Peptide Vinyl Sulfones: Inhibitors and Active Site Probes For the Study of Proteasome Function *In Vivo*

by

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ABSTRACT

Protein degradation plays an important role in the control and regulation of many biological functions, ranging from cell cycle progression to presentation of viral antigens for scrutiny by cells of the immune system. At the heart of many of these catabolic events is the multi-catalytic proteinase complex known as the proteasome. This large barrel-shaped protein complex executes a remarkable set of functions ranging from the complete destruction of abnormal and misfolded proteins to the exquisitely specific proteolytic activation of crucial signaling molecules. Inhibitors of this proteolytic complex have thus been extremely useful for perturbing its function and deciphering its role in these widely diverse biological processes.

The first part of this work describes the synthesis of a class of peptides in which the C-terminal carboxylic acid is replaced with a vinyl sulfone moiety. Although initially described as inhibitors of cysteine proteases, peptide vinyl sulfones containing the core sequence LeuLeuLeu are capable of covalent, irreversible inhibition of proteasomal proteolysis by modification of the catalytic, threonine hydroxyl nucleophile. Peptide vinyl sulfones composed of different amino acid sequences were also synthesized and evaluated for activity against the proteasome. These structure-function studies provide further insight into the proteolytic mechanism and substrate specificity of the proteasome.

The second portion of this thesis describes the *in vivo* application of the peptide vinyl sulfones and in their use in uncovering the role of the proteasome in the Human cytomegalovirus (HCMV)-mediated destruction of MHC class I heavy chains The genome of the Human cytomegalovirus contains two genes, US2 and US11 which are responsible for the rapid degradation of MHC class I molecules. Treatment of HCMV infected cells with peptide vinyl sulfone

inhibitors of the proteasome lead to the accumulation of a heavy chain breakdown intermediate which was devoid of N-linked glycan and was found in the cytosol. Not only does the proteasome play a role in destruction of this ER resident protein but also , the process by which the viral gene products US2 and US11 catalyze proteolytic destruction of MHC class I molecules involves the reverse translocation of a polypeptide from the ER to the cytosol. Further, this process of retrograde translocation in the ER may be a much more general phenomenon used by normal cells to purge the ER of misfolded proteins.

The final portion of this work describes the characterization of a population of lymphoblastoid cells which are adapted to growth in the presence of the peptide vinyl sulfone inhibitors. Normally, prolonged exposure of cells to proteasome inhibitors results in death, presumably due to loss of proteasome function. However, we have found that prolonged exposure of cells to low concentrations of the proteasome inhibitor NIP-L_3VS results in the outgrowth of cells which are able to grow in concentrations of inhibitor which are toxic to previously un-exposed cells. These cells are not the result of a genetic mutation nor are they the result of a multi-drug resistance phenomenon. Biochemical characterization of these cells indicates that these cells are able to survive in the absence of normal proteasome function. Examination of other non-proteasomal proteolytic activities in these cells revealed that adaptation may be the result of up-regulation of additional proteases which are able to compensate for proteasome functions required for cell survival. Thus the use of the peptide vinyl sulfone proteasome inhibitors in living cells has allowed for the study of other proteolytic functions which may otherwise have been overlooked.

Thesis supervisor: Professor Hidde L. Ploegh

Title: Professor of Biology

For Pake: You will always be an inspiration to me

ACKNOWLEDGEMENTS

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I would also like to thank Jun Liu for supporting me in the beginning stages of my quest for the PhD. He has always been an extremely positive influence for me here at the Center for Cancer Research. There are many things that I learned from him during the my year in his lab that will always be a part of the way I do science. I also thank Larry Stern for taking the time to read this massive document and for agreeing to be my joint-advisor.

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I would also like to thank all my fiends in the Chemistry department; Dave, Evan , Ben, Bill, Jeff, Martha, Becky, and Chris. They all helped to make my graduate experience a fun one and many times helped me when I just didn't know what to try next. I thank Evan for letting me move into his lab on occasion and for sharing his incredible wealth of knowledge with me. I thank Sonya for helping me that first day with my mice (I forgive you for killing that one...there was nothing you could do...he had a bad heart) and for being a great friend ever since. I thank Ben for all his help during my first year in Jun's lab and for all the feedback (like rippin' guitar solo, indie rock feedback) he has given me on a number of occasions as well as for always playing the Devil's advocate when I show him my data ("I think you're labeling GST!").

I would also like to thank the members of my family who have made all this possible. I thank my Dad for his help and support and for being one of the few people in the family that I can really answer the question "so what are working on?". I thank Tom Bardos (1st cousin twice removed. for those of you who are interested in genealogy) for all his support throughout my scientific career and my entire life. He has been a strong motivating force in my life and for that I thank him. I would also like to thank my uncle Gerry (WUG) for everything he has done for me. I will never forget the year I spent living in "the bachelor pad" during my first year at MIT. He made what could have been a difficult first year of graduate school seem like a piece of cake.

I would also like to thank my mom. It is hard to follow such a huge list of people with someone as important as my mom. I will never be able to say thanks enough by writing it here at the beginning of my thesis. I feel extremely lucky to have had her love and support throughout this, yet another milestone in my life. It is many of her personal traits, that I see in myself, that have made the daunting tasking of achieving a PhD possible for me.

Finally, I want to thank my future wife (fianceé, there I said it) Becky. She has always been around to cheer me up when I feel like nothing will ever work. She has given me the same advice I gave her when she was writing her thesis "don't worry, it will get done". She is a truly remarkable person and I feel very lucky to have her standing behind me ever step of the way.

TABLE OF CONTENTS

LIST OF FIGURES

CHAPTER 1

CHAPTER 2

LIST OF ABBREVIATIONS

Ab	Antibody
ATP	Adenine tri-phosphate
BFA	Brefeldin A
Boc	t-butoxy-carbonyl
Bpa	p-benzoyl-phenylalanine
β_2m	β_2-microglobulin
βNA	β-nitroanilide
Cbz	Benzyloxycarbonyl
CH_2Cl_2	Dichloromethane
CHO	Carbohydrate
DCC	Di-cyclohexyl-carbodiimide
DIEA	Diisopropylethyl amine
DMEM	Dulbecco's modified Eagle's medium
DMF	Dimethyl formamide
DMSO	Dimethyl sulfoxide
DTT	Dithiothreitol
Et_2O	Di-ethyl ether
EtOAC	Ethyl acetate
FACS	Fluorescence activated cell sorter
HC	Heavy chain
HCMV	Human cytomegalovirus
HLA	Human histocompatibility leukocyte antigen
HOBt	N-hydroxybenzotriazole
HSV	Herpies simplex virus
IC50	50% inhibiting concentration
IEF	Isoelectric focusing
kD	Kilodalton
LLnL	Acetyl-leucinyl-leucinyl-norleucinal
LMP	Low molecular weight protein
MCA	4-methyl-cumaryl-7-amide
MCMV	Murine cytomegalovirus
MHC	Major histocompatibility complex
NEPHGE	Non-equilibrium pH gradient gel electrophoresis
NIP	4-hydroxy-3-iodo-nitropheylacetic acid
NLVS	4-hydroxy-3-iodo-nitrophylacetyl-leucinyl-leucinyl-leucine vinyl sulfone
NP	4-hydroxy-nitropheylacetic acid
NP-40	Nonidet P-40
PBS	Phosphate buffered saline
PGPH	Peptidyl glutamyl peptide hydrolylzing activity
PNGase	Peptide N-glycanase

PPO	2,5-diphenyloxazole
PyBOP	Benzotriazole-1-yl-oxy-pyrrolidino-phosphonium hexafluorophosphate
SDS-PAGE	Sodium dodecylsulfate-polyacrylamide gel electrophoresis
TAP	Transporter associated with antigen presentation
TFA	Trifluoroacetic acid
THF	Tetrahydrofuran
TsOH	Tosic acid
Z	Benzyloxycarbonyl (also Cbz)

I. BACKGROUND

Matthew Bogyo, Maria Gaczynska, and Hidde L. Ploegh. Proteasome Inhibitors and Antigen Presentation. *Peptide Science.* In press.

A. Introduction

The proteasome is a multi-subunit complex responsible for the degradation of many if not all cytosolic proteins (Coux et al., 1996). It plays a crucial role in a variety of biological processes including degradation of key regulatory proteins such as the cyclins and the activation of transcription factors by removal of inhibitory factors (Palombella et al., 1994; Sheaff and Roberts, 1996; Ghislain et al., 1993; Hateboer et al., 1996; Glotzer et al., 1991). The proteasome is also required for the generation of antigens for presentation to cytotoxic T-cells via the major histocompatibility complex (MHC) class I pathway (Goldberg and Rock, 1992; Goldberg et al., 1995). Its role in these diverse biological processes has been uncovered largely through the use of inhibitors and genetic mutants which allow for the controlled blocking of proteolytic function.

B. Structure and Mechanism of the Proteasome

The proteasome is a large, barrel-like structure made up of four stacked rings of 7 subunits each (Coux et al., 1996; Löwe et al., 1995; Lupas et al., 1995). These rings form a tunnel with openings at either end and an inner core where controlled proteolysis takes place. There are two types of subunits termed α and β, with the α-subunits playing mainly a structural role while the β-subunits are endowed with catalytic activity. The simplest type of proteasomes are found in bacteria such as *Rhodococcus* (Lupas et al., 1994)and in the archaebacterium *Thermoplasma acidophilum.* (Lupas et al., 1995). In these organisms the proteasome is made up of only a single type of α subunit and a single type of β subunit. The eukaryotic proteasome, although structurally similar to the eubacterial enzyme, is much more complex, consisting of 14 unique but related α and 14 unique but related β subunits (Coux et al., 1996; Lupas et al., 1995; Dahlmann et al., 1989; Heinemeier et al., 1994). The eukaryotic proteasome exists as a 20S complex made up of two rings of catalytic β-subunits (7 subunits per ring) and two rings of α-subunits (also seven subunits per ring), or as a larger 26S complex in which regulatory subunits (termed the 19S complex) are added to the core 20S complex. These regulatory subunits include ATPase, isopeptidases (discussed below) and several proteins thought to be responsible for the

unfolding of a protein substrate prior to insertion into the proteolytic core of the 20S proteasome. The structure of the 20 and 26S proteasomes is shown in figure 1.1.

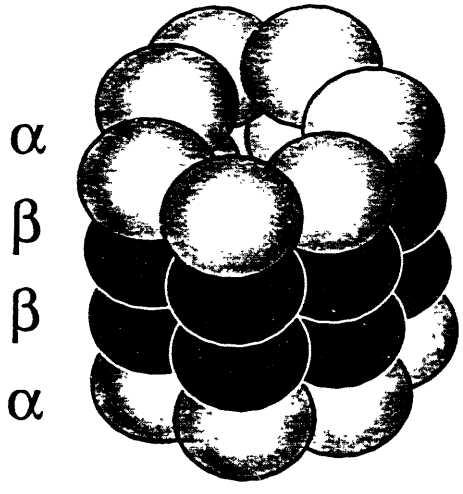
The 26S complex, unlike the 20S complex, binds ATP and is responsible for the degradation of proteins which have been targeted for degradation by conjugation with a 72 amino acid polypeptide known as ubiquitin (Hershko and Ciechanover, 1992). Ubiquitin is attached to a target protein by an isopeptide bond formed between the ε-amino group of lysine on the target and the C-terminal glycine reside of ubiquitin. This conjugation is performed by a series of enzymes called E1, E2 and E3 (Hershko and Ciechanover, 1992). The mechanism by which ubiquitin is conjugated to a protein for degradation is shown in figure 1.2. Ubiquitin conjugating enzymes act in series by transferring a ubiquitin chain from one enzyme to the next by an activated thio-ester linkage to a cysteine residue. The final step is a transfer of the activated ubiquitin chain from the E2 enzyme to the target protein. The mono-ubiquitinated protein is then acted on again and the same ubiquitin conjugating enzymes attach an additional ubiquitin to the previous one at either of two possible lysine residues. Ubiquitin conjugation continues and results in a high molecular weight poly-ubiquitin protein complex. This heterogeneous population of ubiquitin tagged molecules is then the target for rapid degradation by the 26S proteasome (Coux et al., 1996).

Once a ubiquitin-protein conjugate begins to be destroyed by the 26S proteasome, ubiquitin is recycled by removal of the large, branched poly ubiquitin chain (Wilkinson et al., 1995). The resulting polymer is subsequently cleaved to individual monomer units by enzymes called isopeptidases which perform the cleavage of the ubiquitin-isopeptide bond. In some cases an isopeptidase may remove a polyubiquitin chain from a protein , thus saving the potential substrate from destruction by the proteasome (Lam et al., 1997). There are several known isopeptidases in this family of enzymes but still little is known about the mechanism by which these enzymes act (Hadari et al., 1992; Falquet et al., 1995; Lam et al., 1997; Wilkinson et al., 1995). Possible inhibitors of these peptidases could be of great importance in deciphering the role of ubiquitin conjugation in protein degradation.

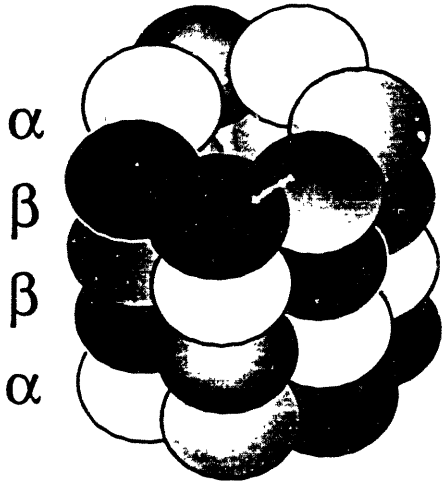
One of the qualities which distinguishes the proteasome from many other proteolytic enzymes is its multiple peptidase activities. Initial studies of the

Figure 1.1. Structure of the eukaryotic and prokaryotic 20S and 26S proteasome. (A) cartoon representation and (B) sketch based on the electron micrograph.

A.

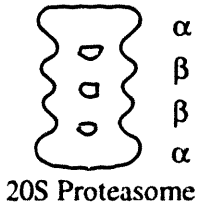


Thermoplasma,
Rhodococcus
20S proteasome

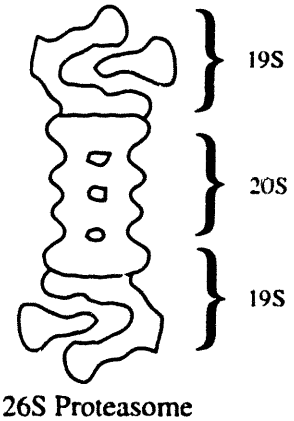


Eucaryotic
20S proteasome

B.

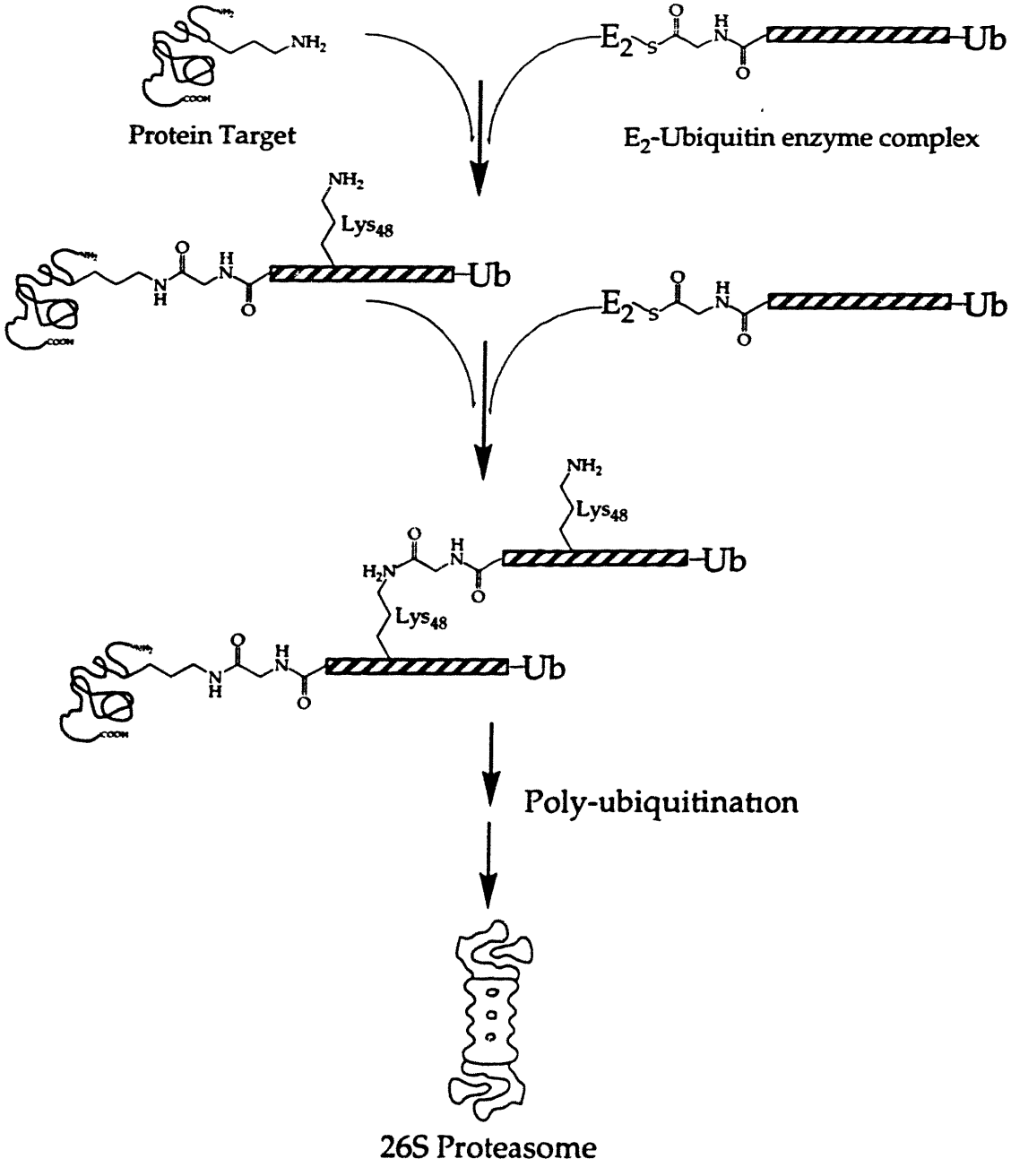


20S Proteasome



26S Proteasome

Figure 1.2. Conjugation of ubiquitin to a target protein for destruction by the 26S proteasome.


