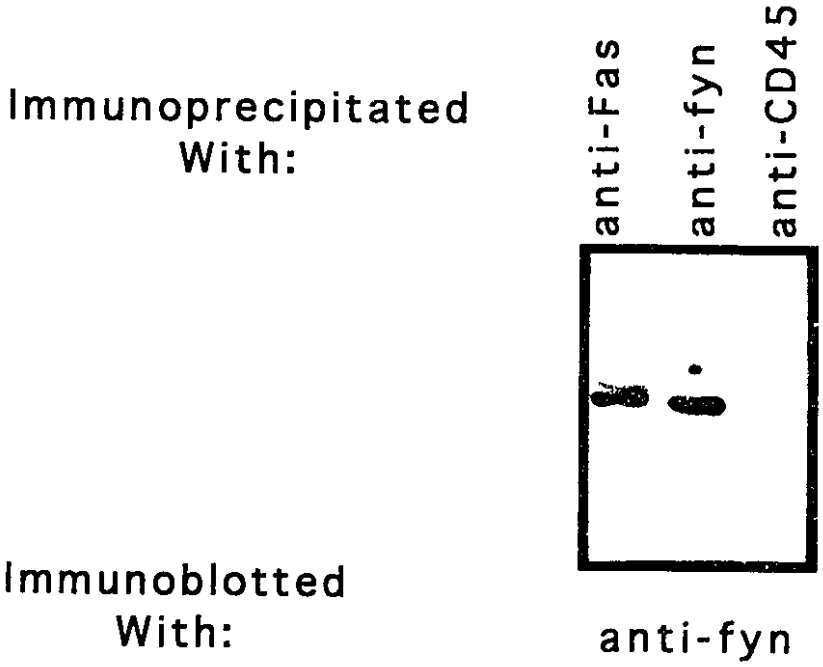
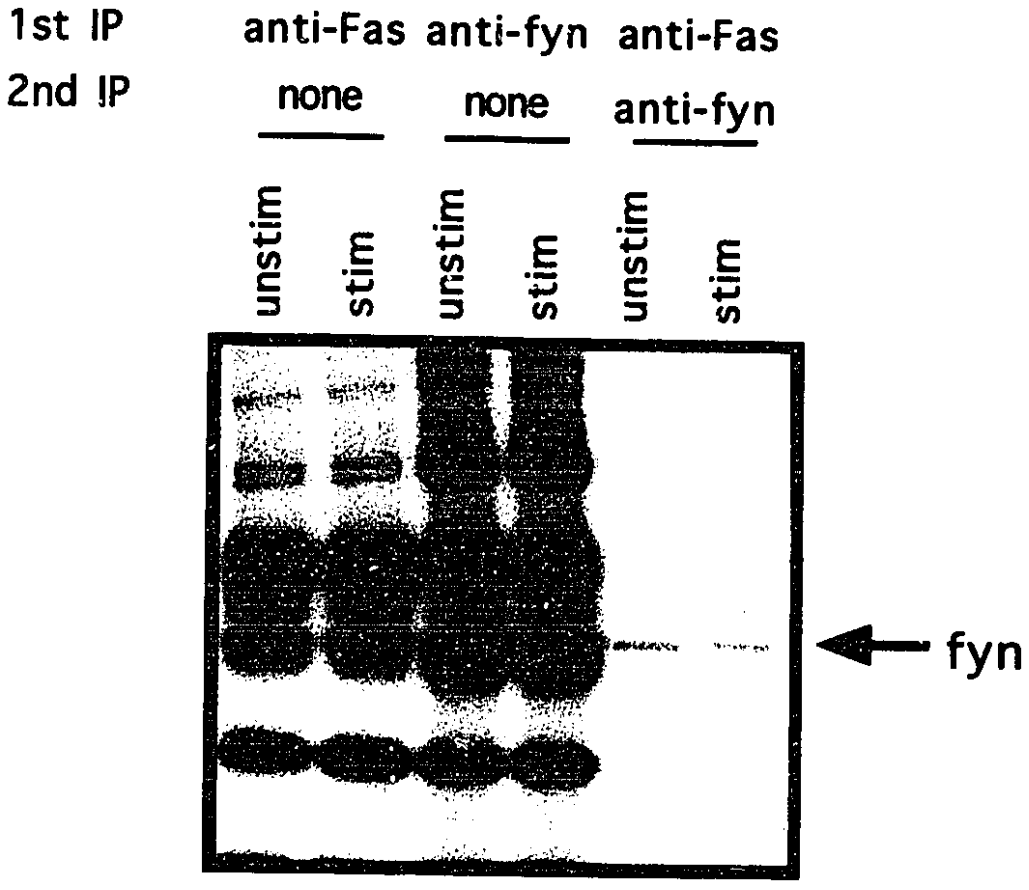

Immunoprecipitated With:
anti-Fas
anti-fyn
anti-CD45
Immunoblotted With:
anti-fyn

Figure 5-8 *In Vitro* Kinase Assays of anti-Fas and anti-fyn Immunoprecipitates

YAC-1 cells were either stimulated with PMM-1 cells (1:1) or not, lysed, precleared, and then subjected to the indicated immunoprecipitations. *In vitro* kinase assays were performed and then secondary immunoprecipitations done as indicated. p59fyn is shown to have been present in the Fas immunoprecipitations/kinase reactions. This experiment has been performed twice, with similar results.


1st IP
2nd IP
anti-Fas
none
anti-fyn
none
anti-Fas
anti-fyn
unstim
stim
unstim
stim
unstim
stim
fyn

employed. It should also be noted that increased fyn activity is not always apparent upon co-immunoprecipitation of this kinase with CD3.

Cellular Substrates May Be Tyrosine Phosphorylated Upon Fas Engagement

In order to determine if physiological stimulation of Fas with Fas-ligand results in increased tyrosine phosphorylation of cellular substrates, lysates of YAC-1 cells unstimulated, or stimulated with PMM1 over a time course were electrophoresed, transferred to nitrocellulose, and then probed with an anti-phosphotyrosine antibody (Figure 5-9A). To keep the amount of protein constant in stimulated and unstimulated samples, lysates of PMM1 cells were mixed with the unstimulated YAC-1 lysates before electrophoresis. As shown in Figure 5-9A, PMM1 stimulation of YAC-1 cells results in the rapid tyrosine phosphorylation of a number of cellular proteins. Therefore, interaction of sensitive Fas-positive target cells with Fas ligand-bearing killer cells results in the stimulation of tyrosine kinase activity.

Many different interactions likely occur when a killer cell bearing the Fas ligand interacts with a target cell, so it is possible that the tyrosine phosphorylation observed upon PMM1 interaction with YAC-1 was a result of non-Fas signals. Since PMM1 possesses a TCR that is directed against H-2^b, and YAC1 cells have downregulated their expression of MHC molecules, it is likely that the tyrosine phosphorylations are not due to TCR/CD3- or MHC-derived signals. Nevertheless, tyrosine phosphorylation due to adhesion receptors and other surface molecules on both the PMM1 and YAC-1 cells cannot be discounted. We therefore investigated the ability of the Jo2 anti-Fas antibody to induce tyrosine phosphorylation in YAC-1 cells. Figure5-9B shows a western blot of lysates of YAC-1 cells stimulated with anti-Fas IgG over a one hour time course. The blot was probed with an anti-phosphotyrosine antibody. There is no indication of any major changes in tyrosine phosphorylation in the YAC-1 cells over the hour they were exposed to anti-Fas. It should be remembered, however, that PMM1 cells kill YAC-1 with much faster kinetics than Jo2, and it is therefore possible that the signal generated by

Figure 5-9 Tyrosine Phosphorylation of Cellular Proteins in Response to Fas Ligation.

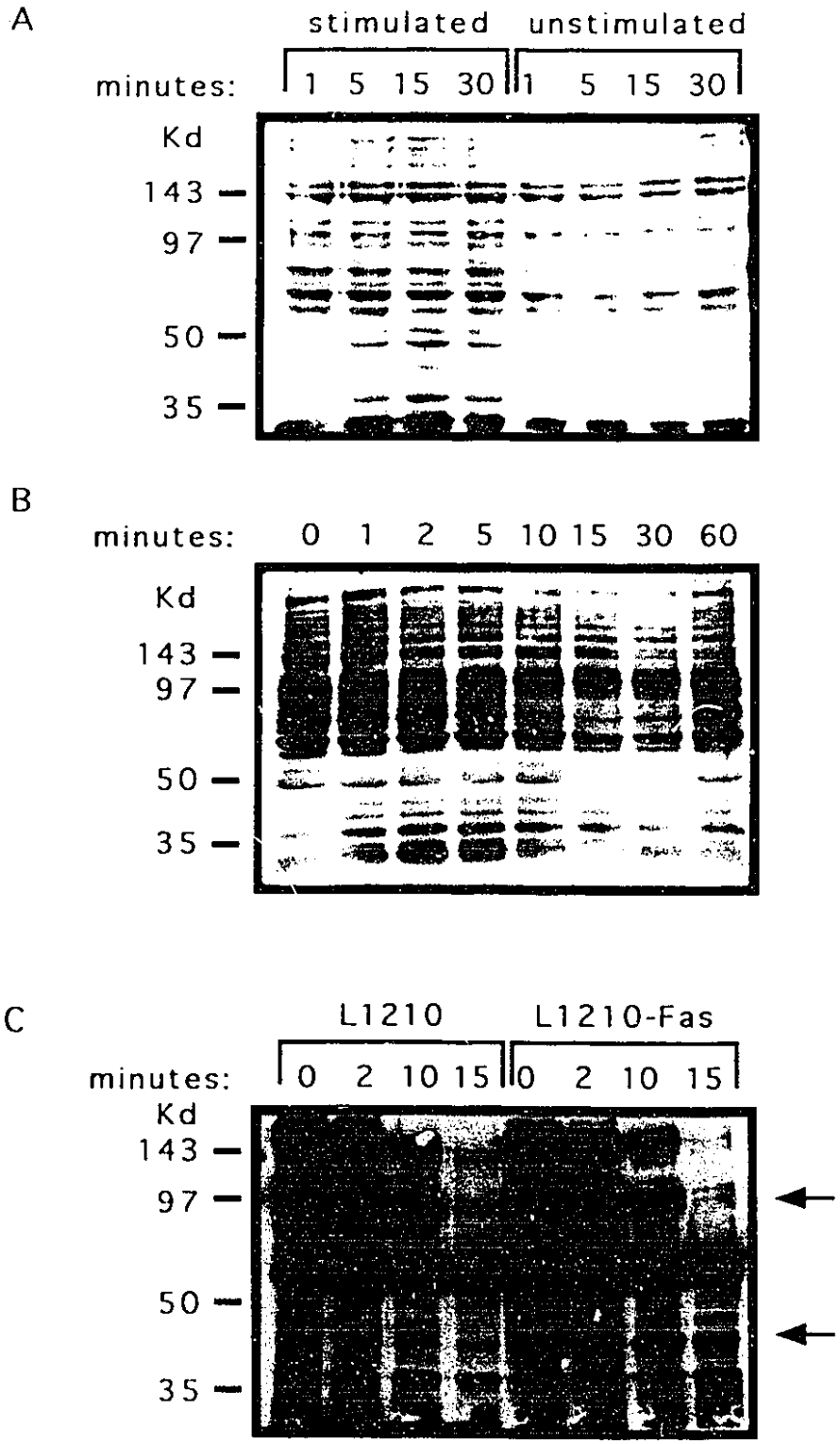
A. Anti-phosphotyrosine western blot of YAC-1 cells stimulated with PMM1.

An equivalent number of YAC-1 and PMM-1 cells in serum-free medium were incubated either alone (unstimulated) or together (stimulated) for the indicated times, after which they were pelleted, the supernatant removed by aspiration, and the cells lysed directly in 200 µl (stimulated samples) or 100 µl (unstimulated samples) SDS sample buffer. The unstimulated YAC-1 and PMM-1 samples of appropriate time points were then mixed together to keep protein concentrations constant. Genomic DNA was sheared by sonication and 40 µl of each sample was separated via 8% SDS-PAGE and transferred to nitrocellulose. Tyrosine-containing proteins were detected using anti-phosphotyrosine antibody 4G10 and enhanced chemiluminescent detection .

B. Anti-phosphotyrosine western blot of YAC-1 cells exposed to anti-Fas antibody.

Anti-Fas was added to YAC-1 cells at a concentration of 1 µg/ml and processed as described above.

C. Anti-phosphotyrosine western blot of L1210 and L1210-Fas cells stimulated with PMM1. Target cells were exposed to an equal number of PMM1 cells for the times indicated and samples were processed as described above. For 0 time points, YAC-1 and PMM1 cells were pelleted and lysed separately, before being mixed together to keep protein concentrations equal for all samples.


A
stimulated
unstimulated
minutes:
1 5 15 30
1 5 15 30
Kd
143
97
50
35
B
minutes:
0 1 2 5 10 15 30 60
Kd
143
97
50
35
C
L1210
L1210-Fas
minutes:
0 2 10 15
0 2 10 15
Kd
143
97
50
35

Jo2 is so weak that any phosphorylations induced cannot be detected. It is also possible that soluble IgG does not cluster Fas to an adequate degree for strong signalling. Indeed, it has recently been reported that a high degree of cross-linking of Fas is required for an efficient death signal to be delivered (Dhein et al., 1992). However, even when Jo2 is bound to the surface of a plate and Fas positive target cells are added, no detectable tyrosine phosphorylations are induced (Dr. Hanne Ostergaard, personal communication). With the reagents available for studying Fas signaling in murine cells, it is therefore difficult to firmly conclude that Fas ligation results in the tyrosine phosphorylation of cellular proteins.

In an attempt to further address this question, we investigated if L1210-Fas target cells differed in tyrosine phosphorylation compared to the parental L1210 line upon exposure to PMM1. Since these two cell lines are identical except for the amount of Fas on their surface and their susceptibility to Fas-induced cell death, any differences in phosphorylation should be due to their level of Fas expression. Figure 5-9C shows an anti-phosphotyrosine western blot of L1210 and L1210-Fas cells exposed to an equal number of PMM1 cells over a 30 minute time course. Some minor differences were seen between the two cell lines. Interestingly, in contrast to YAC-1 cells, L1210 and L1210-Fas cells showed an actual decrease in tyrosine phosphorylated cellular proteins upon exposure to PMM1. Some of the dephosphorylated proteins in the L1210-Fas samples were dephosphorylated to a lesser degree than in the L1210 samples (for instance, the doublet at approximately 97 Kd). Also, a protein running just under the 50 Kd marker actually may have become more phosphorylated on tyrosine at the 10 minute time point and still maintained a significant degree of phosphorylation by 15 minutes in L1210-Fas. This same protein was dephosphorylated to a large degree in L1210 cells at these same times. It is interesting that L1210 and L1210-Fas cells respond differently from YAC-1 cells. The reason for this is not clear, although it is possible that it relates to the relative amounts of p59fyn in the cells. YAC-1 cells express fyn to a much greater degree than do

L1210 cells (data not shown). Therefore, the action of the tyrosine phosphatase FAP-1, which is tethered to the C-terminus of Fas, may be able to more efficiently dephosphorylate its substrates upon Fas engagement. In any event, it does appear that Fas ligation results in changes in the tyrosine phosphorylation of some cellular proteins. Although the lack of a good anti-Fas antibody for the murine system has hindered our analysis of this phenomenon, a paper published while these studies were being performed demonstrated increased tyrosine phosphorylation in human Jurkat cells exposed to anti-human Fas IgM (Eischen et al., 1994). We therefore conclude that one or more tyrosine kinases may play an important role in Fas signal transduction.

Fyn-Deficient Splenocytes Exhibit Reduced Sensitivity to Fas-Based Death

Despite the fact that we were unable to unequivocally demonstrate tyrosine phosphorylation of cellular substrates upon Fas engagement, our findings that fyn associated with Fas were nonetheless compelling. We next sought to directly determine if fyn played a role in Fas-mediated cell death. To do this, we utilized alloantigen-stimulated splenocytes from $fyn^{-/-}$ and $fyn^{+/+}$ mice and determined their susceptibility to anti-Fas antibody. Wells of 96 well plates were coated with either anti-Fas or BSA as a control, and day 5 alloantigen-activated splenocytes were incubated in them overnight. The next day, cell viability was assessed by vital dye exclusion. As seen in Figure 5-10A, the $fyn^{-/-}$ splenocytes were resistant to the effects of anti-Fas antibody while the control cells were susceptible. We also determined the relative sensitivities of $fyn^{-/-}$ and $fyn^{+/+}$ splenocytes to killing by PMM1. Normal and fyn-deficient splenocytes were used as targets in a ^{51}Cr-release lytic assay. The $fyn^{-/-}$ cells were found to be far less sensitive to PMM1 than their normal counterparts (Figure 5-10B). These results provides strong and direct evidence that fyn activity is involved in the Fas death pathway in these cells.