Protein Target
E2-Ubiquitin enzyme complex
Lys48
Ub
Poly-ubiquitination
26S Proteasome

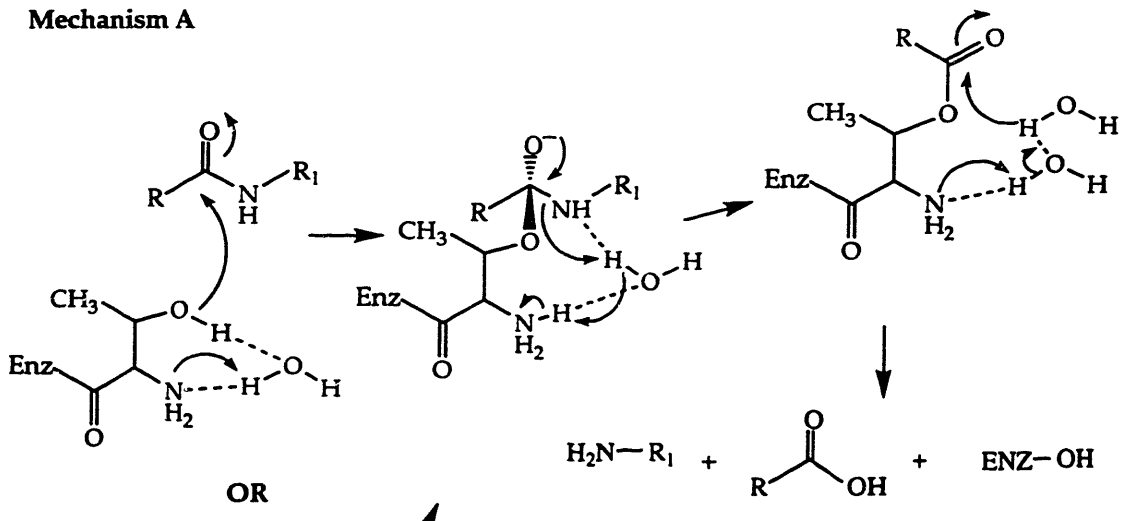
proteasome using fluorogenic substrates consisting of a variety of sequences indicated that the proteolytic activity of the proteasome could be categorized into three main activities: cleavage after hydrophobic side chains (chymotrypsin-like), cleavage after acid side chains (post glutamyl peptidase), and cleavage after basic side chains (trypsin-like)(Orlowski, 1990; Wilk and Orlowski, 1983; Rivett, 1989). These activities were found to be the result of distinct active sites and could be modulated by mutation of β-subunits or by changing the composition of individual subunits (Goldberg et al., 1995; Hilt and Wolf, 1995). In addition to the three major proteolytic activities of the proteasome, two other activities have been discovered as the result of their resistance to inhibition by the serine protease inhibitor 3,4-dichloroisocoumarin (Cardozo et al., 1992). These two proteolytic activities result in cleavage after branched-chain amino acids (BrAAP activity) and cleavage after small neutral amino acids (SNAAP activity)(Cardozo et al., 1992).

Initial studies of the proteasome were unable to classify it into a category with other known proteases mainly due to lack of homology and unusual reactivity with protease inhibitors (Zwickl et al., 1991; Zwickl et al., 1992). It was initially thought to utilize a cysteine or serine as the catalytic nucleophile, but exhaustive mutational analysis found neither of these residues to be required for catalytic activity (Seemüller et al., 1995). Recently mutation analysis of the β subunits of the Thermoplasma proteasome showed the N-terminal threonine residue to be required for hydrolysis (Seemüller et al., 1995). This, combined with the X-ray crystal structure of the 20S proteasome from Thermoplasma with an inhibitor bound in the active site, provided strong evidence that the sidechain hydroxyl of Thr_1 was the catalytic nucleophile (Löwe et al., 1995; Seemüller et al., 1995). The proposed mechanism of peptide hydrolysis by a β-subunit of the proteasome is shown in figure 1.3. Activation of the sidechain hydroxyl of threonine is thought be catalyzed by either the free amino terminus or the amine of a nearby lysine. This lysine residue, in conjunction with a glutamic acid residue, may act as a charge relay system analogous to that found in most serine proteases. Thus the proteasome is an example of a N-terminal hydrolase, of which there are only a few known other examples (Tikkanen et al., 1996; Duggleby et al., 1995; Smith et al., 1994).

Like many proteolytic enzymes, activation of the N-terminal nucleophile found on the catalytically active subunits of the proteasome is a process involving a proteolytic processing step. The proteasome synthesizes all of its catalytic β-subunits as inactive precursor proteins which must first be activated by removal of an N-terminal pro-sequence. This prosequence is remarkably diverse from subunit to subunit and there is also little homology within the pro-sequences of β-subunits from different species (Seemüller et al., 1996). Mutational analysis has shown that the catalytic Thr_1 as well as a conserved glycine residue on the N-terminal side of Thr_1 (termed Gly_{-1}) are both required for efficient processing of the β-subunits (Schmidtke et al., 1996; Chen and Hochstrasser, 1996). It has been reported by multiple laboratories working with yeast and mammalian systems that removal of the prosequence is not required for assembly of subunits into the large 20S and 26S complexes, and therefore it is believed that the pro-sequences prevent catalytic activation of β-subunits before they become part of the larger proteolytic complex (Chen and Hochstrasser, 1996; Schmidtke et al., 1996). The mechanism of pro-sequence hydrolysis, however, still remains unclear. Several reports have proposed an intermolecular mechanism in which another β-subunit is required for processing (Chen and Hochstrasser, 1996; Seemüller et al., 1996), while a purely autocatalytic, intramolecular process remains possible. The proposed mechanism for autocatalytic processing of a β-subunit is shown in figure 1.4. The details of the processing events are difficult to address and the process may in fact be a combination of the two proposed mechanisms.

Figure 1.3. Peptide bond hydrolysis by the proteasome by two possible mechanisms. The side chain hydroxyl of threonine is activated by either the N-terminal amino group (mechanism A) or by a lysine amino group (mechanism B).

Mechanism A



Mechanism B

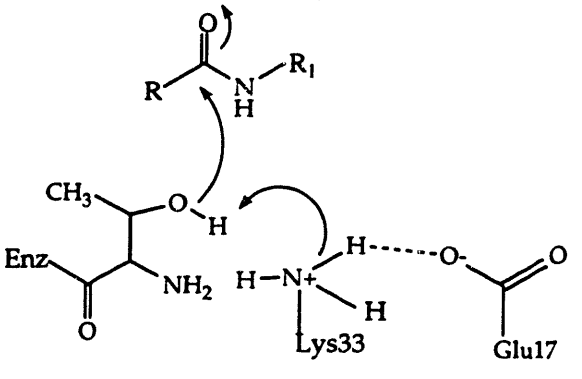
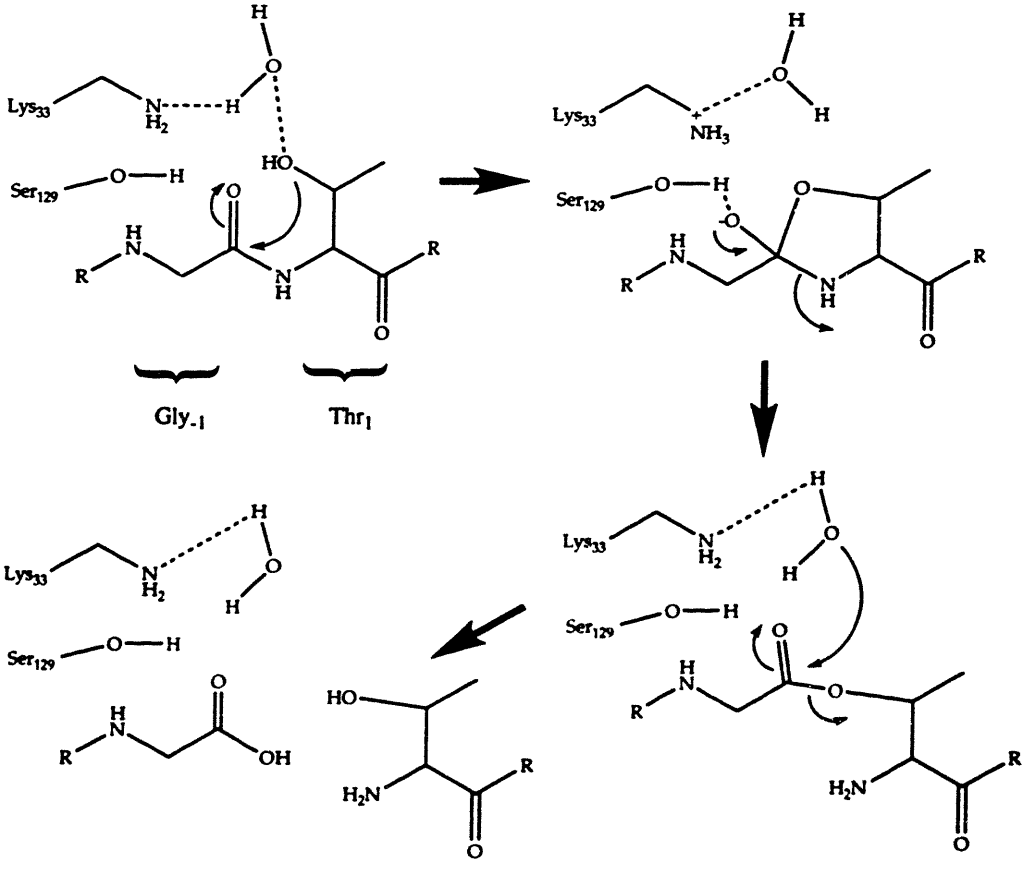


Figure 1.4. Possible mechanism for autocatalytic processing of a proteasomal β-subunit.

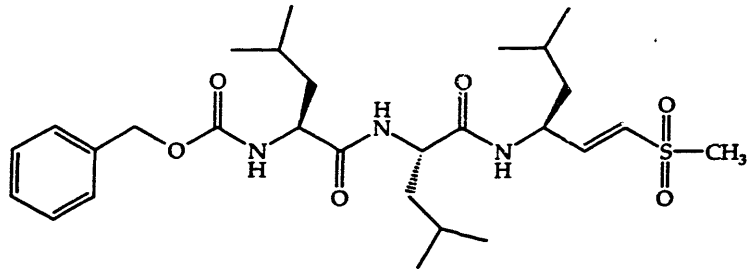

Lys33
Ser129
Gly-1
Thr1

C. Inhibitors of the proteasome

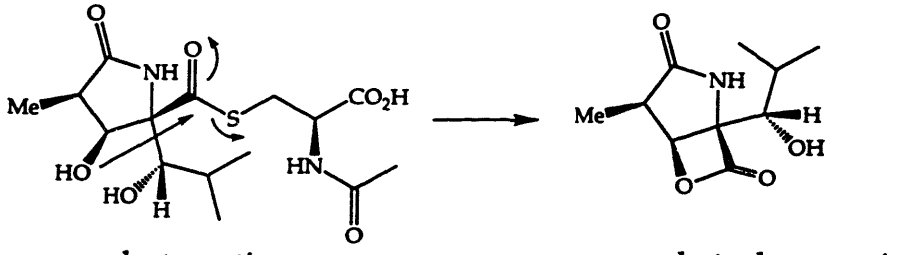
Crucial to understanding proteasome function is the ability to modulate its activity *in vivo*. This goal has been accomplished with the advent of several classes of proteasome inhibitors which are able to penetrate living cells and block proteasome function without affecting normal biological processes such as ATP metabolism and protein synthesis (Iqbal et al., 1995; Fenteany et al., 1995; Harding et al., 1995; Rock et al., 1994). The structures of several classes of proteasome inhibitors are shown in figure 1.5. The first class of compounds to be studied as inhibitors of the proteasome were the C-terminal peptide aldehydes (Rock et al., 1994; Jensen et al., 1995; Iqbal et al., 1995; Wilk and Figueiredo-Pereira, 1993). The C-terminal aldehyde group is capable of forming a covalent hemi-acetal with a threonine hydroxyl and may also form a stable oxizolidine ring by simultaneous reaction with the N-terminal amine and the sidechain hydroxyl. The mechanism of inhibition of the proteasome by the peptide aldehydes as well as several other classes of inhibitors is shown in figure 1.6. These types of covalent adducts, although energetically favored, are reversible and therefore peptide aldehyde inhibitors may be removed, resulting in the return of proteolytic function (Rock et al., 1994).

Initial studies using peptide aldehydes were carried out with compounds previously described to inhibit other proteolytic enzymes. The tri-peptide aldehyde acetyl-leu-leu-norleucinal (Calpain I inhibitor) was found to reversibly inhibit the chymotrypsin like activity of the proteasome while the tri-peptide aldehyde leupeptin was found to be a weak inhibitor of the trypsin-like activity (Cardozo et al., 1992; Wilk and Figueiredo-Pereira, 1993). Because of the therapeutic possibilities of proteasome inhibitors and driven by the need for tools to dissect proteasome function, other more potent inhibitors of the proteasome including the tri-peptide aldehyde, cbz-leu-leu-leucinal (or Z-L_3-H)have been reported (Read et al., 1995; Wiertz et al., 1996a; Wiertz et al., 1996b; Jensen et al., 1995; Rock et al., 1994). This tri-peptide aldehyde has been subsequently used to block proteasome function in living cells and was shown to be a reversible inhibitor of the post glutamyl and trypsin-like as well as the chymotrypsin-like activities of the proteasome. Although these types of inhibitors have found widespread use, caution must be taken when interpreting effects of the peptide

Figure 1.5. Structures of several classes of inhibitors of proteasomal proteolysis.

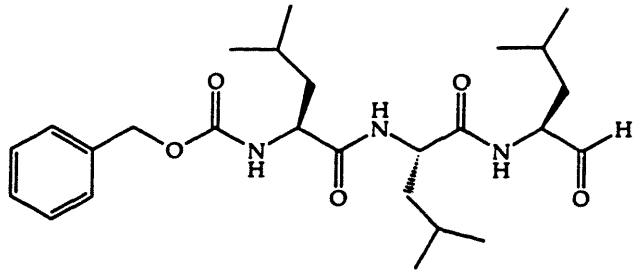


Cbz-leucyl-leucyl-leucine vinyl sulfone



lactacystin

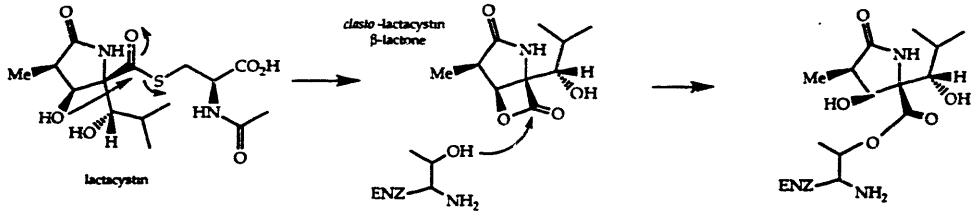
clasto -lactacystin
β-lactone



Cbz-Leucyl-Leucyl-Leucinal

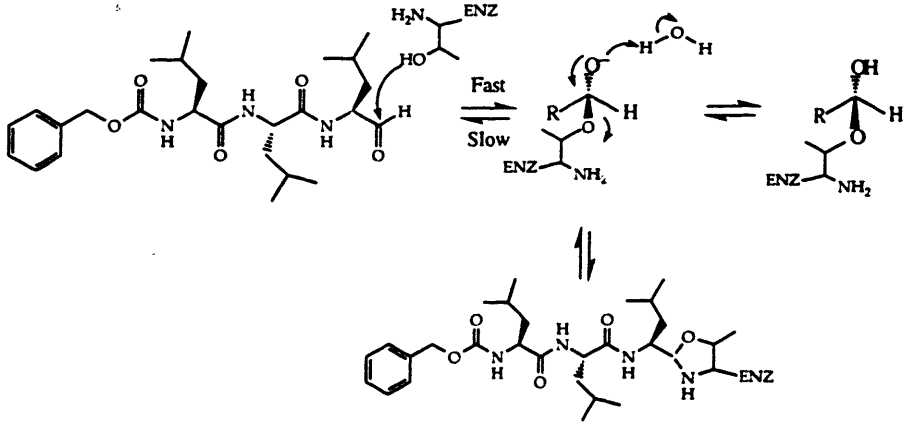
Figure 1.6. Mechanism of inactivation of the N-terminal threonine residue of catalytic β-subunits by proteasomal inhibitors.

Inhibition of the Proteasome by lactacystin

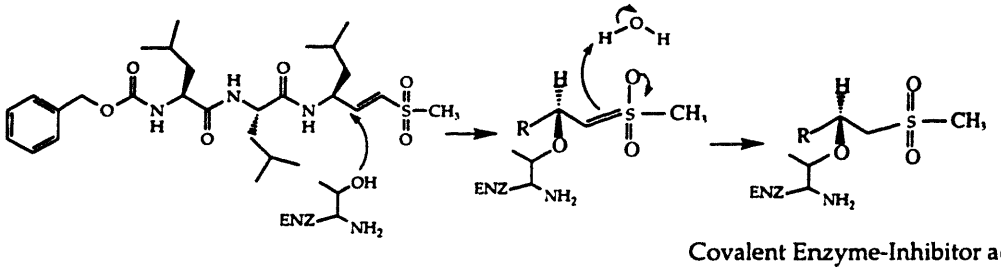


Covalent Enzyme-Inhibitor adduct

Inhibition of the Proteasome by C-Terminal Peptide Aldehydes



Inhibition of the proteasome by C-Terminal Peptide Vinyl Sulfones



Covalent Enzyme-Inhibitor adduct

aldehydes due to their known inhibitory effect on other proteases such as the lysosomal cathepsins and calpains (Coux et al., 1996; Bogyo et al., 1997). In addition, the highly reactive aldehyde functional group allows for possible side effects as the result of Schiff's base formation with circulating free amines.