Analysis of Tyrosines 253 and 262 in Fas Signalling

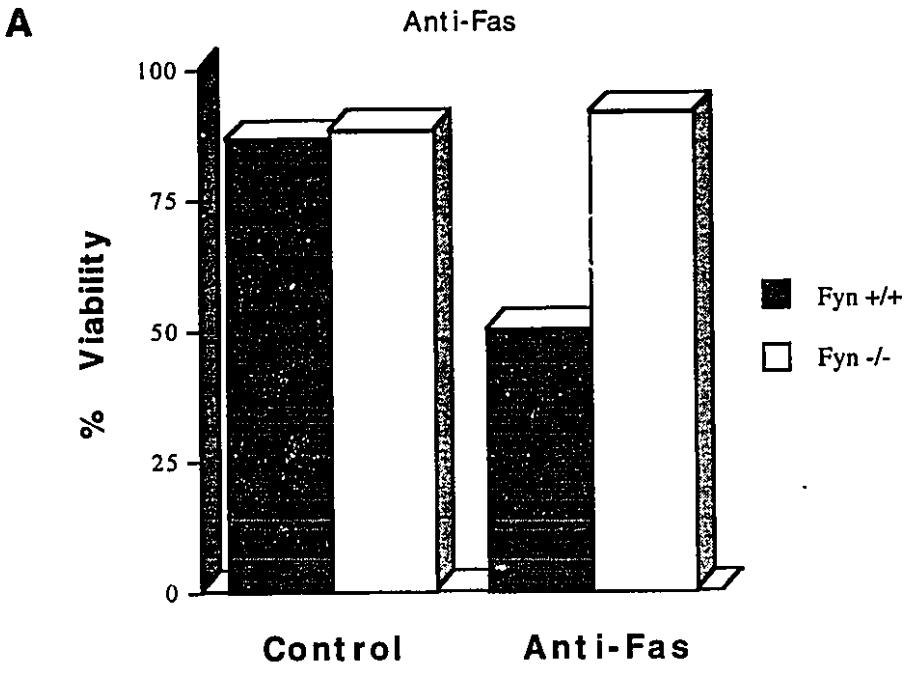
Our results thus far indicated that fyn physically associates with Fas and contributes towards the Fas death signal. Although no known signalling motifs have been

Figure 5-10 Reduced Sensitivity to Fas-Based Death of fyn-Deficient Splenocytes.

Normal or fyn-deficient splenocytes from day 5 MLRs were assessed for their sensitivity to Fas ligation by either anti-Fas (A) or PMM1 (B).

A. Splenocytes were incubated overnight in floppy high protein-binding 96 well plates previously coated with either anti-Fas or BSA as a control. The next day, viable cell numbers were determined by trypan blue exclusion staining and light microscope examination of multiple fields.

B. ^{51}Cr-labelled splenocytes were mixed with PMM1 effector cells at a range of effector to target ratios and incubated for 4 hours before being harvested.


A
Anti-Fas
100
75
50
25
0
% Viability
Fyn +/+
Fyn -/-
Control
Anti-Fas

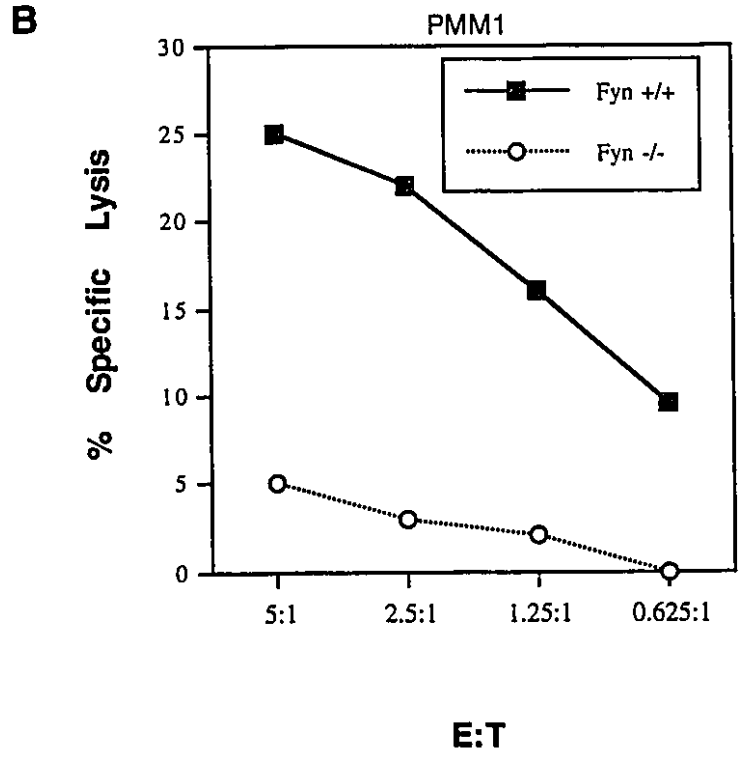

B
PMM1
30
25
20
15
10
5
0
% Specific Lysis
Fyn +/+
Fyn -/-
5:1
2.5:1
1.25:1
0.625:1
E:T

Figure 5-11 Death Domain-Containing Proteins Possess Conserved YXXL Motifs.

The death domains of a number of proteins capable of signaling apoptosis are shown. Conserved YXXL motifs are shaded and boxed, and conserved leucine residues are shaded.The alignment of the death domains is as per Cleveland and Ihle (1995).

Mouse Fas	207	I	A	E	D	M	T	I	Q	E	A	K	K	F	A	R	E	N	N	I	K	E	G	K	I	D	E	I	M
Human Fas	220	I	A	G	V	M	T	L	S	Q	V	K	G	F	V	R	K	N	G	V	N	E	A	K	I	D	E	I	K
Mouse TNFR1	333	V	V	D	G	V	P	P	A	R	W	K	E	F	M	R	F	M	G	L	S	E	H	E	I	E	R	L	E
Human TNFR1	334	V	V	E	N	V	P	P	L	R	W	K	E	F	V	R	R	L	G	L	S	D	H	E	I	D	R	L	E
Mouse RIP	575	I	R	E	N	L	G	R	-	Q	W	K	N	C	A	R	K	L	G	F	T	E	S	Q	I	D	E	I	D
Human RIP	291	I	R	E	N	L	G	K	-	H	W	K	N	C	A	R	K	L	G	F	T	Q	S	Q	I	D	E	I	D
Reaper	1									M	A	V	A	F	Y	I	P	D	Q	A	T	L	L	R	E	A	E	Q	K

Mouse Fas	235	H	D	S	I	Q	D	T	A	E	Q	K	V	-	Q	L	L	L	C	W	Y	Q	S	H	G	-	K	S	D
Human Fas	248	N	D	N	V	Q	D	T	A	E	Q	K	V	-	Q	L	L	R	N	W	H	Q	L	H	G	-	K	K	E
Mouse TNFR1	361	M	Q	N	G	R	-	C	L	R	E	A	Q	Y	S	M	L	E	A	W	R	R	R	T	P	R	H	E	D
Human TNFR1	362	L	Q	N	G	R	-	C	L	R	E	A	Q	Y	S	M	L	A	T	W	R	R	R	T	P	R	R	E	A
Mouse RIP	603	H	D	Y	E	R	D	G	L	K	E	K	V	Y	Q	M	L	Q	K	W	L	M	R	E	G	T	K	G	A
Human RIP	317	H	D	Y	E	R	D	G	L	K	E	K	V	Y	Q	M	L	Q	K	W	V	M	R	E	G	I	K	G	A
Reaper	21	E	Q	Q	I	L	R	-	L	R	E	S	Q	W	R	F	L	A	T	V	V	L	E	T	L	K	Q	Y	T