More recently peptides modified at the C-terminus by a vinyl sulfone moiety have proven to be another class of compounds capable of inhibiting proteasome function (Bogyo et al., 1997). This type of C-terminally modified peptides are Michael acceptors and were therefore initially designed to be reactive towards soft nucleophiles such as thiols in the active site of a lysosomal cysteine proteases (Brömme et al., 1996; Palmer, 1995; Rosenthal et al., 1996). However, this class of inhibitors, when equipped with the proper tri-peptide sequence, is capable of covalent modification of the N-terminal threonine of the proteasome. In addition, since the vinyl sulfones act as "suicide substrates" for the active site nucleophile, attachment of a radioisotope to these peptides results in an active site label which can covalently tag the proteasome in living cells (Bogyo et al., 1997). These compounds have also been found to be less reactive than peptide aldehydes towards lysosomal proteases and have no adverse effects on normal metabolic functions such as protein synthesis (Bogyo et al., 1997).

An inhibitor of the proteasome which is structurally quite different from the peptide based inhibitors is the natural antibiotic lactacystin (Figures 1.6 and 1.7). This compound was initially identified by virtue of its ability to promote neurite outgrowth in cultured neurons (Omura et al., 1991). A radiolabeled form of lactacystin was used to identify the target of the drug as a single catalytic β subunit (named X) of the eukaryotic proteasome (Fenteany et al., 1995). Subsequently, several laboratories have shown that lactacystin in fact modifies all active β-subunits (Bogyo et al., 1997; Craiu et al., in press). Lactacystin is distinct from the peptide aldehydes and peptide vinyl sulfones in its specificity for the proteasome, having little or no effect on lysosomal proteolysis (Fenteany et al., 1994; Coux et al., 1996). Lactacystin is a covalent inhibitor of the chymotrypsin-like and the trypsin-like activities of the proteasome and a weak reversible inhibitor of the postglutamyl peptidase activity (Fenteany et al., 1995). Kinetic and biochemical studies of proteasomal inhibition by lactacystin and related derivatives have uncovered the unusual mechanism by which these compounds covalently modify the N-terminal threonine of the proteasome. Upon prolonged

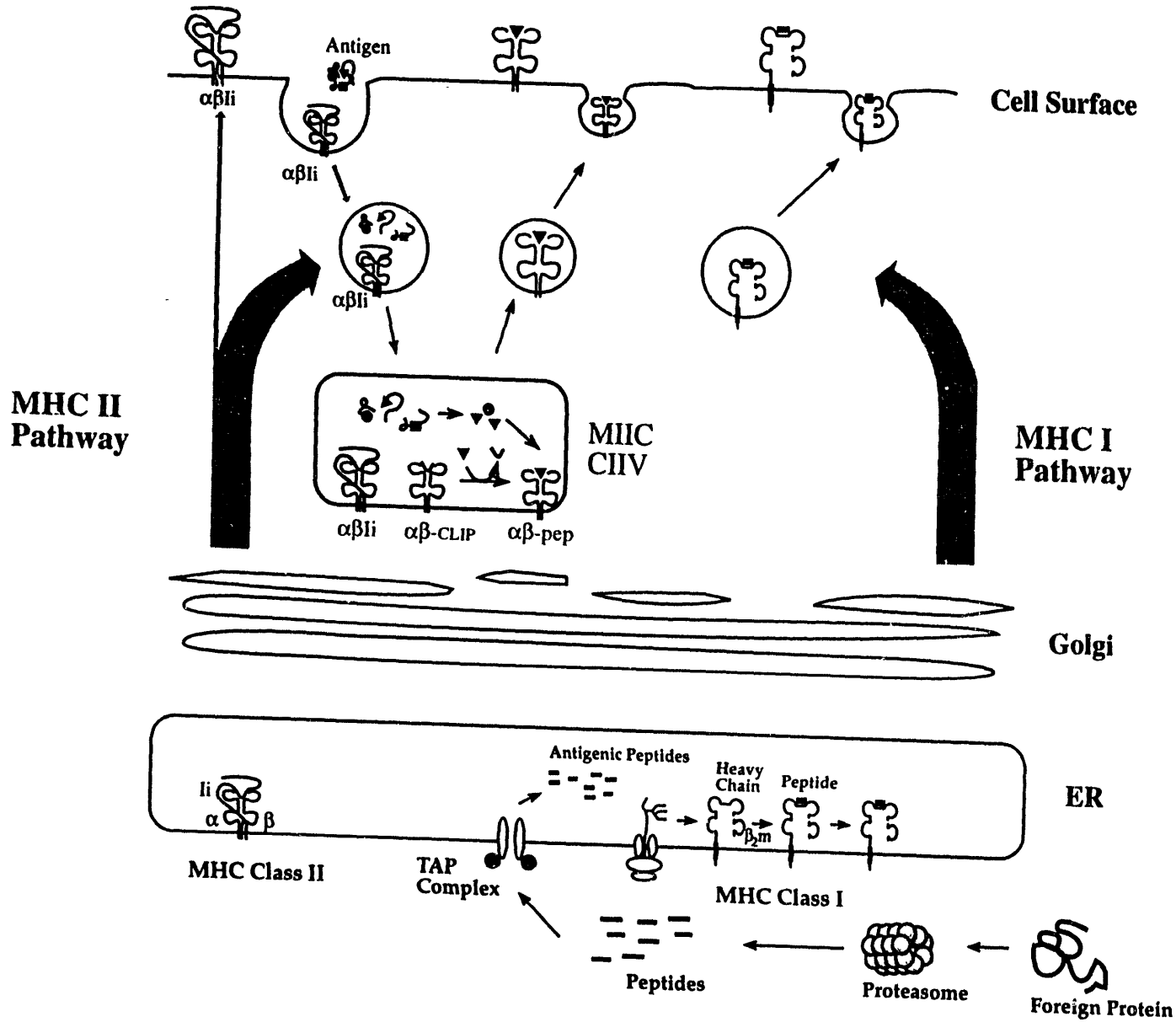
incubation in aqueous media, a hydroxyl group reacts with the thioester functional group resulting in the formation of a β-lactone (figures 1.6 and 1.7) (Dick et al., 1996). It is this highly reactive β-lactone that is thought to be the species which is responsible for the covalent modification of the N-terminal threonine of proteasomal β-subunits.

Still other classes of compounds have been described which are capable of either reversible or irreversible inhibition of the proteasome. These reagents include several di- and tri-peptide aldehydes with sequences different from that of Z-L_3-H (Iqbal et al., 1995), peptide boron esters (Iqbal et al., 1996; McCormack et al., Submitted), peptide α-ketocarbonyls (Iqbal et al., 1996), and tripeptide α,β-epoxyketones (Spaltenstein et al., 1996). These compounds all have potential use in studying proteasome function but have not yet been exploited to their full potential.

D. Antigen Presentation

The topic of antigen presentation has been the subject of many review articles (Heemels and Ploegh, 1995; Wolf and Ploegh, 1995; Germain and Margulies, 1993) and therefore the details of these pathways will not be discussed at length in this introduction. Figure 1.7 shows a general scheme for presentation of peptides via the two main routes. Presentation of peptides via MHC class II molecules begins with the endocytosis of foreign material . Once inside the endocytic pathway, proteins are broken down into peptide fragments by lysosomal proteases known as cathepsins (Germain and Margulies, 1993). These proteases have optimal activity at the acidic pH of the lysosomal compartment. Consequently, neutralization of the endosome leads to a block in antigen presentation via this pathway (Ziegler and Unanue, 1982; Bénaroch et al., 1995). At the same time, MHC class II molecules are transported from the endoplasmic reticulum through the golgi apparatus and are targeted to vesicles containing the foreign peptides. These peptides may then bind in the groove of a class II molecule and move on as a complex to the cell surface for display to CD-4 positive helper T-cells. Engagement of the proper class II-peptide complex with the T-cell receptor of a CD4+ T-cell can lead to an inflammatory response and to the production of antibodies.

Figure 1.7. Presentation of antigenic peptides via MHC class I and class II molecules.


Antigen
αβIi
αβIi
αβIi
Cell Surface
MHC II
Pathway
MIIC
CIIV
MHC I
Pathway
αβIi
αβ-CLIP
αβ-pep
Golgi
Antigenic Peptides
Heavy
Chain
Peptide
Ii
α
β
ER
MHC Class II
TAP
Complex
MHC Class I
Peptides
Proteasome
Foreign Protein

Largely distinct from this pathway is presentation of peptides by MHC class I molecules. In this pathway, class I heavy chains are synthesized and assembled with the light chains, β2-microglobulin (β_2m) in the endoplasmic reticulum. A peptide of 7-9 residues in length is the third and final component of this complex and is required for proper assembly and release of the class I complex from the ER . Peptides presented by class I molecules are in most cases derived from cytosolic proteins by proteolysis, as described below. Thus, the host cell must have a mechanism which allows for the transport of these peptides across the ER membrane. This translocation process is carried out by a dedicated transporter called TAP (for Transporter associated with Antigen Presentation). TAP is a heterodimeric complex consisting of two transmembrane proteins (TAP1 and TAP2) which utilize energy from ATP to translocate peptides from the cytosol to the lumen of the ER. Once peptides bind to Class I molecules in the ER, the complex is allowed to leave the ER and is transported thought the secretory pathway. Eventually this peptide MHC complex is displayed to cytotoxic T-cells at the cell surface. Upon engagement of a T-cell receptor with an MHC class I molecule containing an appropriate peptide, the T-cell becomes activated and may proceed to kill the infected cell.

In order for a cell to advertise the presence of an intruding organism to cytotoxic T-cells, it must first be able to generate peptide fragments from proteins synthesized by the presenting cell. Crucial to presentation of antigens by this pathway is the generation of peptides suitable for transport by TAP and also capable of binding to class I molecules. The proteasome is a key player in the generation of these antigenic peptides and its role in this pathway will be the focus of further discussion throughout this thesis.

E. The Proteasome's Role in Antigen Presentation

Cells of the immune system must be able to recognize when a cell is infected with a foreign pathogen and subsequently deal with the intruder by destruction of the infected cell. This communication between the T-cell and a virus infected cell is mediated by molecules of the major histocompatibility complex, or MHC. MHC proteins carry bits of foreign proteins, in the form of 8-10 residue peptides, to the cell surface to interact with antigen specific receptors

on the surface of circulating T-lymphocytes. These peptides must be generated from intact proteins by the action of proteolytic enzymes such as the proteasome.

Although the existence of a soluble ATP dependent proteolytic complex known as the proteasome was known since the late 70's (Etlinger and Goldberg, 1977), its role in the processing of class I antigens for presentation to cytotoxic T-lymphocytes has only recently been uncovered. Initial studies of genes encoded in the MHC region identified several low molecular weight proteins given the name the LMPs (Monaco and McDevitt, 1986). Since the expression of two of the LMP subunits was inducible by γ-interferon (Monaco and McDevitt, 1986), known to stimulate immune responses, it was proposed that these genes played a role in antigen presentation. Although the LMP subunits were similar in size to proteasomal subunits and formed complexes of high molecular weigh similar to the proteasome, it was not until many years after their discovery that the two were found to be identical (Kelly et al., 1991; Brown et al., 1991; Glynne et al., 1991).

With this initial discovery, many laboratories began examining the details of the proteasome's involvement in antigen presentation. Initial studies focused on the two LMP subunits, LMP-2 and LMP-7, the genes for which were found adjacent to the TAP transporter genes in the MHC class II region and whose expression could be modulated with γ-interferon (Kelly et al., 1991; Brown et al., 1991; Glynne et al., 1991). These two subunits were found to be homologous to the catalytically active β-type subunits of the proteasome (Kopp et al., 1993; Heinemeier et al., 1994). The effect of these subunits on antigen presentation was studied using a mutant human cell line in which the MHC region containing the LMP-2 and LMP-7 genes as well as the TAP-1 and TAP-2 genes had been deleted (Momburg et al., 1992; Arnold et al., 1992). By replacing the TAP genes by transfection, it was possible to examine the effects of loss of LMP-2 and LMP-7 alone. Interestingly, several laboratories showed that the presentation of a variety of epitopes was unaffected by the absence of these subunits, indicating that LMP-2 and LMP-7 were not essential for the generation of epitopes (Yewdell et al., 1994).

Initially, these findings seemed to dispute claims of the proteasome's involvement in antigen presentation. However, these experiments provided no information about more subtle effects of LMP-2 and LMP-7 on proteasome

function. Several laboratories then examined the catalytic activities of the proteasome either containing or lacking the γ-interferon inducible subunits LMP-2 and LMP-7 (Gaczynska et al., 1993; Driscoll et al., 1993). Activity of the proteasome against several different fluorogenic peptide substrates designed to act as substrates for the chymotrypsin-like, the trypsin-like and the post glutamyl peptidase activities was compared for cells which had or had not been exposed to γ-interferon. The results form these experiments showed that the activity of the proteasome changes quite dramatically upon inclusion of LMP2 and LMP7, favoring cleavage after hydrophobic and basic residues and strongly reducing cleavage after acidic residues. Furthermore, the activity of proteasomes isolated from mutant cells lacking the LMP-2 and LMP-7 subunits showed the exact opposite effects of γ-interferon treatment, favoring cleavage after acidic residues and reducing the propensity for cleavage after hydrophobic and basic residues (Gaczynska et al., 1994; Gaczynska et al., 1996). These findings fit surprisingly well with the sequences of peptides known to bind with high affinity to MHC class I molecules. Several studies also showed the importance of hydrophobic or basic residues at the C-terminus of peptides for efficient transport by TAP (Heemels et al., 1993). These data restored the belief that the proteasome played an important role in antigen presentation.

More recently the requirement of the proteasome for generation of antigenic peptides was examined using inhibitors capable of blocking proteasome function (Harding et al., 1995; Rock et al., 1994). Studies performed by several laboratories provided strong evidence that proteasome function was crucial to presentation of epitopes to cytotoxic T-lymphocytes. Several peptide aldehydes comprised of different amino acid sequences were used to block proteasomal proteolysis. These inhibitors were able to cause a block in the rate of break-down of both long and short lived proteins, as well as the degradation of abnormal proteins, all of which were believed to be the result of proteasomal inhibition. Further, when cells were pre-treated with the peptide aldehyde inhibitors and subsequently assembly of class I molecules was examined by metabolic labeling and pulse chase analysis, it was found that inhibition of the proteasome led to a block in the formation of stable MHC class I heterodimers (heavy chain and β_2m). Since studies with cells lacking the TAP transporter showed a similar assembly defect due to a lack of peptides in the ER (Salter and

Cresswell, 1986), this result was presumed to be caused by a loss of peptides capable of binding to and stabilizing class I dimers. These data strongly suggested that proteasomal proteolysis was required for generation of peptides which bind to class I molecules in the ER.

Finally, proteasome inhibitors were found to have a dramatic effect on the ability of cells to present epitopes to cytotoxic T-lymphocytes (Rock et al., 1994). When an antigenic protein was introduced into cells followed by pre-incubation with a peptide aldehyde known to inhibit proteasome function, there was a complete block in lysis by epitope-specific T-lymphocytes. However, when a mini-gene encoding only the epitope required for transport, binding and presentation was introduced into the same cells, lysis by CTLs was resistant to treatment with proteasome inhibitors. Further, when a peptide aldehyde known to inhibit lysosomal and soluble proteases other than the proteasome was used, there was no effect on antigen presentation.

II. COVALENT MODIFICATION OF THE ACTIVE SITE THREONINE OF PROTEASOMAL β-SUBUNITS AND THE *ESCHERICHIA COLI* HOMOLOG HslV BY A NEW CLASS OF INHIBITORS

Matthew Bogyo, John S. McMaster, Maria Gaczynska, Domenico Tortorella, Alfred L. Goldberg, and Hidde L. Ploegh. (1997) *Proc. Natl. Acad. Sci. USA*, **94**, 6629-6634

A. Introduction

Proteasomes are multi-catalytic proteolytic complexes found in almost all living cells and are responsible for the degradation of the majority of cytosolic proteins in mammalian cells (Coux et al., 1996). The 20S proteasome is a 700 kDa barrel-shaped structure composed of four stacked rings with seven-fold symmetry (Löwe et al., 1995). These rings are composed of two types of subunits; α subunits make up the outer two rings of the complex and the catalytic β subunits the inner two rings. Proteasomes of the archaebacterium, Thermoplasma acidophilum , and certain eubacteria are comprised of 14 identical α and 14 identical β subunits. In contrast, the α rings of the eukaryotic proteasomes contain 7 different but homologus α subunits and the β rings contain 7 distinct but related β subunits (20S proteasome) (Dahlmann et al., 1989). The 20S proteasome functions as the catalytic core of the larger, ATP-dependent, 26S complex which is responsible for the degradation of ubiquitin conjugated proteins ((Hershko and Ciechanover, 1992)). Further complexity arises from the possible replacement of the catalytic β subunits X,Y, and Z with the IFN-γ -inducible, MHC-encoded subunits LMP-2, LMP-7, and MECL-1 (Gaczynska et al., 1993).

Initial attempts to classify the proteasome's catalytic mechanism into a category with known proteases were unsuccessful mainly due to lack of homology with known peptidases (Zwickl et al., 1992). Mutational studies and the x-ray crystal structure of the Thermoplasma proteasome in a complex with a tri-peptide aldehyde inhibitor provided evidence that the proteasome employs a novel catalytic mechanism, involving an N-terminal threonine residue as the catalytic nucleophile (Löwe et al., 1995; Seemüller et al., 1995). In this mechanism, either the free-amino terminus or the ε-amino group from a conserved, nearby lysine residue activates the side-chain hydroxyl group for nucleophilic attack on the peptide bond (Seemüller et al., 1995). This mechanism thus does not involve a charge relay system utilized by most cysteine and serine proteases.

The ubiquitin proteasome pathway plays a crucial role in the degradation of highly abnormal and many short lived regulatory proteins and has been shown to be involved in many diverse cellular functions including cell cycle

progression, antigen presentation, and activation of transcription factors (Ghislain et al., 1993; Harding et al., 1995; Palombella et al., 1994). Inhibitors of the proteasome are thus of considerable interest as tools for studying the detailed molecular mechanistic aspects and physiological consequences of proteasome activity. Peptide aldehydes are potent, reversible inhibitors that inactivate the proteasome's multiple active sites by forming a transient, covalent hemi-acetal with the catalytic N-terminal threonine hydroxyl (Harding et al., 1995; Rock et al., 1994). Peptide aldehydes are active against proteasomal proteolysis both in vitro and in cells grown in tissue culture. However, this class of inhibitors can also inhibit cellular thiol proteases (e.g. calpains or lysosomal cathepsins) which can complicate the interpretation of certain studies (Coux et al., 1996; Rock et al., 1994).