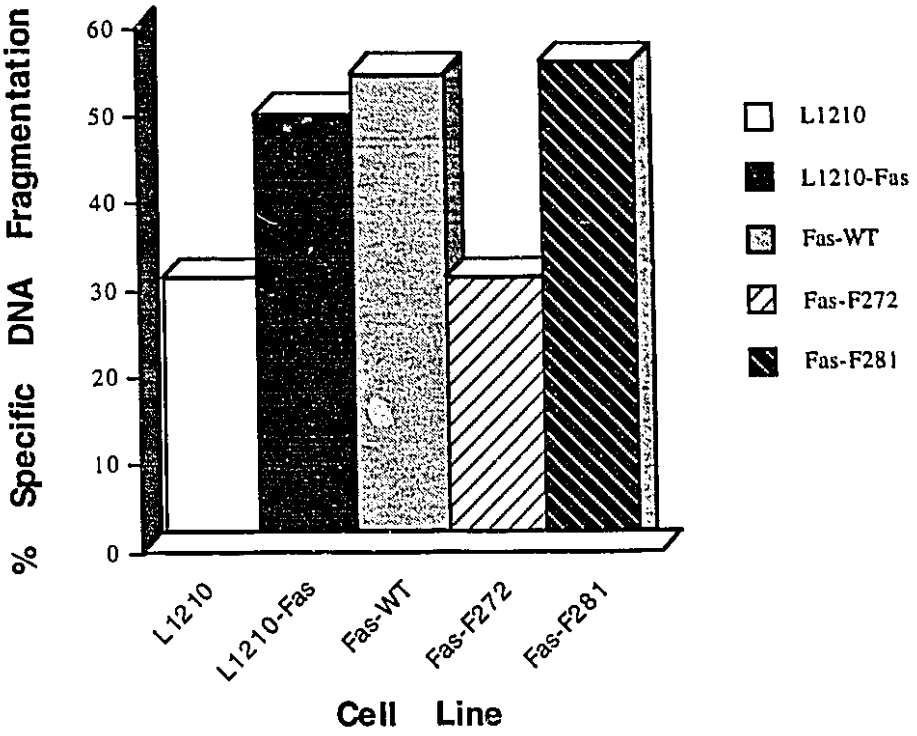
Mouse Fas	261	A	Y	Q	D	L	I	K	G	L	K	K	A	E	-	C	R	R	T	L	D	K	F	Q	D
Human Fas	274	A	Y	D	T	L	I	K	D	L	K	K	A	N	L	C	T	L	A	-	E	K	I	Q	T
Mouse TNFR1	388	T	L	E	V	V	G	L	V	L	S	K	M	N	L	-	A	G	C	L	E	N	I	L	E
Human TNFR1	409	T	L	E	L	L	G	R	V	L	R	D	M	D	L	-	L	G	C	L	E	D	I	E	E
Mouse RIP	630	T	V	G	K	L	A	Q	A	L	H	Q	C	-	-	C	R	I	D	L	L	N	H	L	T
Human RIP	346	T	V	G	K	L	A	Q	A	L	H	Q	C	-	-	S	R	I	D	L	L	S	S	L	T
Reaper	48	S	C	H	P	K	T	G	R	K	S	G	K	Y	R	K	P								

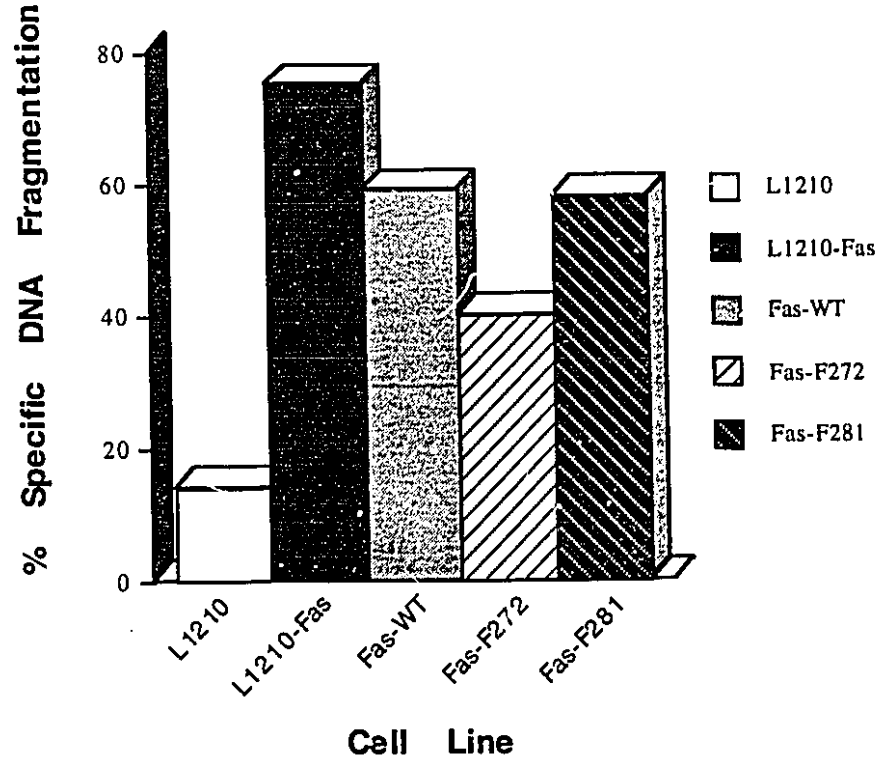
identified in the cytoplasmic domain of Fas, we wondered if a closer examination of this domain might yield clues as to how fyn interaction and/or influence might be achieved. We studied the context of the tyrosine residues in the cytoplasmic domains of both human and mouse Fas for any similarities to those in other signalling molecules that are known to interact with tyrosine kinases. Interestingly, one of the tyrosines is found in the Fas death domain within a conserved YXXL motif. This sequence is reminiscent of half an Immunoreceptor Tyrosine-based Activation Motif (ITAM) found in the T cell receptor CD3 complex chains and elsewhere (see Chan et al., 1994 for review). It is also suggestive of a binding site for proteins with SH2 domains (Songyang et al., 1993). It is also interesting to note that like Fas, other proteins with cytoplasmic death domains and which can signal apoptosis contain a similar amino acid sequence. Human and mouse TNFR1 as well as human and mouse RIP proteins all contain conserved YXXL motifs within their putative death domains, and the leucine residues of this motif in all three proteins are highly conserved among almost all the known proteins with death domain-like sequences (Fig. 5-11).

The combination of the high degree of conservation of this motif, its similarity to signaling motifs in the CD3 complex, and the fact that it contained a tyrosine residue which could potentially be a target for fyn phosphorylation, led us to hypothesize that the YXXL motif might be an important structural determinant in Fas signal transduction. We therefore decided to investigate the importance of the tyrosine residue in this motif to the Fas death signal. Site-directed mutagenesis was employed to change the tyrosine residue (amino acid 262) to a phenylalanine. As a control, the next closest tyrosine at position 253 was also converted to a phenylalanine residue. These mutations were introduced into murine Fas cDNA within the Fas expression vector utilized by Golstein and colleagues in their construction of the L1210-Fas cell line (Rouvier et al, 1993). L1210 cells were electroporated with wildtype Fas, Fas-F262, and Fas-F253 expression vectors. Transfected cells were selected by growing the electroporated cultures in the

Figure 5-12 Effect of Mutating The Tyrosine Residues in the Death Domain of Fas on Fas-Induced Apoptosis.

The ability of mutant and wildtype Fas to cause apoptosis in transfected L1210 cells when ligated with either anti-Fas antibody (A) or PMM1 (B) was assessed in DNA fragmentation assays. Anti-Fas was used at 1 μg/ml and PMM1 was used at an E:T of 0.5:1. The results are representative of 2 separate experiments.


% Specific DNA Fragmentation
60
50
40
30
20
10
0
L1210
L1210-Fas
Fas-WT
Fas-F272
Fas-F281
Cell Line


% Specific DNA Fragmentation
80
60
40
20
0
L1210
L1210-Fas
Fas-WT
Fas-F272
Fas-F281
Cell Line

presence of G418. Bulk-selected cells were then compared in a DNA fragmentation assay for their susceptibility to Fas ligation by either anti-Fas antibody or PMM1 cells (Fas ligand). L1210 and L1210-Fas cell lines were used as negative and positive controls, respectively. As seen in Figures 5-12 A and B, mutation of tyrosine 262 to phenylalanine had no impact on the ability of Fas to transmit a death signal initiated by either anti-Fas or PMM1. The amount of DNA fragmentation obtained in L1210 cells transfected with Fas-F262 was identical to that obtained in cells transfected with the wild-type Fas expression vector, and comparable to that seen in the original L1210-Fas cell line. This indicates that tyrosine 262, found within the conserved YXXL motif, does not need to be phosphorylated in order for a Fas death signal to be generated. It does not, however, rule out the possibility that p59fyn associates with Fas via interactions with this motif.

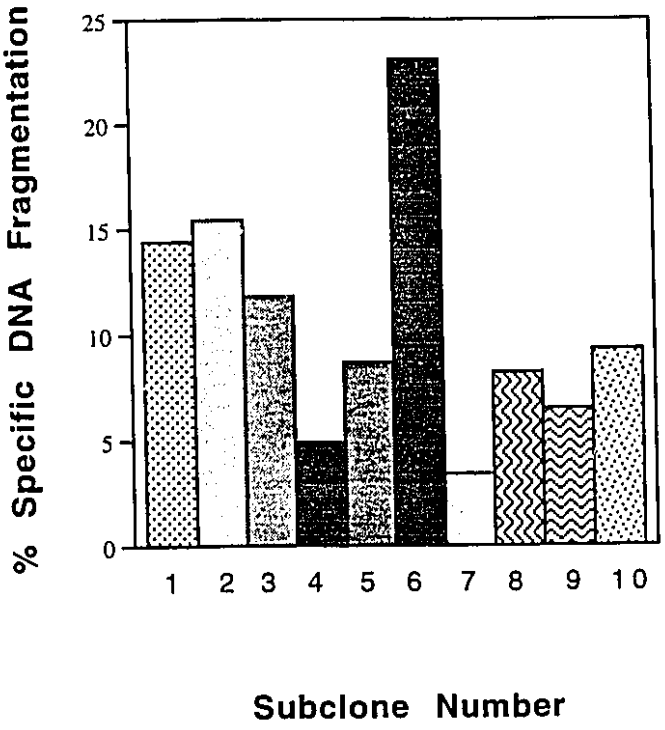
A surprising result was obtained with the Fas-F253 transfected cells. This tyr to phe mutation was made as a negative control and was expected to have no impact on Fas signalling. However, when cells transfected with this construct were exposed to anti-Fas antibody, the amount of DNA fragmentation achieved was identical to that of untransfected L1210 cells. Both untransfected and Fas-F253 transfected cells fragmented approximately 30% of their DNA during a 20 hour incubation with anti-Fas antibody. Even more surprising, the level of sensitivity of Fas-F253 transfected cells was intermediate between wild-type Fas transfectants and untransfected cells. Untransfected L1210 cells exhibited approximately 18% specific DNA fragmentation in response to PMM1 cells, whereas cells transfected with wild-type Fas fragmented approximately 60% of their DNA. Fas-F253 transfected cells responded to PMM1 by fragmenting about 40% of their DNA within the assay time period. In order to verify that all transfected cultures did indeed express elevated levels of Fas, each culture was analyzed for Fas surface expression via flow cytometry. It was found that all transfected cultures expressed much higher levels of Fas than L1210 cells. Surprisingly, Fas-F253 transfected cultures actually showed slightly higher Fas staining than wild-type or F262 Fas transfectants

(data not shown). Therefore, it appears as though tyrosine 253 may be an important residue in Fas signaling, but the impact of altering this residue to a non-phosphorylatable phenylalanine is more severe when Fas is ligated by anti-Fas antibody than when it is physiologically ligated by Fas-ligand bearing killer cells. The significance of this finding will be discussed later in this chapter.

We were puzzled by the finding that conversion of tyrosine 253 to phenylalanine had a significant impact on the ability of Fas to signal apoptosis but a similar mutation of the conserved tyrosine at position 262 had no effect. This is especially true because tyrosine 253 is not conserved in human Fas, so it seemed unlikely that it would be an important target for a critical phosphorylation. Analysis of surface Fas expression by flow cytometry indicated that the parental L1210 cell line into which we had transfected our Fas constructs had drifted in terms of Fas expression. Two types of cells appeared to be present in the cultures: one with very low Fas expression and one with somewhat more Fas, although substantially less than that expressed on the surface of L1210-Fas (data not shown). It was possible, then, that different levels of endogenous Fas might be influencing the previous results with the mutant Fas constructs. We therefore decided to reclone the L1210 cell line and select a subclone that showed low sensitivity to Fas ligation. Figure 5-13 shows the results of a DNA fragmentation assay testing the sensitivity of ten of the clones obtained to Fas-based killing by PMM1. The differences in Fas sensitivity obtained further underscore the heterogeneity that was present in the original culture. Although most clones showed low sensitivity, fragmenting only 10% of their DNA or less in response to PMM1 attack, clone number 6 fragmented over 20% of its DNA. Since clone number 4 showed relatively low sensitivity to PMM1, fragmenting less than 5% of its DNA in this assay, this clone was expanded and used as the recipient of the various Fas constructs in a new transfection. Clones 4 and 6 will be referred to as L1210-4 and L1210-6 in subsequent discussions.

Figure 5-13 Analysis of Sensitivity to PMM1 of Ten L1210 Subclones.

The parental L1210 cell line was cloned by limiting dilution. The ability of PMM1 (E:T=1:1) to cause apoptosis in ten of the subclones was assessed in a 4 hour DNA fragmentation assay.


% Specific DNA Fragmentation
25
20
15
10
5
0
1 2 3 4 5 6 7 8 9 10
Subclone Number

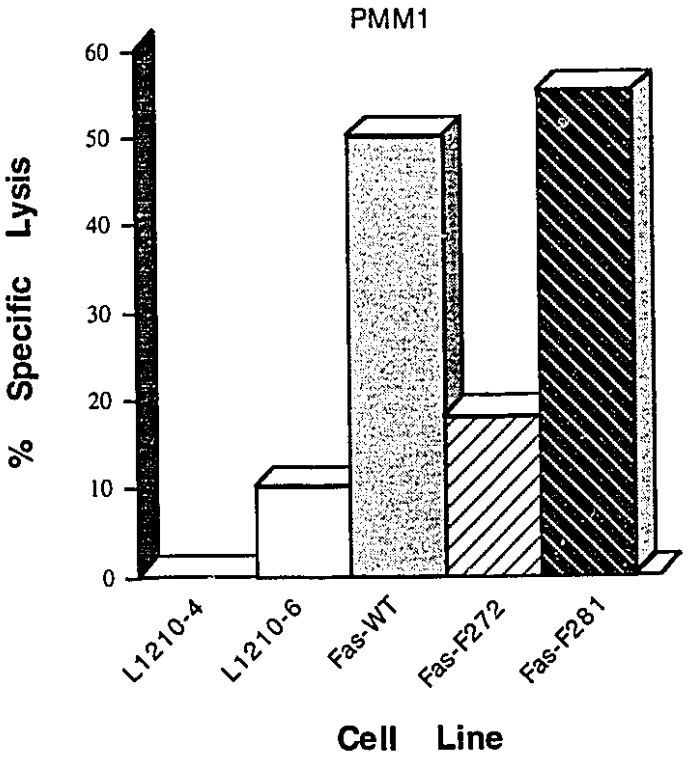
Prior to electroporating L1210-4 with the Fas expression vectors, we resequenced all three Fas vectors to ensure that the mutations we thought we were studying had not been mislabeled at any point. Having verified this, L1210-4 cells were electroporated with the wildtype, F262, and F253 Fas expression vectors and selected once again with G418. A ^{51}Cr-release lytic assay was performed on the various drug-selected cultures, using PMM1 as the cytolytic effector (Figure 5-14). Untransfected L1210-4 and L1210-6 were used as negative controls. Once again, the wildtype Fas$^+$ and F262 mutant transfectants gave comparable levels of killing, 55% and 50%, respectively. The F253 mutant again resulted in a higher level of death than in the untransfected cell line (which was entirely resistant to PMM1 in this assay) but substantially less than the wildtype or F262 constructs, giving 18% specific lysis. Analysis of surface Fas expression again showed that if anything, the cultures transfected with the F253 constructs had slightly higher levels of Fas than the other two transfected cultures (data not shown). The tyrosine at position 253 in murine Fas therefore does appear to be important in generation of the Fas death signal, and the tyrosine at position 262 that is found within the conserved YXXL motif does not appear to be critical. Studies are currently being undertaken to more closely analyze the effects of these tyr to phe mutations in cloned cell lines with comparable levels of surface Fas. As well, the effects of mutating the remaining two tyrosine residues in the Fas cytoplasmic domain are being analyzed. Determination of key residues that are important for Fas signaling will greatly aid in the elucidation of the signal transduction mechanism utilized by this important molecule.

DISCUSSION

Elimination of activated T cells is important in regulating immune responses and maintaining homeostasis. Within the last two years, the mechanism whereby this is accomplished has begun to become more clearly elucidated. It is now believed that a few days after activation, mature T cells upregulate the expression of both Fas and Fas ligand. Interaction of these cognate molecules causes T cell death via apoptosis. The importance

Figure 5-14 Re-analysis of the Effect of Mutating Tyrosine Residues in the Death Domain of Fas on Fas-Based Death.

The tyr to phe site-directed mutants of Fas (Fas-F72 and Fas-F281), as well as wildtype Fas (Fas-WT), were transfected into the PMM1-insensitive subclone L1210-4. Bulk-selected cultures were used in a four hour ^{51}Cr-release assay, using PMM1 effectors at E:T=1:1.