The natural product lactacystin is an irreversible, covalent inhibitor of the chymotrypsin-like and trypsin-like activities and a weak, reversible inhibitor of the peptidylglutamyl peptidase activity of the proteasome (Fenteany et al., 1995). The ability of lactacystin to covalently inactivate its target led to its use as a probe to examine the specificity of binding. A radiolabeled analog of lactacystin, was reported to uniquely label a single proteasomal β subunit, MB-1 or X (Fenteany et al., 1995). Its exquisite specificity has made it a very useful reagent for studying proteasome function in mammalian cells, but its modest activity against proteasomes from archaebacterium and certain eubacterial homologs has limited its use in studies of these related proteolytic enzymes.

We report here a new class of inhibitors of the proteasome: peptide vinyl sulfones. The vinyl sulfone acts as a Michael acceptor for soft nucleophiles such as thiols, leading to the formation of a covalent bond (Palmer, 1995) (figure 2.1B). However, these compounds are not susceptible to attack by free thiols which can cause inactivation of other classes of protease inhibitors (Palmer, 1995). We show that the tripeptide vinyl sulfone Z-L_3-VS and related derivatives quite unexpectedly inhibit the trypsin-like, the chymotrypsin-like and, unlike lactacystin, the peptidylglutamyl peptidase activity of the proteasome in vitro by covalent modification of the N-terminal threonine of the catalytically active β subunits. They are more easily synthesized than lactacystin and can be conveniently tagged with either biotin for purposes of affinity chromatography (Bogyo & Ploegh, unpublished observation), or a nitro-phenol moiety for

subsequent radiolabeling. We show that a ^{125}I-labeled vinyl sulfone of the tri-peptide sequence Leu-Leu-Leu selectively modifies β-subunits in purified proteasome preparations as well as in whole cell homogenates and in living cells of widely different origin.

Recently an ATP-dependent protease complex from *E. coli* comprised of two heat shock proteins has been discovered (Rohrwild et al., 1996). The HslV gene product is a peptidase which shows homology to β-type subunits of the proteasome, including the presence of a N-terminal threonine (Lupas et al., 1994; Rohrwild et al., 1996). HslV is co-transcribed with the adjacent HslU gene which encodes an ATPase with homology to other known *E.coli* ATPases such as ClpX (50% identity) (Chuang et al., 1993). Together the HslV and HslU gene products make up a complex with an ATP-dependent proteolytic activity similar to that of the eukaryotic proteasome (Rohrwild et al., 1996). The peptide vinyl sulfones were used as probes to examine the proteolytic mechanism of this peptidase complex. We show that peptide vinyl sulfones covalently modify HslV only in the presence of HslU and ATP, consistent with the reported nucleotide dependence of the activity of this complex (Rohrwild et al., 1996). These observations provide experimental support for the HslU/HslV complex's proposed functional homology to the proteasome and indicate that ATP influences the formation of the active site of this enzyme complex.

B. Materials and Methods

1. Cells and Cell Culture

The human cell lines HOM-2, T2, and US11 transfectants prepared from the astrocytoma cell line, U373-MG have been described (Wiertz et al., 1996a). The US11 transfectants were cultured in Dulbecco's modified Eagle medium supplemented with 10% fetal calf serum and puromycin (Sigma, St. Louis, MO) at a final concentration of 0.375 μg/ml. All other cells were grown in RPMI media supplemented with 10% fetal calf serum.

2. Antibodies

Proteasomes were precipitated using the mono-clonal antibody which recognizes an α subunit as part of the mature 720 kDa proteasome complex (Organon Teknika, Turnhout/Belgium) (Briane et al., 1992). MHC class I molecules were precipitated using a rabbit anti-class I heavy chain serum described elsewhere (Beersma et al., 1993).

3. Biochemical Methods and Materials

Peptide inhibitors (Z-L_3H, Z-L_3VS, and NIP-L_3VS) were dissolved in DMSO and diluted in cell culture media to the desired concentration, keeping the final concentration of DMSO to be < 1%. Cells (1.5×10^6), purified proteasomes (0.1 μg for rabbit, *S. cerevisiae,* and *Thermoplasma acidophilum;* 0.1 μg HslV and 0.4 μg HslU) or lysates (100 μg total protein) were labeled with ^{125}I-NIP-L_3VS, diluted to a final concentration of 1.8×10^4 Bq/ml in tissue culture medium (cells) or reaction buffer (50 mM Tris pH 7.4, 2 mM DTT, 5 mM $MgCl_2$, 2mM ATP, purified proteasomes), at 37° C for two hours. For labeling in the absence of ATP, apyrase (5 units/ml) was used instead of ATP. Labeling of cells was quenched by washing 3X with PBS followed by addition of 1X SDS-Laemmli sample buffer. Labeling of purified proteasomes and total lysates was quenched by addition of 4X Laemmli sample buffer (to 1X). All samples were resolved by

SDS-PAGE, NEPHGE-PAGE or IEF-PAGE . Preparation of lysates and immuno-precipitations were performed as described ((Beersma et al., 1993)).

4. Kinetics Analysis of inhibition of proteasomal peptidase activity

Purified mixtures of 20S and 26S proteasomes were isolated from U373-MG cells by differential centrifugation and anion-exchange chromatography as previously described (Gaczynska et al., 1993; Gaczynska et al., 1994). 20S proteasomes were separated from these mixtures by native PAGE (4% polyacrylamide gel). Mixtures of 20S and 26S proteasomes or pure complexes were used to determine kinetics of inhibition of the proteasomal peptidases. Fluorogenic peptide substrates (100μM final conc.) were used as substrates for the hydrophobic (Suc-LLVY-MCA), basic (Boc LRR-MCA) and acidic (Cbz-LLE-βNA) peptidase activities as described (Gaczynska et al., 1993; Gaczynska et al., 1994)). The rates of association (K_{assoc}) of Z-L_3VS were determined as previously described (Fenteany et al., 1995).

5. Synthesis of the Peptide Vinyl Sulfones

The peptide vinyl sulfones were synthesized by standard solution chemistry as described below. The synthetic route is shown in figure 2.1 along with the mechanism of inhibition of the proteasome by this class of inhibitors.

a. Synthesis of Boc-leucinyl-leucinyl-methyl ester (I)

Boc-Leu-OH (12.4 g, 50 mmol) was dissolved in DMF. To the flask was added dicyclohexylcarbodiimide (12.3 g, 60 mmol) and HOBT (8.1g, 60 mmol). The reaction was then stirred for 30 min until a precipitate formed. Finally, leucine methyl ester hydrochloride salt (6.0 g, 33 mmol) and diisopropylethyl amine (6.3 ml, 36 mmol) were added and the reaction stirred overnight. The reaction mixture was then filtered to remove solids and the DMF was removed by rotary evaporation. The resulting crude oil was dissolved in dichloromethane and washed with three volumes of 0.1 N HCl and three volumes of saturated sodium bicarbonate. The organic layer was dried over magnesium sulfate and

then concentrated to dryness by rotary evaporation. The pure dipeptide ester was obtained by recrystallization of the crude solid from ethyl acetate/hexane. Yield (8.4g, 23.5 mmol, 71%).

Boc-Leucinyl-leucine-methyl ester 1H-NMR (300 MHz, CDCl3) d 6.45 (1H, d), 4.89 (1H, d), 4.62 (1H, m), 4.10 (1H, q), 3.75 (3H, s), 1.50-1.78 (6H, m), 1.45 (9H, s), 0.88-099 (12H, m).

b. Synthesis of Boc-leucinyl-leucine (II)

Boc-Leucinyl-leucine methyl ester (I; 4.0 g, 11.2 mmol) was dissolved in 50 ml of methanol. To the flask was added 50 ml of an aqueous solution of 20% potassium carbonate and the reaction stirred overnight. The reaction was quenched by the addition of 3N HCl. The resulting aqueous solution was then extracted with three volumes of methylene chloride. The organic phases were combined, dried over $MgSO_4$, and evaporated to dryness and used without further purification. Yield (2.7 g, 7.8 mmol, 71%).

c. Synthesis of Boc-leucine-dimethyl hydroxyl amide (III)

Boc-leucine (2.0 g, 5.6 mmol) was dissolved in dichloromethane. PyBOP (2.9 g, 5.6 mmol) and triethyl amine (782 µl, 5.6 mmol) were then added and the reaction was allowed to stir for 10 minutes. Dimethylhydroxyl amine (0.61g, 6.2 mmol) was then added followed immediately by more triethylamine (860 µl, 6.2 mmol). The reaction was stirred for 1 hour and then quenched by addition of three volumes of 0.1 N HCl. The remaining aqueous phase was extracted with three volumes of methylene chloride. The organic layers were combined, washed with one volume of saturated sodium bicarbonate and one volume of saturated sodium chloride, dried over $MgSO_4$ concentrated to an oil. The crude oil was purified by flash column chromatography over silica gel using hexane/ethyl acetate (3:1 V:V) as the mobile phase. Yield (2.2 g, 4.5 mmol, 80%).

Boc-Leucine-dimethyl hydroxyl amide 1H- NMR (300 MHz, CDCl3) d 5.05 (1H, d), 4.72 (1H, m) 3.80 (3H, s), 3.20 (3H, s), 1.70 (1H, m), 1.45 (9H, s) 1.40 (2H, m), 0.95 (3H, d), 0.90 (3H, d).

d. Synthesis of Boc-leucinal (IV)

Boc-leucine-dimethyl hydroxyl amide (III) (1.8 g, 4.5 mmol) was dissolved in 10 ml of anhydrous diethyl ether under argon. Lithium aluminum hydride (210 mg, 5.6 mmol) was added as a solid and the reaction stirred for 1 hour. The reaction was quenched by the addition of potassium hydrogen sulfate (1.0 g, 7.9 mmol) in 30 ml of water. The reaction mix was stirred for 20 minutes and then the organic layer was removed. The remaining aqueous phase was washed with three volumes of diethyl ether. The organic layers were combined, washed with three volumes each of 3N HCl, saturated sodium carbonate, and saturated sodium chloride, dried over $MgSO_4$ and then evaporated to dryness. Due to problems of racemizaton upon column chromatography, Boc-leucinal was used without further purification. Yield (1.3 g, 3.9 mmol, 87%).

Boc-Leucinal ^{1}H-NMR (300 MHz, $CDCl_3$) d 9.60 (1H, s), 4.95 (1H, d), 4.25 (1H, dd), 1.70 (1H, m), 1.45 (9H, s), 1.40 (2H, m), 0.95 (3H, d), 0.90 (3H, d).

e. Synthesis of Boc-leucine-vinyl sulfone (V)

Diethyl (methylthiomethyl) phosphonate was oxidized to the corresponding phosphonate sulfone using peracetic acid in aqueous dioxane. The product was isolated by crystallization from warm ethyl acetate. Diethyl phosphonate sulfone (802 mg, 3.5 mmol) was dissolved in anhydrous THF. Sodium hydride (128 mg, 3.2 mmol) was added and the reaction is stirred for 30 minutes. Finally, Boc-leucinal (IV; 500 mg, 2.3 mmol) was dissolved in anhydrous THF and added slowly by canula to the reaction mix. The reaction was then allowed to stir for 2 hours. The reaction was quenched with water and the resulting aqueous phase washed three times with dichloromethane. The organic phases were combined, dried over $MgSO_4$ and evaporated to dryness. the crude oil was purified by flash chromatography over silica gel using a

hexane/ethyl acetate (3:1 v:v) as the mobile phase. Yield (785 mg, 1.9 mmol, 82%).

Boc-leucine-vinyl sulfone ^{1}H-NMR (300 MHz, $CDCl_3$) d 6.78-6.88 (1H, dd), 6.41-6.52 (1H, dd), 4.60 (1H, d), 4.40 (1H, m), 2.92 (3H, s), 1.60-1.75 (1H, m), 1.45 (9H, s), 1.35-1.45 (2H, m), 0.95 (3H, d), 0.89 (3H, d).

f. Synthesis of leucine-vinyl sulfone tosic acid salt

p-Toluenesulfonic acid monohydrate (386 mg, 2.0 mmol) was dissolved in 5 ml isopropanol with gentle heating. Toluene (20 ml) was then added and the solution rotary evaporated to dryness. The resulting oil was further dried in vacuo for several hours. Boc-leucine-vinyl sulfone (V; 200 mg, 677 µmol) was dissolved in 10 ml diethyl ether and the added to the flask containing the anhydrous p-toluenesulfonic acid. The reaction was stirred overnight and the resulting amine salt precipitated. The precipitate was isolated by centrifugation and the pellet was washed two times with diethyl ether. Yield (237 mg, 643 µmol, 95%).

Leucine-vinyl sulfone tosic acid salt ^{1}H-NMR (300 MHz, $CDCl_3$) d 7.75 (2H, d), 7.15 (2H, d), 6.85-6.95 (1H, dd), 6.5-6.6 (1H, dd), 3.60 (1H, m), 2.95 (3H, s), 1.60-1.78 (1H, m), 1.35-1.42 (2H, m), 0.95 (3H, d), 0.86 (3H, d).

g. Synthesis of Boc-Leucinyl-leucinyl-leucine-vinyl sulfone

Boc-leucinyl-leucine (II) (400 mg, 1.2 mmol) was dissolved in dichloromethane. PyBOP (726 mg, 1.3 mmol) and triethylamine (180 µl, 1.3 mmol) were then added and the reaction was allowed to stir for 10 minutes. Leucine-vinyl sulfone tosic acid salt (VI; 500 mg, 1.4 mmol) was then added followed immediately by additional triethylamine (195 µl, 1.4 mmol). The reaction was stirred for 1 hour and then quenched by addition of three volumes of 0.1 N HCl. The remaining aqueous phase was extracted with three volumes of methylene chloride. The organic layers were combined, washed with one volume of saturated sodium bicarbonate and one volume of saturated sodium

chloride, dried over $MgSO_4$, and concentrated to an oil. The crude oil was purified by flash column chromatography over silica gel using hexane/ethyl acetate (2:1 V:V). A small amount of a slightly less polar compound was isolated and found to have a nearly identical NMR as the desired product except for a slight change in the splitting of the vinyl resonance adjacent to the alpha carbon of leucine at P1. This minor product is likely to be the tripeptide containing a D-leucine in the P1 position and was the result of a small amount of racemizaton during the synthesis of leucine-vinyl sulfone. Yield (600mg, 1.15 mmol, 96%).

Boc-Leu_3-VS ^{1}H-NMR (300 MHz, $CDCl_3$) d 7.95 (1H, d), 6.78-6.88 (1H, dd), 6.52-6.62 (1H, dd), 6.48 (1H, d), 2.95 (1H, d), 4.71 (1H, m), 4.46 (1H, m), 4.0 (1H, m), 2.95 (3H, s), 1.50-1.80 (6H. m), 1.45 (9H, s), 1.40-1.50 (3H, m), 0.80-0.99 (18 H, m).

FAB Mass Spectrum: [M+H] Calculated for $C_{25}H_{47}N_3O_6S$: 518.7 Found: 518.3

h. Synthesis of carboxylbenzyl-leucinyl-leucinyl-leucine-vinyl sulfone (Z-L_3VS)

The synthesis of Z-L_3VS was performed exactly as described for Boc-leucinyl-leucinyl-leucine-vinyl sulfone except that carboxylbenzyl-leucine was used instead of Boc-leucine in the first step of the synthesis.

Z-L_3-VS ^{1}H-NMR (300 MHz, $CDCl_3$) d 7.33-7.45 (5H, m), 7.80-7.90 (1H, dd) 7.85 (1H, d), 6.52-6.60 (1H, dd), 6.35 (1H, d), 5.20 (1H, d), 5.12 (2H, s), 4.71 (1H, m), 4.38 (1H, m), 4.08 (1H, m), 2.95 (3H, s), 1.45-1.85 (9H, m), 0.88-1.00 (18H, m).

FAB Mass Spectrum: [M+H] Calculated for - $C_{28}H_{45}N_3O_6S$: 552.7 Found: 552.5

i. Synthesis of leucinyl-leucinyl-leucine-vinyl sulfone tosic acid salt (VIII)

p-Toluenesulfonic acid monohydrate (299 mg, 1.7 mmol) was dissolved in 1.5 ml isopropanol with gentle heating. Toluene (8 ml) was then added and the solution rotary evaporated to dryness. The resulting oil was further dried in vacuo for several hours. Boc-leucinyl-leucinyl-leucine-vinyl sulfone (VII; 300 mg,

580 μmol) was dissolved in 2 ml diethyl ether and then added to the flask containing the anhydrous p-toluenesulfonic acid. The resulting amine salt precipitated as the reaction proceeded and was isolated by centrifugation. The pellet was washed two times with diethyl ether and dried under vacuum. Yield (278 mg, 470 μmol, 81%).

Leucinyl-leucinyl-leucinyl-vinyl sulfone tosic acid salt ^{1}H-NMR (300 MHz, $CDCl_3$) d 7.78 (2H, d), 7.75 (1H, d), 7.18 (2H, d), 7.87 (1H, d), 7.75-7.84 (1H, dd), 6.49 (1H, dd), 4.68 (1H, m), 4.34 (1H, m), 3.40 (1H, m), 2.95 (3H, s), 1.50-1.80 (6H, m), 1.40-1.50 (3H, m), 0.82-1.00 (18H, m).

FAB Mass Spectrum: [M+H] calculated for $C_{20}H_{39}N_3O_4S$- 418.6 Found: 418.2

j. Synthesis of biotin-leucinyl-leucinyl-leucine-vinyl sulfone (Biotin-L_3VS)

Biotin-nitrophenyl ester (37 mg, 102 μmol) and leucinyl-leucinyl-leucine-vinyl sulfone (VIII) (50 mg, 85 μmol) were weighed into a dry flask. Dimethylformamide (1 ml) was then added followed by diisopropylethyl amine (16 μl, 85 μmol). The reaction was stirred overnight. A white precipitate formed and the reaction mixture was diluted with ten volumes of diethyl ether. The precipitate was collected by centrifugation and the pellet was washed two times with diethyl ether. The pure solid was dried in vacuo. Yield (45 mg, 84 μmol, 82%).