PMM1
% Specific Lysis
60
50
40
30
20
10
0
L1210-4
L1210-6
Fas-WT
Fas-F272
Fas-F281
Cell Line

of an intact Fas pathway is underscored by the identification of the lymphoproliferative defect in lpr and gld mice as being caused by mutations in Fas and Fas ligand, respectively. Also, a recent study has reported that a number of children afflicted with lymphoproliferative disease had mutations in either Fas or Fas ligand. Intriguingly, there is also evidence which suggests that loss of CD4+ cells in HIV-infected individuals may occur in the context of upregulated Fas expression. Therefore, strict control of Fas expression and Fas-based signals is likely of paramount importance to both lymphoproliferative and lymphodeficiency diseases. Understanding the biochemistry of the Fas death pathway is obviously relevant in that it may open avenues for pharmacological intervention in disease states caused by defects in this pathway.

Little is currently known about the nature of the Fas death signal. Susceptibility to Fas ligation lags behind actual upregulation of Fas, suggesting a further complexity in the control of the death process beyond simple control of expression. It has been suggested that the level of the anti-death proteins belonging to the ever-growing bcl-2 family may influence the susceptibility to Fas-based death. Our previous findings that activated fyn-deficient splenocytes appeared to live longer in culture than their normal counterparts caused us to hypothesize that the fyn tyrosine kinase was involved in the signal transduction pathway utilized by Fas. The experiments described in this chapter provide support for this suggestion.

We have shown that target cell killing by a CTL cell line that kills exclusively via the Fas-based death pathway is sensitive to a tyrosine kinase inhibitor. Because the inhibitor was required to be present throughout the entire killing assay, it is possible that it was affecting the killer cells and not the target cells. This seems unlikely for a number of reasons. First, although tyrosine kinase inhibitors have been shown to inhibit activation of Fas-based killer cells, this was demonstrated when the cells were activated via their TCR/CD3 complex in the presence of the inhibitor. Since CD3-mediated signaling is dependent upon tyrosine kinases, inhibition at this stage would entirely eliminate any

downstream activation event, including induction of Fas ligand expression. In our experiments, however, the PMM1 killer cells were always fully activated with PMA and ionomycin for 3 hours and should have maximally expressed Fas ligand by the time they were used. Also, calcium mobilization and activation of protein kinase C are events that are downstream of tyrosine kinase activation in CD3 signalling, so even if the PMM1 cells were not fully activated by the time they were added to the assays, the type of pharmacological induction employed should not have been influenced by tyrosine kinase inhibitors. Second, an actual requirement for Fas ligand-derived signals transduced into the killer cell also seems unlikely, since soluble Fas ligand can kill Fas-bearing targets, and since COS cells can be transformed into Fas-dependent killer cells by transfection with a Fas ligand expression vector alone. It would appear, then, that clustering of Fas at the surface of a target cell is all that is required for death, and special signaling by the killer cell is not necessary.

Support for our findings that tyrosine kinase inhibition acts to inhibit Fas-mediated apoptosis was provided by a report that was published as our experiments were being performed. Eischen et al (1994) demonstrated that DNA fragmentation in human Jurkat cells exposed to anti-Fas IgM could be inhibited by the tyrosine kinase inhibitors herbimycin A, genistein, and staurosporine in a dose-dependent manner. Interestingly, a high dose of genistein (100 µg/ml) inhibited DNA fragmentation by only 60%. This is essentially the same degree of inhibition we observed when 90 µg/ml genistein was used in a DNA fragmentation assay in which human MOLT-4 cells were exposed to anti-Fas IgM. More complete inhibition was seen by Eischen et al. when they used herbimycin A. This is in contrast to our own findings, as we found this inhibitor to be ineffectual at inhibiting DNA fragmentation in murine cells exposed to PMM1 (data not shown). The reason for this discrepancy is not known, but may be due to some differences existing between the human and mouse systems.

Our finding of a physical association between Fas and p59fyn is potentially very important. This represents the first identification of a known signal transducing molecule with a defined catalytic activity interacting with Fas and provides some insight into the signal transduction mechanism employed by Fas. The biological relevance of the observed interaction is underscored by our observations that Fas-dependent death is compromised in $fyn^{-/-}$ lymphocytes. Both natural Fas-mediated death (i.e., normal death seen in cultures of activated splenocytes resulting from Fas/Fas ligand interactions) and anti-Fas antibody-induced death are reduced in fyn-deficient lymphocytes. We therefore propose that p59fyn activity is involved in Fas signal transduction, and that this is likely achieved by a direct interaction of fyn with Fas.

The exact nature of the fyn/Fas interaction will be important to determine in the future. Interestingly, it would appear that this interaction is somewhat stronger than that between fyn and the cytoplasmic domains of the CD3 chains. In order to co-immunoprecipitate fyn with CD3, a digitonin-based lysis buffer was necessary. Triton-X100-based buffers were found to disrupt this interaction. Later, a chemical cross-linker was employed to verify the existence of the fyn/CD3 association. In preliminary *in vitro* kinase experiments, we found no difference between lysates prepared in either digitonin- or Triton-X100-based buffers (data not shown). Our ability to co-immunoprecipitate fyn with Fas from triton-X100 lysates confirms the strong fyn/Fas interaction.

The presence of a conserved YXXL motif in human and mouse Fas death domains, and the similarity of this sequence to half of an ITAM caused us to hypothesize that this motif might be important in Fas signaling, either as a binding site for fyn or as tyrosine phosphorylation target for fyn or some other kinase. An intact ITAM consists of two YXXL sequences seperated by approximately 10 amino acids. Two tyrosine kinases important in TCR/CD3 signal transduction, ZAP-70 and fyn, have been shown to interact with CD3 chains via this motif, but the nature of these interactions differs for the two molecules. Binding of ZAP-70 to the CD3-ε ITAM has recently been shown to be

dependent on very specific sequence criteria (Gauen et al., 1994). These include the requirement for an intact ITAM, accurately spaced tyrosine residues, and prior phosphorylation of the tyrosine residues. Binding of ZAP-70 to the ITAM is likely achieved via its 2 SH2 domains. Binding of fyn to an ITAM is much more flexible, however (Gauen et al., 1994). Even mutating the tyrosine residues in the ITAM or altering their spacing does not affect the interaction of p59fyn, which is thought to be mediated by its unique amino terminal region (Gauen et al., 1992).

Our finding that changing the tyrosine residue within the conserved YXXL motif (tyr 262) did not affect the ability of Fas to transmit a death signal, indicates that this residue is likely not a critical target for phosphorylation. However, it does not rule out the possibility that fyn does interact within this region, since tyrosine to phenylanalanine conversions within intact ITAM sequences still allow fyn binding to CD3-ε (Gauen et al., 1994). A more thorough analysis of mutations within this region is obviously required to fully assess the question of its importance to Fas function. Mutation of the conserved leucine residues, which are also very important in ITAM signaling function, will be done in the future. Also, careful mapping of the site of fyn interaction should be carried out by deletion analysis.

It was surprising that tyrosine 253 appears to be involved in signal transduction of mouse Fas. This residue exists within the death domain, but is not conserved in human Fas. Mouse Fas contains two additional tyrosine residues within its cytoplasmic domain. Both of these occur N-terminal to the death domain. Only one of these tyrosine residues is conserved in human Fas. Interestingly, though, not only is the tyrosine residue conserved, but so are a number of surrounding residues, raising the possibility that this tyrosine may also be involved in Fas function. The remaining two tyrosine residues in the mouse Fas cytoplasmic domain have already been mutated to phenylalanines and the effects of these changes on the ability of Fas to induce apoptosis will be determined in the

near future. For all four tyr-phe mutants, cloned cell lines should be established which have comparable surface Fas expression. This is also ongoing.

Given the plasticity of the sequence requirements for fyn binding to YXXL-containing motifs, it is tempting to speculate that the region of Fas (and perhaps TNFR1 and RIP) that contains the two highly conserved leucine residues, one of which is contained within the YXXL motif, could represent an intact "ITAM-equivalent" for fyn interaction. The interaction of Fas with FADD and RIP (and TNFR1 and Fas with themselves) has been postulated to occur via dimerization of the death domains (Chinnaiyan et al., 1995; Stanger et al., 1995; Boldin et al., 1995; Cleveland and Ihle, 1995). Also the YXXL motif of Fas encompasses the C-terminal leucine of the putative ITAM-equivalent sequence, whereas the YXXL motifs of RIP and FADD encompass the N-terminal leucine (see Fig. 11). It seems possible, therefore, that a tight heterodimeric interaction of these proteins (possibly via the multiple conserved leucine residues) could reconstitute an intact ITAM consisting of 2 YXXL motifs existing in trans.