FAB Mass Spectrum: [M+H] Calculated for $C_{30}H_{53}N_5O_6S_2$: 644.8 Found: 644.3

k. Synthesis of 5-iodo-4-hydroxy-3-nitrophenyacetyl-leucinyl-leucinyl-leucine-vinyl sulfone (NIP-L_3VS)

5-iodo-4-hydroxyl-3-nitrophenylacetic acid (37 mg, 115 μmol) was dissolved in dichloromethane. PyBOP (70 mg, 127 μmol) and triethyl amine (23 μl, 127 μmol) were then added and the reaction was allowed to stir for 10 minutes. Leucinyl-leucinyl-leucine-vinyl sulfone tosic acid salt (VIII; 75 mg, 127 μmol) was then added followed immediately by additional triethyl amine (21 μl,

115 mol). The reaction was stirred for 1 hour and then quenched by addition of three volumes of 0.1 N HCl. The remaining aqueous phase was extracted with 3x1 volume of methylene chloride. The organic layers were combined, washed with 2x1 volume of 0.1 N HCl, one volume of saturated sodium chloride, dried over $MgSO_4$, and concentrated to dryness. The crude solid was purified by crystallization from warm ethyl acetate. Yield (71.6 mg, 100 µmol, 87%).

NIP-L_3-VS ^{1}H-NMR (300 MHz, DMSO-d_6) d 10.98 (1H, s), 8.38 (1H, d), 8.15 (1H, d), 8.05 (1H, s), 8.00 (1H, d), 7.85 (1H, s), 6.65 (1H, dd), 6.55 (1H, dd), 4.55 (1H, m), 4.25 (1H, m), 3.50 (2H, d), 2.95 (3H, s), 1.20-1.60 (9H, m), 0.70-0.90 (18H, m).

FAB Mass Spectrum: [M+H] Calculated for $C_{28}H_{43}N_4O_8SI$: 723.6 Found: 723.3

l. Synthesis of 4-hydroxyl-3-nitrophenyacetyl-leucinyl-leucinyl-leucine-vinyl sulfone (VIV)

Compound VIV was synthesized using the same conditions described for NIP-L_3VS except 4-hydroxyl-3-nitrophenylacetic acid was used instead of 5-iodo-4-hydroxyl-3-nitrophenylacetic acid.

4-hydroxyl-3-nitrophenylacetyl-leucinyl-leucinyl-leucine vinyl sulfone ^{1}H-NMR (300 MHz, DMSO-d_6) d 10.95 (1H, s), 8.30 (1H, d), 8.25 (1H, t), 8.05 (1H, d), 7.80 (1H, s), 7.45 (1H, d), 7.10 (1H, d), 6.65 (1H, dd), 6.55 (1H, dd), 4.55 (1H, m), 4.25 (1H, m), 3.50 (2H, d), 2.95 (3H, s), 1.20-1.60 (9H, m), 0.70-0.90 (18H, m).

m. Synthesis of radio labeled 4-hydroxyl-3-nitrophenyacetyl-leucinyl-leucinyl-leucine-vinyl sulfone (^{125}I-NIP-L_3VS)

A glass test tube was coated with 100 µg of Iodo-gen® (Pierce, Rockford, IL). The tube was placed on ice and compound VIV (25 µL of a 10 mM solution in Ethanol/phosphate buffer pH 7.5 (v:v)) was added to the tube. Finally, 1 mCi of Na^{125}I (10 µL) was added to the tube and incubation continued on ice for 15 minutes. The labeled inhibitor was purified by application to a sep-pak® (Waters, Milford, MA) column containing a C_{18} stationary phase. After the

sample was applied to the column, the column was washed with 30 ml of phosphate buffer pH 7.5. ^{125}I-NIP-L_3VS was then eluted using 100% acetonitrile. Fractions of 1 ml were collected and the fractions with peak radioactivity were pooled. Aliquots of the acetonitrile stock were evaporated to dryness before use in biological systems.

6. Pulse Chase Analysis

Cells were detached by trypsin treatment and incubated in methionine-free Dulbecco's modified Eagle's medium with or without added inhibitors for 1 hr. Two million cells (10 min. pulse; 250 μCi of label) were used per sample. Incorporation was terminated by the addition of non-radioactive methionine to a final concentration of 1 mM. Immediately after the chase, samples were placed on ice and lysed in 1 ml of ice-cold NP-40 lysis mix. Preparations of lysates and immuno-precipitations were performed as described (Beersma et al., 1993).

7. Gel Electrophoresis

SDS-PAGE, two dimensional IEF-PAGE, fluorography and autoradiography were performed as described (Mozdzanowski et al., 1995; Wiertz et al., 1996a). Two dimensional NEPHGE-PAGE was performed as described (Monaco and McDevitt, 1982).

C. Results

1. Synthesis of peptide vinyl sulfones

Tripeptide vinyl sulfones were synthesized containing the sequence Leu-Leu-Leu (Z-L_3VS, figure 2.1). Analogs of Z-L_3VS in which the carboxylbenzyl group was replaced with biotin (Biotin-L_3VS), the hapten 4-hydroxyl-5-iodo-3-nitrophenyl acetate (NIP-L_3VS), and the corresponding radiolabeled hapten (^{125}I-NIP-L_3VS) were also synthesized.

2. Peptide vinyl sulfones irreversibly inhibit proteasome activity in vitro

The ability of the peptide vinyl sulfones to inhibit proteasomal proteolysis was determined in vitro using a partially purified mixture of 20S and 26S proteasomes with fluorogenic peptides as substrates. Strongest inhibition was observed for the chymotrypsin-like peptidase activity of the proteasome with somewhat lesser inhibition of the trypsin-like and peptidylglutamyl peptidase activities, as shown for Z-L_3VS (Fig. 2.2). The peptide vinyl sulfones, including the biotin and NIP derivatives (data not shown), were only slightly less potent inhibitors than the peptide aldehyde Z-L_3H (Fig. 2.2A) and were more active towards all three peptidase activities than was lactacystin upon simultaneous addition with substrates. When each of the three classes of inhibitors was pre-incubated for one hour with proteasomes and then diluted ten-fold, the extent of inhibition by the aldehyde was reduced as expected for a reversible inhibitor, while the extent of inhibition by both lactacystin and Z-L_3VS increased, consistent with time dependent, irreversible inhibition (Fig. 2.2B). Furthermore, Z-L_3VS caused inhibition of the trypsin-like peptidase activity to a greater extent than lactacystin (Fig. 2. 2B), indicating that Z-L_3VS binds to a subunit or binding site more effectively than lactacystin.

Figure 2.1. (A) Structure of inhibitors of the proteasome. (B) mechanism of inhibition of the proteasome by the peptide vinyl sulfones.

(A) PyBop, DIEA, CH_3NHOCH_3, CH_2Cl_2; (B) LAH, Et_2O; (C) $(EtO)_2P(O)CH_2S(O)_2CH_3$, NaH, THF; (D) TsOH, Et_2O; (E) DCC, HOBt, DMF; (F) 20% K_2CO_3/MeOH; (G) PyBOP, DIEA, CH_2Cl_2; (H) TsOH, Et_2O; (I) Biotin-nitrophenyl ester, DMF, DIEA; (J) 4-hydroxy-3-iodo-5nitrophenylacetic acid, PyBOP, DIEA, CH_2Cl_2; (K) 4-hydroxy-3-nitrophenylacetic acid, PyBOP, DIEA, CH_2Cl_2; (L) $Na^{125}I$, Iodogen®.

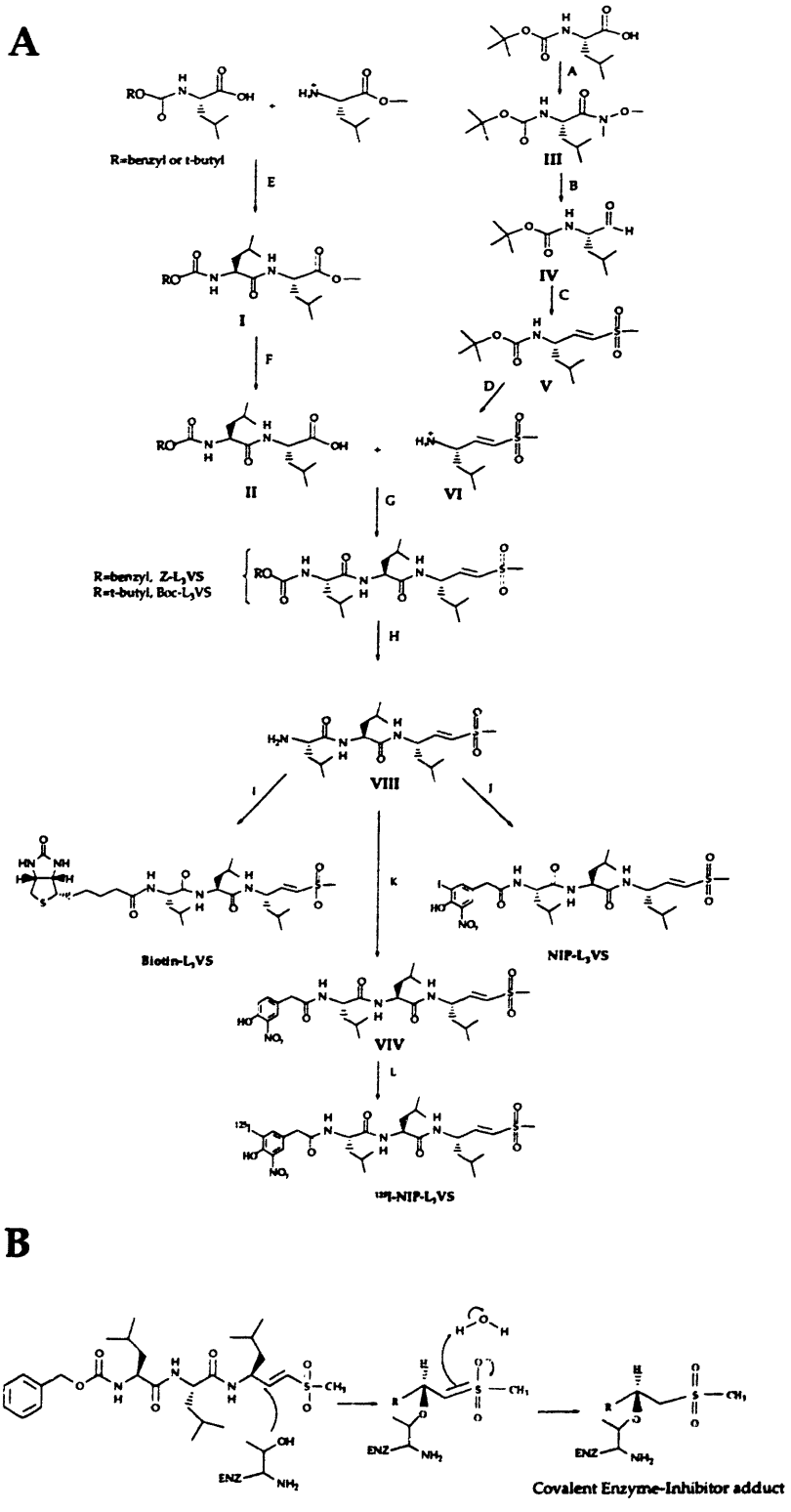
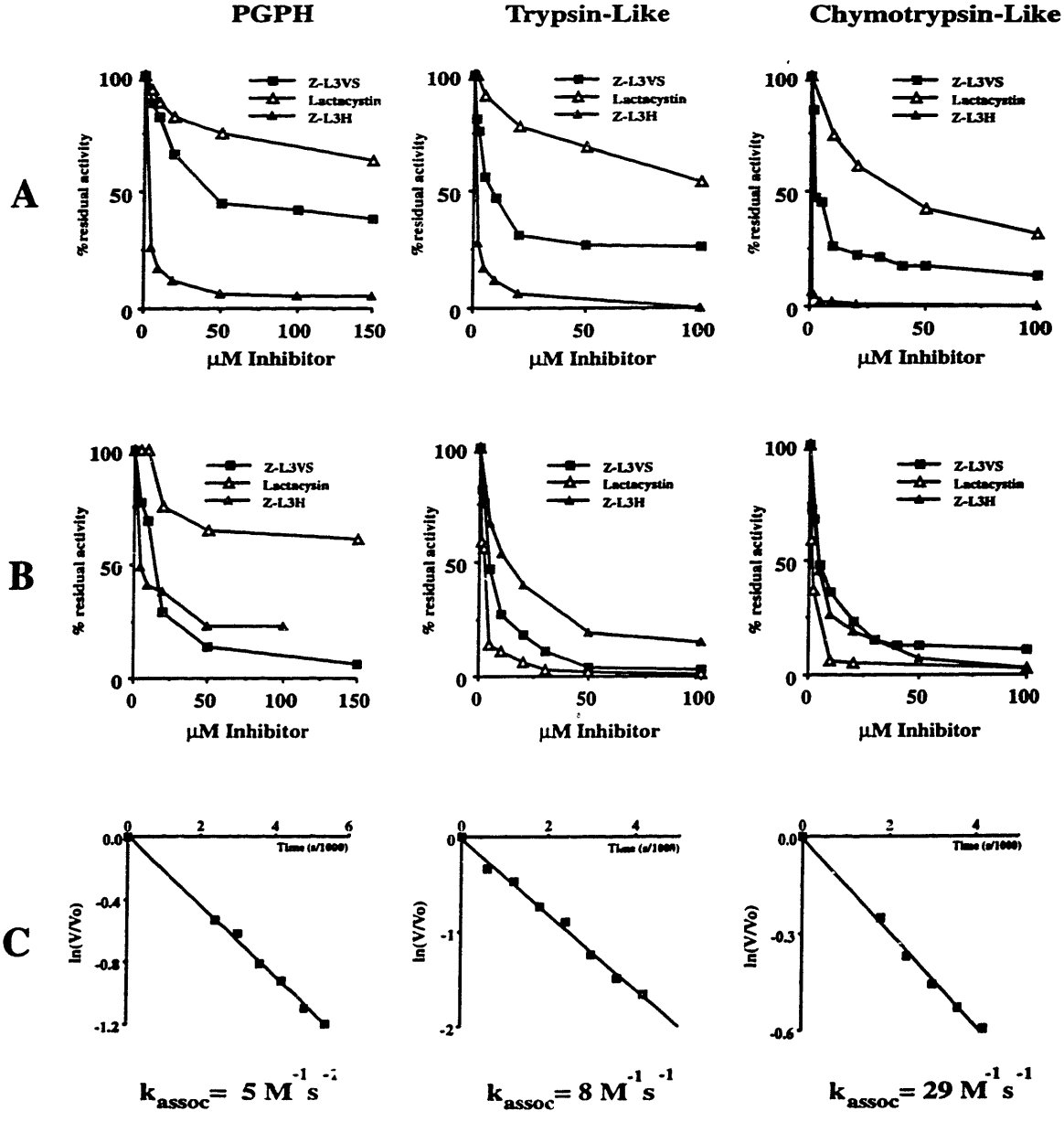

A
R=benzyl or t-butyl
E
A
III
B
IV
C
V
D
F
I
II
VI
G
R=benzyl, Z-L2VS
R=t-butyl, Boc-L2VS
H
VIII
I
J
K
Biotin-L2VS
NIP-L2VS
VIV
L
125I-NIP-L2VS
B
ENZ
Covalent Enzyme-Inhibitor adduct

Figure 2.2. The peptide vinyl sulfones inhibit the proteasome *in vitro*.

Purified preparations of 20S and 26S proteasomes were incubated with increasing concentrations of the three inhibitors; Z-L_3H, Z-L_3VS and lactacystin, either simultaneously with substrates (A) or samples were pre-incubated for 1 hour (37°C) and diluted ten-fold prior to addition of substrates (B). Hydrolysis of substrates corresponding to the acidic (Cbz-LLE-βNA), basic (Boc-LRR-MCA), and hydrophobic (Suc-LLVY-MCA) activities of the proteasome were measured by fluorescence spectroscopy. Association constants (K_{assoc}) for the compound, Z-L_3VS, were determined for the three peptidase activities of purified 20S proteasomes (C). V represents velocity of the reaction at the time t, and V_o is the velocity at time 0. $K_{assoc} = \ln(V/V_o)/[I]$ where [I] is the concentration of inhibitor (5 μM for hydrophobic activity, 50 μM for basic and acidic activity).


PGPH
Trypsin-Like
Chymotrypsin-Like
A
B
C
Z-L3VS
Lactacystin
Z-L3H
% residual activity
µM Inhibitor
ln(V/Vo)
Time (s/1000)
k_{assoc}= 5 M^{-1} s^{-1}
k_{assoc}= 8 M^{-1} s^{-1}
k_{assoc}= 29 M^{-1} s^{-1}

3. ^{125}I-NIP-L_3VS covalently modifies multiple β subunits in purified proteasomes preparations and in total cell extracts

A radiolabeled version of the peptide vinyl sulfone NIP-L_3VS was used to examine the ability of this class of compounds to react with proteasomes of diverse origin. ^{125}I-labeled NIP-L_3VS was incubated with purified 20S proteasomes and with total cell homogenates from rabbit muscle and yeast (Fig. 2.3A, 2.3B). A single band of 23 kDa was labeled both in crude cell extracts and in purified proteasomes. However, in both cases this band could be resolved into at least two polypeptides of similar molecular weight but different isoelectric points.