A model in which an intact ITAM is formed by a tight interaction of Fas with another protein would predict that Fas could be co-clustered with other death domain-containing proteins at the cell surface. This hypothesis has not yet been suggested by others, and although we have not directly tested it, some of the data could be explained if they were interpreted within the context of such a model. For instance, the fact that genistein was shown to be effective at inhibiting apoptosis when Fas was ligated physiologically by Fas ligand provided by PMM1 killer cells, but actually enhanced the degree of apoptosis achieved when Fas was ligated by anti-Fas antibody suggests a fundamental difference between these two types of Fas activation. Also, the kinetics of death are much slower when Fas is ligated by optimal amounts of immobilized anti-Fas antibody than when even low levels of PMM1 are used. Both observations could be explained if PMM1 was supplying another signal, perhaps by introducing another molecule into the activated Fas cluster. Anti-Fas IgG would only cluster Fas with itself,

and while this may allow the generation of a death signal, it may not result in as strong a signal as that which is obtained physiologically.

Another important difference that we have observed between death induced by anti-Fas versus PMM1 is seen in the effect of the tyrosine 253 mutants. When this residue is changed to phenylalanine, apoptosis caused by anti-Fas antibody appears to be abolished, and only background levels of DNA fragmentation were observed. However, although this mutation does result in reduced apoptosis in response to Fas ligation by PMM1, significant amounts of DNA fragmentation above background are still achieved. Again, this could be explained if ligation by PMM1 was causing a heterogeneous complex to form, consisting of Fas plus one or more different cell surface molecules possessing cytoplasmic domains that could interact with Fas and/or fyn. If another molecule that possessed a cytoplasmic tyrosine residue that could act as a phosphate acceptor was brought into the clustered Fas complex, elimination of an important tyrosine residue on Fas may be less severe.

A multi-component activated Fas cluster could be achieved by Fas ligand-bearing cytotoxic cells in a number of ways. It is possible that another as yet unidentified Fas-like molecule exists. This would be analogous to the existence of the two distinct TNF receptors, which belong to the same NGF superfamily as Fas. Since Fas ligand exists as a trimer, this could result in the formation of a heterotrimeric cluster being formed, which would have different signaling characteristics than clusters consisting of just one molecular species. Alternatively, Fas-based killer cells could express another type of ligand that is co-clustered with Fas ligand upon binding Fas. This would be similar to the CD4 or CD8 coreceptors with lck tethered to their cytoplasmic domains being brought into the proximity of the TCR/CD3 complex upon antigen/MHC binding by a T cell. If this were the case, it would predict that either an additional Fas ligand exists on the surface of some cytotoxic cells, or an additional protein on the target cell surface can cooperate in Fas death.

Finally, it is interesting to point out that in some circumstances, Fas ligation can cooperate with T cell receptor/CD3 stimulation in signaling T cell mitogenesis (Alderson et al., 1993), and that T cells from Fas-defective *lpr* mice exhibit reduced responsiveness to antigen (Singer and Abbas, 1994). Our observation that p59fyn binds to Fas provides an explanation for both of these phenomena, by identifying a specific tyrosine kinase that is common to the two systems. Fas-bound p59fyn may, under some conditions, be brought into close proximity with the CD3 chains, and may be able to contribute additional tyrosine phosphorylation of CD3 or other complexed proteins, leading to enhanced T cell stimulation. It will be interesting to determine if the *lpr* mutation disrupts the association between Fas and p59fyn.

Although we believe that p59fyn plays an important role in Fas signal transduction, it is clearly not absolutely essential. Apoptosis still occurs, albeit at a reduced rate, in activated $fyn^{-/-}$ splenocytes. It is likely that other tyrosine kinases may be able to partially compensate for p59fyn in its absence, as may be the case in CD3 signal transduction (Appleby et al, 1992). To our knowledge, fyn-deficient mice have not been shown to exhibit an *lpr*-like phenotype, with large numbers of T lymphocytes accumulating with age. This has not yet been specifically studied, however, and our data would predict that in older $fyn^{-/-}$ mice, there may be some degree of lymphoproliferative disease apparent. Our observations are also important in that they predict that some types of human lymphoproliferative and autoimmune diseases could result in mutations that affect fyn-Fas interactions or p59fyn activity. Clearly, the identification of a physiologically relevant kinase associating with the apoptosis-inducing Fas antigen opens up numerous new avenues of investigation for elucidating the entire nature of the death signal.

CHAPTER SIX: SUMMARY AND FUTURE DIRECTIONS

SUMMARY OF RESULTS

Gene Targeting of Granzyme B

In an attempt to knock out the granzyme B gene in mouse embryonic stem cells, two different targeting vectors were constructed. The first vector utilized a genomic DNA clone from nonisogenic DNA which had been previously isolated and sequenced in our laboratory. Concurrently, a granzyme C targeting vector was also constructed in a similar way. After several unsuccessful attempts were made at targeting the granzyme B and C genes with these constructs, it was decided to design and construct a new granzyme B targeting vector, taking into consideration recently published information regarding factors which influence the efficiency of homologous recombination. The second generation granzyme B targeting vector differed from the first in that it was substantially larger (8.3 kb of total homology with the native locus versus 4 kb), utilized isogenic DNA and a re-engineered neo cassette, and had a much longer short arm (3.4 kb versus 0.9 kb). This vector was used to successfully target the granzyme B gene in germline-competent D3J8 ES cells, with a targeting efficiency of 1 in 64 utilizing the positive/negative enrichment protocol of Mansour et al. (1988). Granzyme B-deficient mice have not been generated by us, since Heusel et al. (1994) reported that they had also knocked out this gene and already had preliminary results of the mutant phenotype.

p59fyn^T and CTL Activation

I have investigated the possibility that the src-family non-receptor tyrosine kinase p59fyn^T plays a role in the signal transduction mechanism which results in the acquisition of cytotoxic potential of CTL. This question was addressed utilizing splenocytes obtained from fyn^T-deficient mice generated by Appleby et al. (1993). Little difference was seen between normal and fyn-deficient lymphocytes activated by either alloantigen or anti-CD3 antibody in terms of their ability to kill appropriate target cells or in the induction of the transcripts for the cytotoxic proteins perforin and granzyme B. Shortly after these experiments were performed, Lancki et al. (1995) reported that both

lytic pathways of CTL remained fundamentally intact in fyn-deficient T cell clones, in agreement with our own findings. However, they found that cytolytic induction of T cell clones stimulated via the glycosyl-phosphatidylinositol-linked surface molecule Thy-1 was greatly impaired in fyn-deficient cells, suggesting a unique role for this tyrosine kinase in signaling by this protein. Fyn had previously been shown to interact with Thy-1 (Thomas and Samelson, 1992).

Curiously, we observed an increased lifespan *in vitro* and an elevated cloning efficiency of $fyn^{-/-}$ CTL concomitant with a decreased level of apoptosis. This led to the hypothesis that fyn might be involved in the downregulation of immune responses, perhaps by contributing towards the signals generated upon ligation of the recently identified Fas death protein.

p59fyn and Fas Signal Transduction

While investigating the possibility that fyn is involved in Fas signal transduction, it was shown that Fas-dependent target cell death was inhibited by the tyrosine kinase inhibitor genistein when Fas was physiologically ligated by Fas ligand on PMM1 killer cells. However, genistein actually caused an enhancement of anti-Fas antibody-mediated apoptosis. This likely indicates a complex transduction cascade, with tyrosine kinases being involved at various levels. It could also suggest that Fas-dependent killer cells may cause target cell death by ligating Fas in conjunction with other cell surface proteins, resulting in a death signal that is biochemically distinct from that achieved via simple Fas clustering.

Fyn was shown to specifically interact with Fas. The physiological relevance of this observation was provided by the demonstration that fyn-deficient lymphocytes exhibit a decreased sensitivity to the Fas-dependent death pathway, induced by either anti-Fas antibody or PMM1 killer cells. Experiments involving site-directed mutagenesis of tyrosine residues within the Fas death domain indicated that the tyrosine residue located within an ITAM-like YXXL motif was not necessary for the Fas death signal.

However, mutation of a nonconserved tyrosine residue within the Fas death domain did result in reduced levels of Fas-dependent apoptosis. The significance of this observation remains unclear and demands additional experiments to fully investigate the role this tyrosine residue plays in Fas signal transduction. Nevertheless, the results presented in this thesis provide evidence that the p59fyn tyrosine kinase contributes towards the Fas death signal.

FUTURE DIRECTIONS

The Role of Granzyme B in Target Cell Destruction

Recent experiments from our lab have shown that an important physiological substrate for granzyme B is the ICE-like proteinase CPP-32 (Darmon et al., 1995). This enzyme is responsible for the cleavage of PARP that occurs early in the apoptotic process (Nicholson et al., 1995; Tewari et al., 1995). Results from the granzyme B knockout mice (Heusel et al., 1994) as well as the *in vitro* fragmentin assays of Greenberg's group (Shi et al., 1992a; 1992b) and the RBL transfection experiments of Henkart's lab (Nakajima and Henkart, 1994; Nakajima et al., 1995b) indicate that granzyme B contributes significantly towards the DNA fragmentation that occurs in the target cell upon CTL attack. However, the relevence of PARP cleavage to DNA fragmentation is not clear, and PARP cleavage is likely not central to apoptosis, since PARP-deficient mice develop normally (Wang et al., 1995). The search for granzyme B and CPP-32 substrates is therefore not over, and more work is required to define how granzyme B and/or CPP-32 lead to DNA fragmentation.

The present model for granule-mediated destruction of target cells suggests that perforin lesions on the target cell membrane allow the entry of other granule proteins, including granzymes, into the cell and these proteins act on intracellular substrates. Extracellular effects that synergize with a perforin signal have not been ruled out for granule-localized lytic molecules, however. The availability of perforin- and granzyme B-knockout mice allows this alternative possibility to be tested. The perforin-facilitated

entry model of granzyme B action requires that both proteins reside in the same cell, if not in the same granule. If granzyme B does not need to enter the target cell to contribute to apoptosis, but instead can provide a signal that cooperates with that given by perforin, or if it can enter the cells in a perforin-independent way, one would predict that co-incubation of target cells with a combination of CTL lacking one or the other lytic molecule would result in an equivalent amount of apoptosis as is achieved by normal CTL. This hypothesis has recently been addressed in our lab. Preliminary results suggest that some of the deficiencies of target cell lysis seen with the single knockout CTL can be compensated for when the two mutant CTL lines are used together. Interestingly, this includes the ability of the granzyme B- and perforin-deficient CTL to work together in causing the cleavage of CPP-32, a phenomenon which does not occur when either of the CTL lines is used as the sole effector (E.Atkinson, A. Darmon and R.C. Bleackley, unpublished results). Clearly then, the present perforin/granzyme model of CTL function has room for refinement.

The Contribution of DNA Fragmentation to CTL-Mediated Apoptosis

The contribution that DNA fragmentation makes towards target cell death is also still a contentious issue and needs further study. Although Henkart and others have shown that enucleated cells are as efficiently destroyed via the granule and Fas pathways as intact cells possessing nuclei (Schulze-Osthoff et al., 1994; Nakajima et al., 1995a) this is not a satisfying way to address this issue. Enucleated cells are not normal, viable cells and so cannot be used to investigate the role that DNA fragmentation makes towards cell death. An interesting physiological system in which this question may be studied is that of the developing eye lens. During development, the cells that make up the lens undergo apoptosis-like DNA fragmentation and enucleation, a process that results in making the cells translucent (reviewed in Sanders and Wride, 1995). The cells themselves are left intact, with no morphological changes such as membrane blebbing or disintegration occurring. The phenomenon of DNA fragmentation is thus separated from the membrane

effects of apoptosis in these cells. If we accept the model of granule-mediated killing that suggests that CTL lytic molecules cause the destruction of their targets by activating a pre-existing suicide program, it would be interesting to study the characterisitics of CTL-induced cell death using pre-lens cells as targets. Are these cells capable of initiating the membrane effects of apoptosis? Or are they restricted to the nuclear events? If the suicide program endogenous to these cells precludes the morphological and membrane blebbing events characteristic of apoptosis, then nuclear death can be distinguished from cell death and DNA fragmentation cannot be considered as being merely an epiphenomenon of CTL-mediated apoptosis, as suggested by Henkart (Nakajima et al., 1995a).

The Role of p59fyn in Fas Signal Transduction

Our observations that the p59fyn tyrosine kinase associates with Fas and contributes towards the death signal delivered by this protein are intriguing, but many questions remain. It is clear that fyn is not absolutely required for apoptosis in activated lymphocytes, since fyn-deficient splenocytes still undergo significant, albeit reduced, apoptosis *in vitro*. Other components of the Fas death signal are likely important. More work is therefore needed on the mechanism of Fas signal transduction, including the characterization of the proteins found to associate with the Fas death domain (Boldin et al., 1995a; Chinnaiyan et al., 1995; Stanger et al., 1995; Sato et al., 1995). Also, *in vivo* elimination of activated T cells in response to tumour or virus challenge should be studied in fyn-deficient mice to see if they develop a *lpr/gld*-like lymphoproliferative disease over time.

Further study on the characteristics of the fyn/Fas association are required. The regions of both fyn and Fas that are necessary for their interaction should be mapped, as has been done for the physical requirements of the association of fyn with the CD3 chains (Gauen et al., 1992; Gauen et al., 1994). Interestingly, although the CD3 ITAM motifs are the sites of fyn interaction, no single mutation of any residues of this motif in CD3ε were found to disrupt fyn binding, and the spacing between the two YXXL squences was

also not critical (Gauen et al., 1994). This is in contrast to ZAP-70 binding to ITAMs, which requires both of the intact YXXL sequences of the motif, as well as their accurate spacing (Gauen et al., 1994). Our results demonstrating no effect of mutating the tyrosine residue of the conserved YXXL motif found in the Fas death domain to a phenylalanine may reflect this plasticity in fyn binding. However, further analysis of the ability of this mutant to interact with fyn is required. Also, a more thorough study of the effects of additional mutations in this motif should be performed, especially of the conserved leucine residue.

Since we found a surprising reduction in Fas-based death when the nonconserved tyrosine residue in the death domain was mutated, we have embarked on a project to mutate the two additional tyrosine residues in the Fas cytoplasmic region. We have not been able to demonstrate tyrosine phosphorylation of Fas itself upon ligation, although this needs to be studied more thoroughly, especially in the context of the effects of the tyrosine site-directed mutants. Our results thus far with the Fas mutants have been with bulk-selected cultures of transfected cells and analysis of several clones with equivalent Fas expression is required before any firm conclusions as to the importance of any of the mutated residues to Fas signaling can be made.

Finally, several of our observations suggested that the Fas death signal generated by ligation with anti-Fas differs from that induced by Fas ligand on PMM1 cells. It will be interesting to address the possibility that Fas-dependent killer cells co-cluster a non-Fas molecule with Fas. This would be analogous to the co-clustering of CD3 with the CD8 or CD4 coreceptors on T cells by antigen/MHC. It would also be similar to the two different TNF receptors being co-clustered by trimeric TNF. Since death domains are able to interact with each other (Boldin et al., 1995b), it is intriguing to speculate that Fas might be brought into the context of other death domain-containing proteins by additional ligands on Fas-dependent killer cells. It might therefore be possible that different types of protein-protein interactions could influence the type of signal generated by Fas ligation.

Interestingly, a recent paper provides evidence that apoptosis of activated lymphocytes can occur by two distinct molecular mechanisms: one involving Fas and one involving the type II TNF receptor (TNFR-II) (Zheng et al., 1995). Unlike the TNFR-I, the TNFR-II does not contain a death domain, and has not been generally regarded as having cytotoxic signaling capabilities. Although Zheng et al. (1995) have interpreted their findings as indicating that the TNF- and Fas-based elimination of activated T cells are distinct, this has not been convincingly demonstrated and some of their results could be explained if Fas and TNFR-II acted in cooperation with each other. For instance, in some circumstances apoptosis of activated lymphocytes can be decreased by blocking either Fas or TNFR-II whereas at other times blocking of one is more efficient than blocking of the other. This would be consistent with co-clustering of Fas and TNFR-II resulting in an apoptosis signal under some conditions.

Concluding Remarks

Much work is still required to elucidate both the granule-mediated and Fas-dependent mechanisms of CTL lysis. However, new technologies such as gene targeting are allowing several basic questions to be addressed that were impossible to even ponder a short time ago. The explosion of growth in this field and its intersection with other fast-moving areas of research such as programmed cell death and ICE family proteases have resulted in a number of long-standing mysteries of CTL function being elucidated in the last few years. The turn of the century and the dawn of a new millennium will surely find many more aspects of cell-mediated cytotoxicity being understood, opening the door for the development of new methods of therapeutic intervention for immune deficiencies and autoimmune diseases. The "killing field" will remain competitive, frustrating and exciting, for years to come.

CHAPTER SEVEN: BIBLIOGRAPHY

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