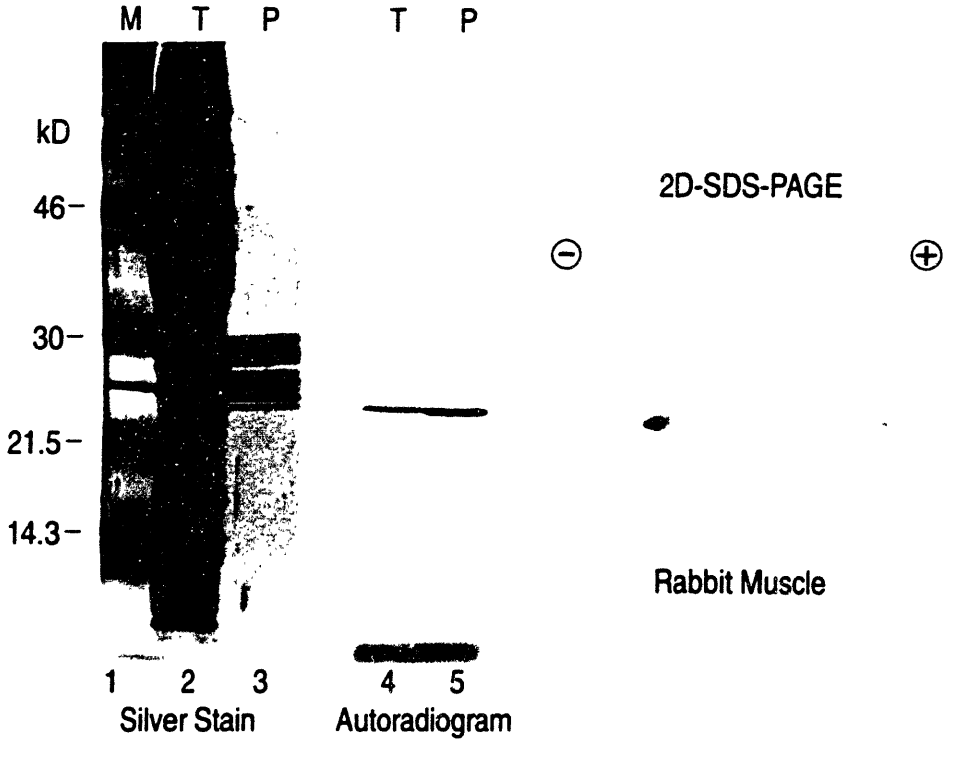
4. The peptide vinyl sulfones are reactive against the archaebacterial proteasome from Thermoplasma

Recombinant mutant and wild type proteasome β subunits from the archaebacterium, Thermoplasma, were used to directly examine whether the N-terminal threonine is required for covalent modification by a peptide vinyl sulfone. Labeling of total cell lysates from Thermoplasma was compared to three preparations of recombinant proteasomes in which the catalytic N-terminal threonine was replaced with either serine or alanine. Recent studies have shown that β-subunits in which the N-terminal threonine is replaced by serine retain full activity against peptide substrates, while replacement with alanine leads to complete loss of peptidase activity. ^{125}I-NIP-L_3VS labeled not only the β subunits in total extracts, but also in the mutant complex in which the N-terminal threonine is replaced by serine (Fig. 2.3C). The N-terminal alanine mutant was refractory to labeling indicating that covalent modification of β subunits requires the catalytic N-terminal threonine of the proteasome. The slight difference in apparent molecular weight between the β subunits from total cell extracts and the recombinant forms of β subunits is due to the presence of a His tag.

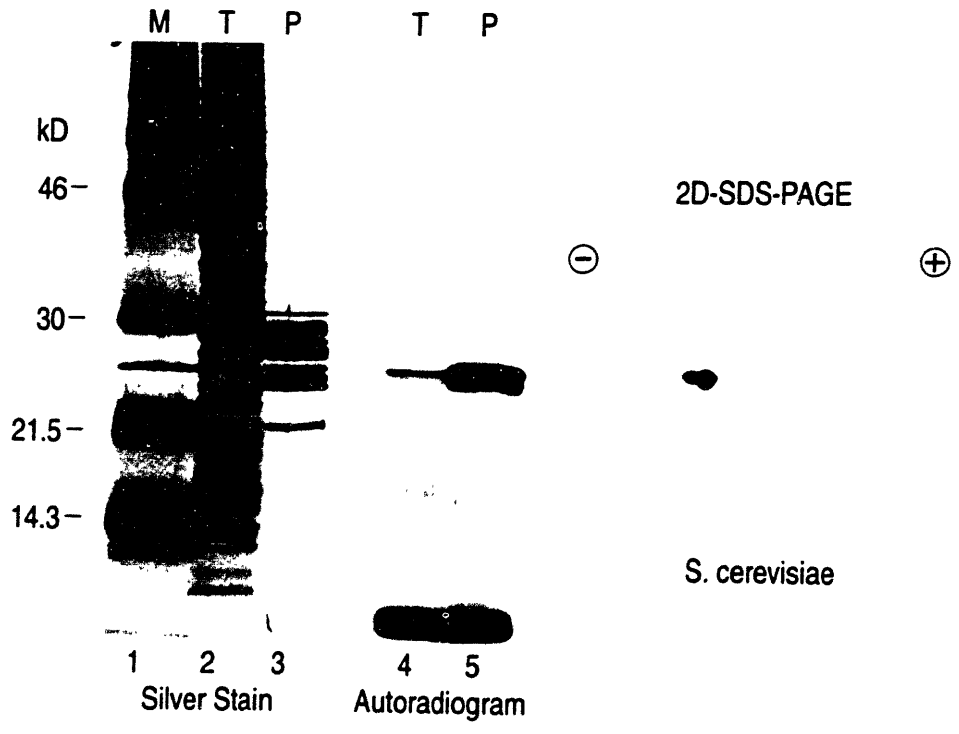
Figure 2.3. The peptide vinyl sulfone, ^{125}I-NIP-L_3VS labels multiple β subunits of the proteasome in total cell extracts and in purified proteasome preparations.

Total cell extracts (T) and purified preparations of proteasomes(P) were incubated with 125I-NIP-L3-VS (1.8×10^4 Bq/ml) at 37°C for 2 hours, resolved on a 12.5% polyacrylamide gel and visualized by silver staining, by autoradiography or by two-dimensional IEF/SDS-PAGE for rabbit muscle (A), S. Cerevisiae (B), or T. acidophilum (C). For Thermoplasma acidophilum recombinant β subunits containing an N-terminal Thr, Ser, or Ala were compared, as indicated.

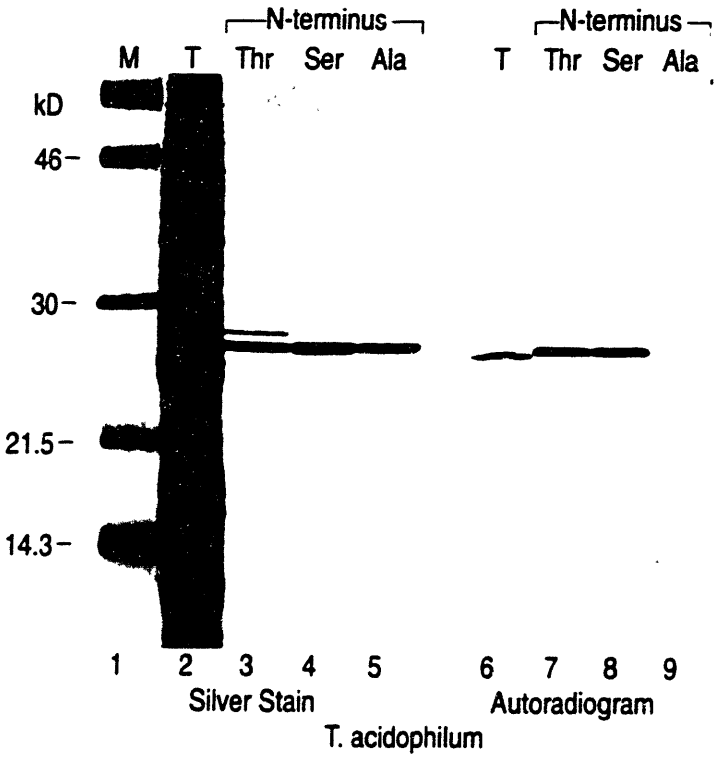
(A)


M T P
T P
kD
46
30
21.5
14.3
2D-SDS-PAGE
⊖
⊕
Rabbit Muscle
1 2 3
4 5
Silver Stain
Autoradiogram

(B)


M T P
T P
kD
46
30
21.5
14.3
2D-SDS-PAGE
⊖
⊕
S. cerevisiae
1 2 3
Silver Stain
4 5
Autoradiogram

(C)


N-terminus
N-terminus
M T Thr Ser Ala T Thr Ser Ala
kD
46–
30–
21.5–
14.3–
1 2 3 4 5 6 7 8 9
Silver Stain
Autoradiogram
T. acidophilum

5. The bacterial protease complex HslV/HslU from *E.coli* is a target for the peptide vinyl sulfones

The proteasome-like protease complex from *E.coli* is composed of two types of heat shock protein subunits, HslV and HslU (Rohrwild et al., 1996). The β-like subunit HslV contains an N-terminal threonine but direct evidence for a catalytic role for this residue is lacking (Lupas et al., 1994). To address the catalytic mechanism of this complex, purified preparations of HslV/HslU were treated with the ^{125}I-labeled peptide vinyl sulfone. ^{125}I-NIP-L_3VS covalently modified HslV, but only in the presence of HslU and ATP (Fig. 2.4), consistent with the reported requirement for ATP of the HslV/HslU complex (Rohrwild et al., 1996). Combined, our results show that the peptide vinyl sulfones are capable of inhibiting proteases of the proteasome family found in a wide variety of species, including the eubacterial species *E. coli.*, by targeting the catalytic, active site threonine residue.

6. ^{125}I-NIP-L_3VS covalently modifies β subunits of the proteasome in living cells in a time and dose dependent manner

The ability of the tri-peptide vinyl sulfones to penetrate cells and specifically modify proteasomal β subunits was established using several different cell lines (Fig. 2.5A). Incubation of ^{125}I-NIP-L_3VS with the human astrocytoma cell line U373-MG, the human B-cell line HOM-2 and the human T2 cell line (Salter and Cresswell, 1986) lacking the two interferon inducible β subunits LMP-2 and LMP-7 showed labeling of a 23 kDa species similar to that described for rabbit muscle preparations. The labeling increased linearly with time and with concentration of ^{125}I-NIP-L_3VS (data not shown). The labeled polypeptides could be immuno-precipitated using a monoclonal antibody raised against an α subunit common to the 20S and 26S proteasome, but not with an irrelevant antibody, thus establishing that the labeled polypeptides are part of the proteasome complex.

The identity of the proteasome β subunits modified by the radiolabeled vinyl sulfone was determined using the mutant human cell line, T2, in which the MHC region coding for the two interferon inducible proteasome β subunits,

Figure 2.4. The peptide vinyl sulfones covalently modify the HslV gene product from the E.coli protease complex HslV/HslU.

Purified preparations of HslV and HslU were incubated with ^{125}I-NIP-L_3VS (1.8×10^4 Bq/ml) independently or as a 1:4 mixture (HslV:HslU) in the presence of 2 mM ATP (+ATP) or 5 units/ml apyrase (-ATP).

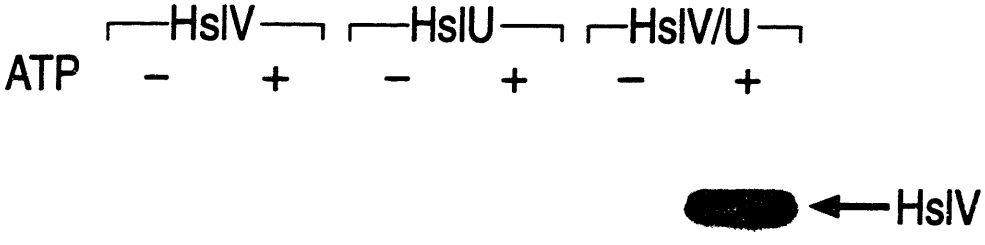
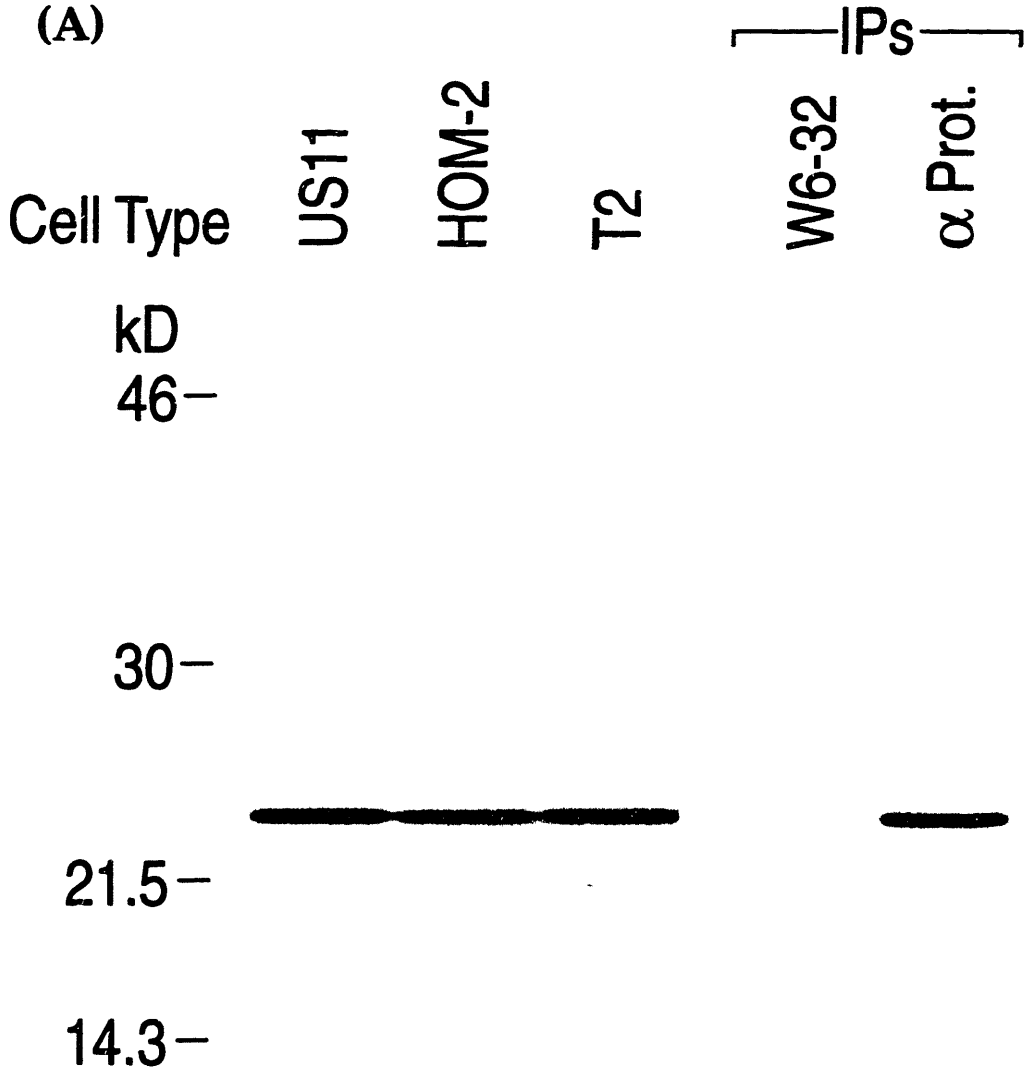
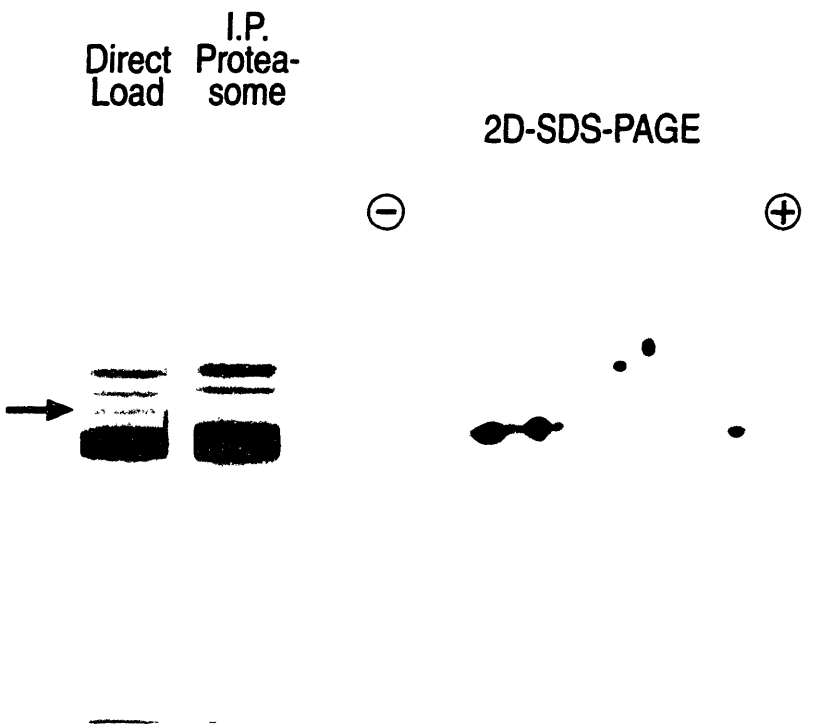

HslV
HslU
HslV/U
ATP
– + – + – +
HslV

Figure 2.5. The peptide vinyl sulfones covalently modify multiple β subunits of the proteasome in living cells, including the γ-IFN inducible subunits LMP-2 and LMP-7.

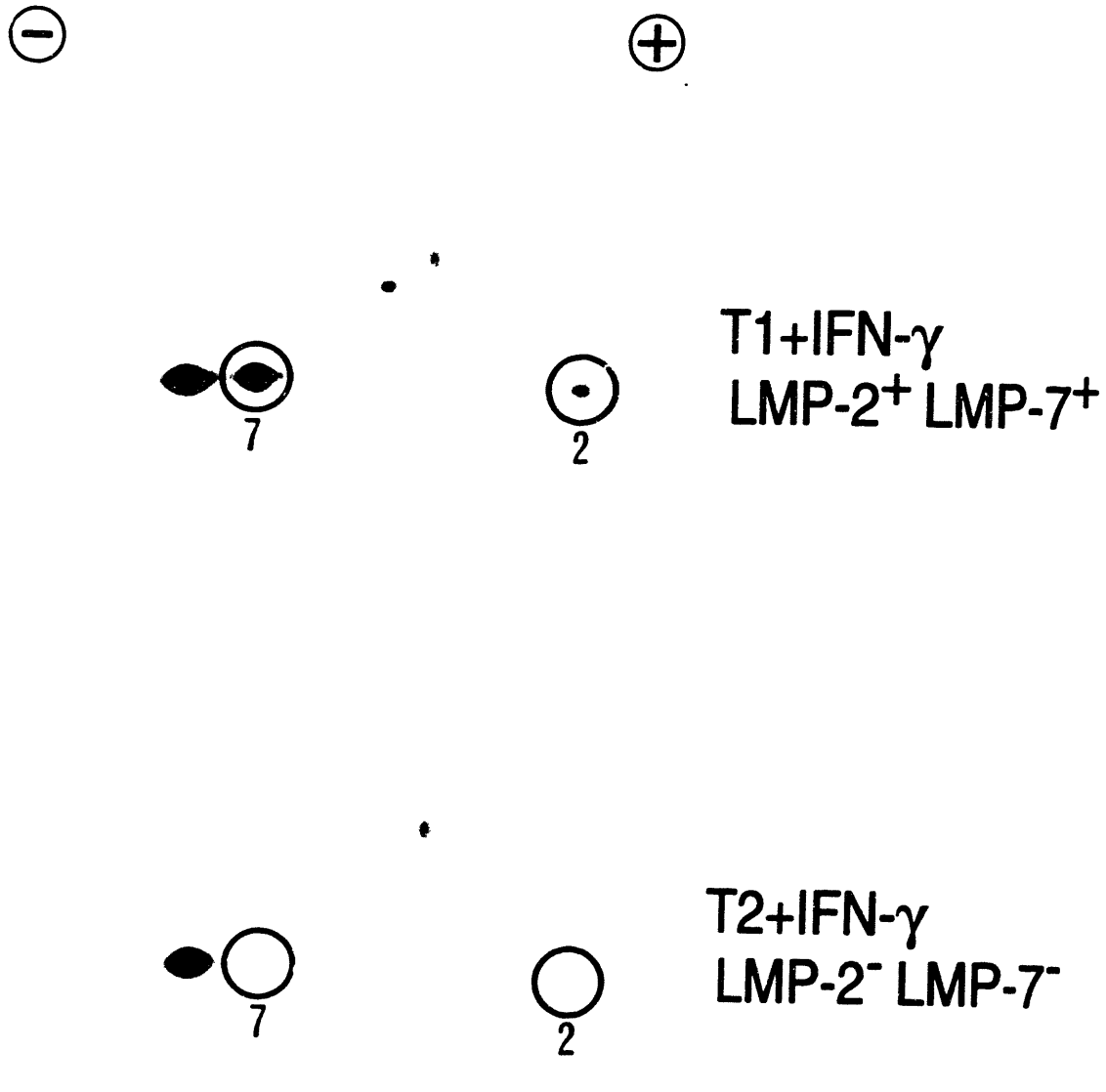
(A) US11+, HOM-2, and T2 cells (1.5×10^6 cells) were incubated with ^{125}I-NIP-L_3VS (1.8×10^4 Bq/ml) for 2 hours at 37°C, washed several times and lysed. Total cell extracts were resolved on a 12.5% polyacrylamide gel and visualized by autoradiography. Immuno-precipitations were performed on lysates from ^{125}I-NIP-L_3VS labeled HOM-2 cells using monoclonal antibodies recognizing MHC class I molecules (W6-32) or a proteasomal α subunit (α prot.)
(B) HOM-2 cells were incubated with ^{125}I-NIP-L_3VS, lysed and analyzed by autoradiography (Direct Load) or proteasomes were immuno-precipitated (I.P. Proteasome) as in A. Note the absence of a single labeled polypeptide (26 kDa) in the proteasome immuno-precipitation. A total cell lysate from ^{125}I-NIP-L_3VS labeled HOM-2 cells was also separated by two-dimensional NEPHGE-PAGE, (11% polyacrylamide gel) and visualized by autoradiography.
(C) T1 and T2 cells (1.5×10^6 cells) were incubated with ^{125}I-NIP-L_3VS (1.8×10^4 Bq/ml) for 2 hours at 37°C, lysed, resolved by two dimensional NEPHGE-PAGE and analyzed by autoradiography. Note the presence of labeled LMP-2 and LMP-7 in lysates from T1 cells only.


(A)
IPs
Cell Type
US11
HOM-2
T2
W6-32
α Prot.
kD
46
30
21.5
14.3

(B)



(C)



LMP-2 and LMP-7 has been deleted (Salter and Cresswell, 1986). We could thus determine which of the major species labeled with ^{125}-I-NIP-L_3VS were LMP-2 and LMP-7 by comparison with the wild type cell line T1 (Figure 2.5C). The most intensely labeled, basic polypeptide corresponding to the subunit X was present in similar quantities in both cell lines, as were the two minor species of higher molecular weight. The more acidic polypeptide was absent in the labeling of T2 cells. Based on the known pIs of the β subunits (Coux et al., 1996), the latter spot was identified as LMP-2. Similarly, a polypeptide of a molecular weight identical to X but with a slightly more acidic pI was present in the T1 cells and absent from T2 cells, identifying it as the LMP-7 subunit of the proteasome. These assignments were confirmed by comparison of mouse LMP-2 and LMP-7 obtained from different H2 haplotypes, where these subunits can be resolved based on differences in their molecular weight and isoelectric point (Nandi et al., 1996) (data not shown).

Upon longer exposure of autoradiographs from the labeled cell extracts, three less intensely labeled polypeptides of 26, 28 and 29 kDa were visible (Fig. 2.5B). Two of these species were co-immunoprecipitated with the predominant 23 kDa band when using a proteasome-specific antibody. The third, minor species is a non-proteasomal protease modified to some extent by the peptide vinyl sulfones (see below). Analysis by two-dimensional gel electrophoresis (Fig. 2.5B) shows that the predominantly labeled, 23 kDa band is composed of three polypeptides of different isoelectric points, while the higher molecular weight species each represent individual subunits.

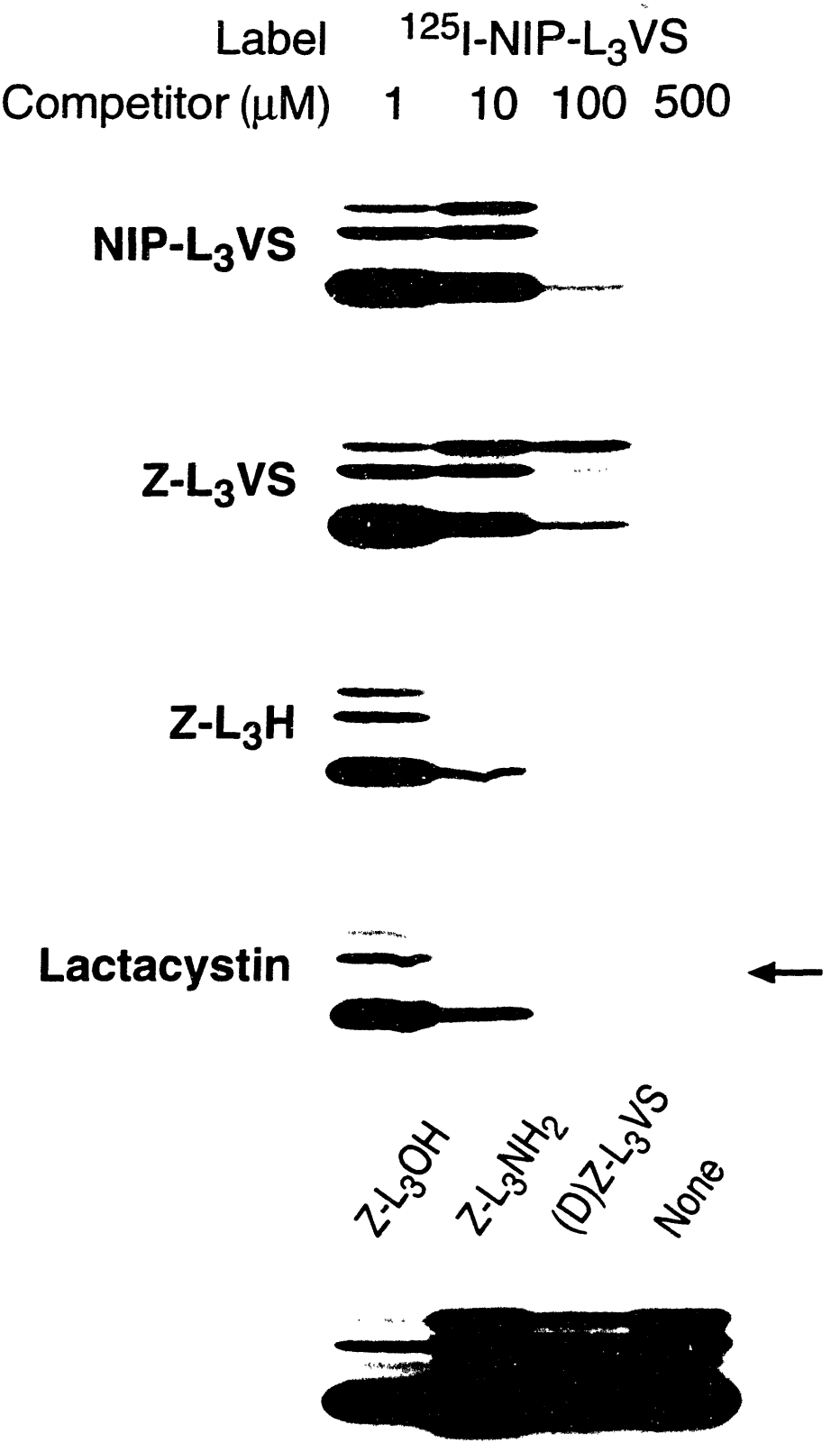
7. All three classes of proteasome inhibitors compete for binding with ^{125}I-NIP-L_3VS

To address the specificity of the radiolabeled inhibitor for the proteasome, competition experiments were performed on intact mammalian cells (Fig. 2.6A). The peptide aldehyde Z-L_3H blocks covalent binding of the labeled inhibitor to all β subunits at a concentration of 10 μM. The compounds NIP-L_3VS, as well as the structurally related compound, Z-L_3VS, also competitively reduced subunit modification by^{125}I-NIP-L_3VS, but were slightly less effective than the aldehyde Z-L_3H and lactacystin. The tripeptide vinyl sulfone in which the COOH-terminal

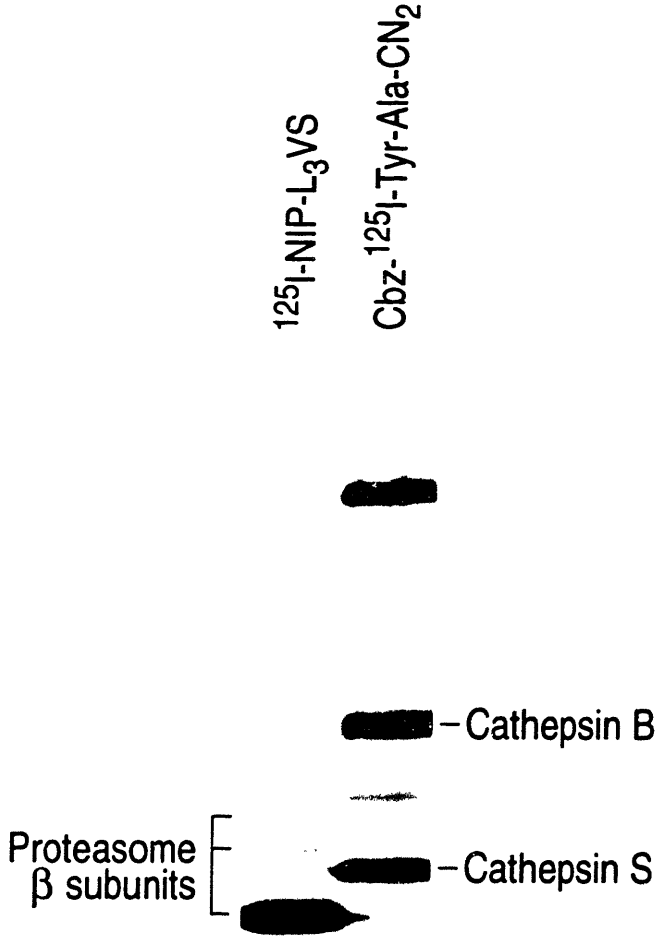
Figure 2.6. The peptide vinyl sulfones, the peptide aldehyde, and lactacystin compete for binding to all proteasome subunits modified by ^{125}I-NIP-L_3VS.

(A) HOM-2 cells were incubated simultaneously with ^{125}I-NIP-L_3VS (1.8×10^4 Bq/ml) and with increasing concentrations of NIP-L_3VS, Z-L_3VS, lactacystin, or with 500 μM of the control peptides Z-L_3-OH, Z-L_3NH_2, or (D)Z-L_3VS for 2 hours at 37°C. Note that the 26 kDa polypeptide shown in figure 5B remains resistant to competition by lactacystin (see arrow).
(B) HOM-2 cells were labeled with ^{125}I-NIP-L_3VS as described or with the cysteine protease inhibitor Cbz-^{125}I-Tyr-Ala-CN_2 for two hours at 37°C. Lysates were separated on a 12.5 % polyacrylamide gel and analyzed by autoradiography.
(C) HOM-2 cells were incubated simultaneously with Cbz-^{125}I-Try-Ala-CN_2 and increasing concentrations of Z-L_3H, NIP-L_3VS, and lactacystin as indicated for 2 hours at 37°C. Lysates were resolved on a 12.5% polyacrylamide gel and analyzed by autoradiography.

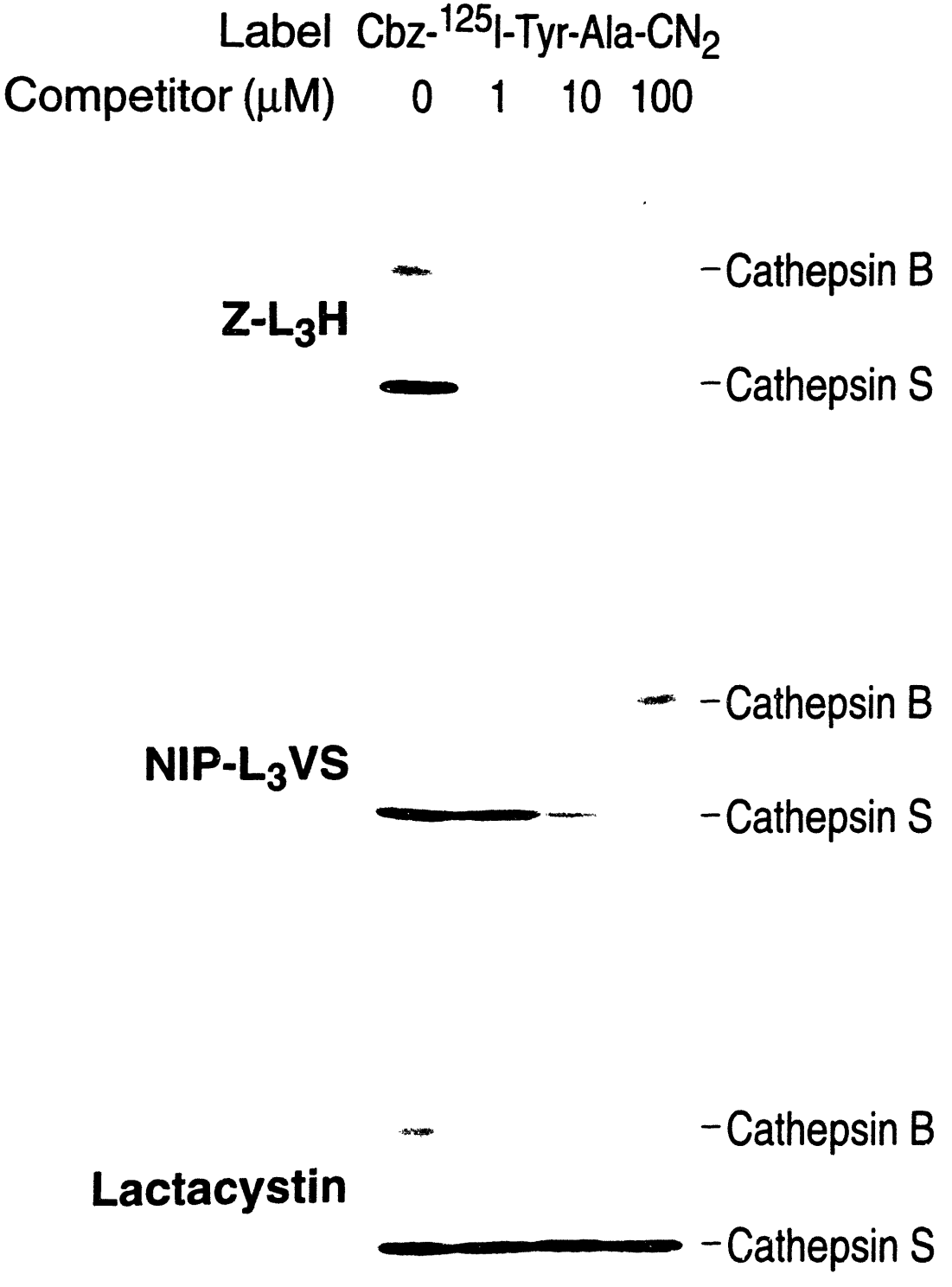
(A)


Label
^{125}I-NIP-L$_3$VS
Competitor (μM)
1
10
100
500
NIP-L$_3$VS
Z-L$_3$VS
Z-L$_3$H
Lactacystin
Z-L$_3$OH
Z-L$_3$NH$_2$
(D)Z-L$_3$VS
None

(B)


^{125}I-NIP-L$_3$VS
Cbz-^{125}I-Tyr-Ala-CN$_2$
Cathepsin B
Cathepsin S
Proteasome
β subunits

(C)


Label Cbz-^{125}I-Tyr-Ala-CN_2
Competitor (μM) 0 1 10 100
Z-L_3H
Cathepsin B
Cathepsin S
NIP-L_3VS
Cathepsin B
Cathepsin S
Lactacystin
Cathepsin B
Cathepsin S

leucine is replaced with the D-isomer ((D)ZL_3-VS) did not compete for binding even at 500 μM.

Of the species modified by the radiolabeled vinyl sulfone, only one rather minor polypeptide (seen only in mammalian cells) was resistant to competition by lactacystin (Fig. 2.6A; arrow). Because peptide aldehydes such as Z-L_3H are known to inhibit other classes of proteases (Coux et al., 1996; Rock et al., 1994), while lactacystin does not, we presumed this minor polypeptide to be a non-proteasomal protease .

To identify the lactacystin-resistant, 26 kDa species, cells were labeled with the cysteine protease-selective probe, Cbz-^{125}I-Tyr-Ala-N_2 (Fig. 2.6B). This modified di-peptide covalently modifies cysteine proteases of the cathepsin family, predominantly cathepsins B and S (Riese et al., 1996). Co-migration of the weakly labeled 26 kDa species with the polypeptide identified as the lysosomal protease cathepsin S confirmed its identity. We further examined the ability of all three classes of proteasome inhibitors to compete with the Cbz-^{125}I-Tyr-Ala-CN_2 probe for binding to cathepsins B and S (Fig. 2.6C). The peptide aldehyde, Z-L_3H, was able to prevent labeling of both cathepsin S and B at concentrations as low as 1μM, while lactacystin showed no inhibition of labeling of either, at concentrations as high as 100 μM. NIP-L_3VS showed only partial inhibition of labeling of cathepsin S and no inhibition of labeling of cathepsin B even at the two highest concentrations tested. This result strongly suggests that Z-L_3H could, in principle, block lysosomal proteases involved in antigen presentation.

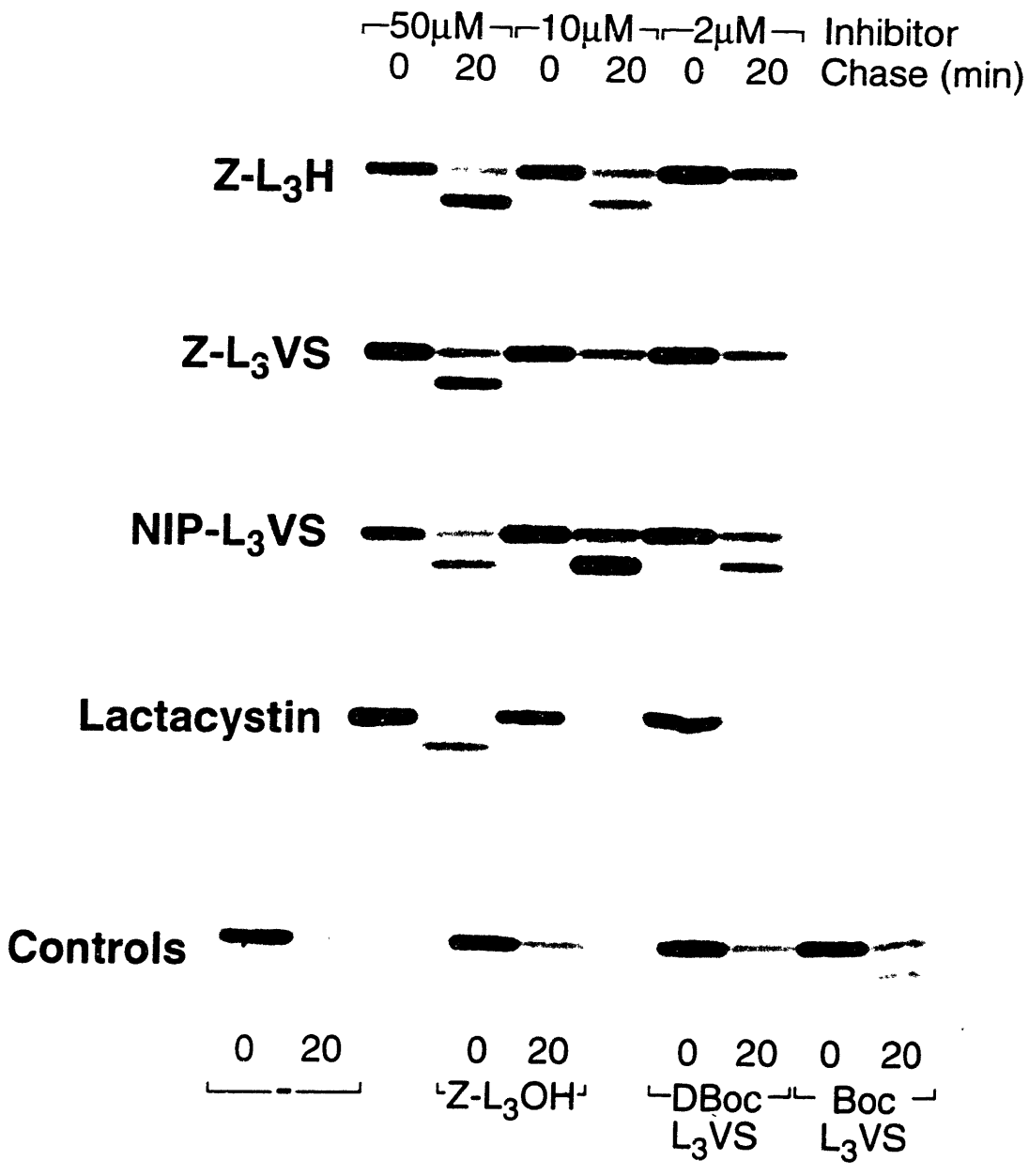
8. Peptide vinyl sulfones inactivate the proteasome *in vivo*

The in vivo effects of the peptide vinyl sulfones were compared to the other two classes of proteasome inhibitors Z-L_3-H and lactacystin. The peptide vinyl sulfones were less toxic to cells than the peptide aldehyde Z-L_3H as assessed by their inhibitory affects on protein synthesis (data not shown). Proteasomal inhibition was examined using an assay in which a viral protein promotes destruction of MHC class I heavy chains in a proteasome-dependent manner (Wiertz et al., 1996a; Wiertz et al., 1996b). The HCMV gene US11 causes the dislocation of free class I heavy chains from the ER to the cytosol, at which

point the single N-linked glycan is removed and the class I heavy chain is degraded by the proteasome. Thus, in the presence of US11 and an active proteasome inhibitor, the transient appearance of a cytosolic, deglycosylated breakdown intermediate results (Wiertz et al., 1996a; Wiertz et al., 1996b). Z-L_3VS caused the characteristic build-up of this heavy chain intermediate. The peptide vinyl sulfone analog containing the hapten NIP at the N-terminus showed activity at 2 μM, better than that of lactacystin, Z-L_3H and the related compound, Z-L_3VS. The peptide vinyl sulfone in which the COOH-terminal leucine was replaced with the D-isomer (DBocL_3VS) was inactive in this assay.

Figure 2.7. The peptide vinyl sulfones block proteasome function *in vivo*.

US11+ cells were pre-incubated in methionine-free medium without (minus) or with the indicated concentrations of Z-L_3H, Z-L_3VS, NIP-L_3VS or lactacystin for 1 hour. Cells were pulse labeled for 10 min., and chased for 20 min. Class I molecules were immuno-precipitated with rabbit anti-heavy chain serum, resolved on a 12.5% polyacrylamide gel and analyzed by fluorography. Note the presence of a class I heavy chain band devoid of carbohydrates, which migrates faster than the glycosylated species, and accumulates in the presence of an active proteasome inhibitor.


50μM
10μM
2μM
Inhibitor
0 20 0 20 0 20 Chase (min)
Z-L3H
Z-L3VS
NIP-L3VS
Lactacystin
Controls
0 20
0 20
Z-L3OH
0 20
DBoc L3VS
0 20
Boc L3VS

D. Discussion

Inhibitors of the proteasome have been instrumental in deciphering its role in a diverse assortment of cellular processes (Harding et al., 1995; Jensen et al., 1995; Palombella et al., 1994). We report here a class of inhibitors , the peptide vinyl sulfones, which inactivate the proteasome by covalent modification of the active site threonine of the catalytic β subunits of widely different origin. The ability of the peptide vinyl sulfones to permeate cells and covalently modify proteasome β subunits also makes them useful active site probes for studying the function of the proteasome in living cells.

Although originally characterized as mechanism-based inhibitors of cysteine proteases, the peptide vinyl sulfones inhibit the chymotrypsin-like, trypsin-like and petidylglutamyl peptidase activities of the proteasome both in vitro and in vivo. The activity of the vinyl sulfones against a sidechain threonine hydroxyl nucleophile is surprising considering that these types of Michael acceptor structures were designed as mechanism-based inhibitors for soft nucleophiles such, as cysteine (Palmer, 1995). The peptide vinyl sulfones therefore provide new information about the reactivity of a threonine residue as an active site nucleophile. The ability of the vinyl sulfone to covalently modify a sidechain hydroxyl of serine when placed at the N-terminus of a proteasomal β-subunit but not when part of the active site of a serine protease suggests basic differences in the way the proteasome and serine proteases utilize a hydroxyl group as a catalytic nucleophile.

The reactivity of the peptide vinyl sulfones against the ATP-dependent eubacterial proteasome homologue, HslU/HslV, provides solid evidence that this multi-component protease complex utilizes a catalytic mechanism similar to that of the proteasome. Moreover, modification required the presence of the peptidase subunit (HslV), the regulatory ATP-ase (HslU) and ATP, all of which are necessary for the cleavage of a peptide substrate. These findings suggest that the vinyl sulfones inactivate their target via a mechanism resembling that involved in peptide hydrolysis.

Competition experiments using the radiolabeled peptide vinyl sulfone was used to assess the activity and specificity of three different classes of proteasome inhibitors. The inability of the tri-peptide vinyl sulfone containing a

D-leucine in the P1 position to inhibit labeling of any of the β subunits labeled by ^{125}I-NIP-L_3VS indicated the proteasome's stereochemical selectivity at the site of hydrolysis. The labeling of a rather minor 26 kDa polypeptide, identified as cathepsin S, was completely blocked by Z-L_3H but was unaffected by lactacystin at all concentrations tested. This finding, combined with the competition data obtained for Z-L_3H, NIP-L_3VS, and lactacystin using the ^{125}I-Tyr-Ala probe, fits not only the known specificity of lactacystin for the proteasome, but also suggests that the peptide aldehyde, Z-L_3H, should be a potent competitive inhibitor of lysosomal proteases. Related peptide aldehydes are known to inhibit lysosomal and Ca+-dependent cysteine proteases, and such possible effects must be considered in studies using these agents on intact cells (Coux et al., 1996; Rock et al., 1994).

The finding that lactacystin competes for labeling of all five β subunits by ^{125}I-NIP-L_3VS, is inconsistent with the initial identification of a single β subunit, X (also known as MB-1), as the target of the drug (Fenteany et al., 1995). While the predominant species modified by lactacystin is X, modification of other subunits which require higher concentrations of drug may have escaped detection due to the relatively low concentration of labeled compound used as confirmed in subsequent work (Craiu et al., in press). We conclude that ^{125}I-NIP-L_3VS labels at least 5 distinct β subunits, all of which are also targets for lactacystin. Thus, the peptide vinyl sulfones are useful diagnostic tools which provide detailed information about proteasomal inhibition by other classes of compounds. Their ease of synthesis and ability to covalently inactivate proteasomal β subunits in intact cells makes peptide vinyl sulfones powerful new tools for the study of proteasome function.

III. STRUCTURE-FUNCTION STUDIES OF TRI- AND TETRA-PEPTIDE VINYL SULFONES

A. Introduction

The proteasome is a large, multi-subunit complex which is involved in many of the catabolic events taking place in the cell. The proteasome employs a rather unique catalytic mechanism and is a member of the small family of proteolytic enzymes known as N-terminal Hydrolyses. This class of enzyme utilizes an N-terminal amino acid as the catalytic residue, which in the case of the proteasome is threonine. The proteasome's N-terminal threonine hydroxyl sidechain is activated by a single water molecule involving a charge relay system with other important internal amino acids such as lysine (Seemüller et al., 1995; Lupas et al., 1995). As a threonine protease, it does not fall into a standard category with other proteolytic enzymes in terms of its reactivity towards different classes of inhibitors. Its reactivity with serine as well as cysteine protease inhibitors complicated its initial characterization (Coux et al., 1996). Quite surprising is the number of types of C-terminally modified peptides which are reactive with the proteasome's N-terminal catalytic residue (Cardozo et al., 1992; Harding et al., 1995; Iqbal et al., 1996; Spaltenstein et al., 1996; Rock et al., 1994; Bogyo et al., 1997).

Central to understanding the mechanism of action of this enzyme complex is the ability to modulate its activity experimentally with small molecule inhibitors. The natural product lactacystin irreversibly inhibits the proteasome by covalent modification of the catalytic threonine hydroxyl (Dick et al., 1996; Fenteany et al., 1995). To this day, the proteasome remains the only known proteolytic enzyme inhibited by lactacystin. Lactacystin is also the only example of a non-peptide-based inhibitor with potent activity against the proteasome. Its lack of structural similarity to the C-terminal peptide aldehyde proteasome inhibitors (see chapter 1 figure 1.5 for structure) suggests that it may bind to the proteasome active sites differently. The peptide aldehydes are likely to more closely mimic a physiological protein substrate.

In fact, this suggestion has been verified with the advent of the crystal structure of the Thermoplasma 20S proteasome (Löwe et al., 1995) and more recently, the yeast 20S proteasome (Groll et al., 1997). Crystals obtained of proteasomes in a complex with either the peptide aldehyde Cbz-Leucinyl-leucinyl-norleucinal (LLnL or Calpain I inhibitor), or with lactacystin indicate

that the two inhibitors do not interact with the proteasome equivalently. While LLnL was found bound to all three catalytically active β-subunits (PUP1 or Z, PRE2 or X, and PRE3 or Y), lactacystin predominantly targeted the X (PRE2) β subunit (Groll et al., 1997).

Because of the proteasome's role in the production of antigenic peptides and in the activation of signaling molecules required for inflammation, work is in progress to develop potent inhibitors of the proteasome as potential drugs for use as anti-inflammatory agents and for treatment of auto-immune diseases. The usefulness of such inhibitors depends on criteria such as bioavailability and cell permeability, criteria which favor small, hydrophobic, non-peptide like compounds. However these compounds, while possibly useful as therapeutic agents, may fail to accurately mimic a protein substrate and therefore fail to provide information about issues such as catalytic mechanism and substrate specificity.

The unveiling of the yeast 20S proteasome crystal structure uncovered the possibility of other active sites within the large multi-subunit complex that do not employ the catalytic N-terminal threonine residues (Groll et al., 1997). Multiple magnesium binding sites were discovered which create a very acidic environment within the center of the proteasome's internal cavity. Thus, bound water molecules may serve as catalytic nucleophiles similar to the mechanism of hydrolysis by a generic aspartic acid protease. Short inhibitors and fluorogenic substrates may not access such a secondary binding pocket and therefore may not accurately mimic substrate binding. Possible additional proteasome-substrate interactions may therefore only be addressed using longer peptides.

We describe here the synthesis and characterization of a series of peptide vinyl sulfones composed of different amino acid sequences, lengths, and C and N-terminal modification. These inhibitors are all equipped with a C-terminal vinyl sulfone, shown previously to covalently modify the catalytic N-terminal threonine residue of the proteasome (Bogyo et al., 1997). Tri-peptide vinyl sulfones containing a free amino-terminus fail to inhibit proteasomal proteolysis, but the addition of a fourth, hydrophobic, aliphatic, or aromatic amino acid (at position P4) results in a dramatic increase in inhibitor potency. Interestingly, when these peptide inhibitors are equipped with a ^{125}I radiolabel, it is possible to directly assess binding to proteasomal subunits by 1- and 2-dimensional

electrophoretic methods. The increase in inhibitory potency by addition of a P4 amino acid is accompanied by a change in the pattern of polypeptides labeled by the inhibitor. This change in labeling profile is likely to shed some light on previously undescribed substrate binding interactions, and may allow for a correlation of potency of inhibition of fluorogenic substrate hydrolysis with the subunit/subunits covalently modified.

Still little is known about how the proteasome is able to control the proteolytic processing of the large number of possible protein substrates into the proper peptide fragments. Many favor an interpretation in which a protein substrate enters the proteasomal cavity and where the sites of hydrolysis are designated by physical location of the polypeptide within the cavity. To address this question we examined a series of tri-peptide vinyl sulfones that differ in amino acid sequence and N- and C-terminal modifications for their ability to inhibit the hydrolysis of a series of fluorogenic substrates. ^{125}I-labeled derivatives were then used to explore subunit binding. Marked differences in the labeling patterns were observed for the different inhibitors, indicating that individual proteasome subunits may in fact discriminate between substrates based on their sequence. This result may begin to explain how substrates are differentially processed by the proteasome.

B. Materials and Methods

1. Inhibitor Synthesis

NIP-L_3-VS, ^{125}I-NIP-L_3-VS, L_3-VS and Z-L_3-VS were synthesized as described in chapter 2. The synthesis of all other inhibitors are described below. All inhibitors were dissolved in DMSO and used in biological assays at the concentrations specified. Stock solutions were prepared such that the concentration of DMSO never exceeded 1%.

2. Synthesis of Tetra-Peptide Vinyl Sulfones Containing the L_3 Core Sequence

The tri-peptide free amine L_3-VS described in chapter 2 was used as a building block for the synthesis of the tetra-peptide vinyl sulfones containing the sequences: YL_3-VS, (D)YL_3-VS, BpaL_3-VS, and GL_3-VS. The synthesis of these tetra-peptides was carried out by the coupling of the desired Boc-amino acid to L_3-VS using the same protocol for all four derivatives. The Boc- protecting group was then removed from the tetra-peptides by treatment with TFA. All tetra-peptides were characterized as the free amine salts only and are described below.

a. Synthesis of Boc-tyrosine-leucinyl-leucinyl-leucine vinyl sulfone (YL_3-VS)

Boc-tyrosine (5.2 mg, 18.6 μmol) and PyBOP (10.2 mg, 18.6 μmol) were dissolved in 1 ml of dichloromethane. Diisopropylethyl amine (3.3 μl, 18.6 μmol) was then added. the reaction was stirred for 5 minutes and L_3-VS tosic acid salt (10 mg, 16.9 μmol) was added, followed by diisopropylethyl amine (3.0 μl, 16.9 μmol). The reaction was stirred for 30 min at room temperature and the solvent was removed by rotary evaporation. The resulting crude reaction mixture was purified by flash column chromatography using ethyl acetate:hexane (2:1) as the mobile phase. Yield (10.4 mg, 15.3 μmol, 90%).

b. Synthesis of tyrosine-leucinyl-leucinyl-leucine vinyl sulfone trifloroacetic acid salt (YL_3-VS)

Boc-YL_3-VS (9 mg, 13.2 μmol) was dissolved in 1 ml of dichloromethane. Trifloroacetic acid (1ml) was then added and the reaction stirred at room temperature for 1 hour. The reaction was quenched by the addition of 5 ml of toluene followed by removal of the solvent by rotary evaporation. A second portion of toluene was then added to the crude solid and again evaporated to dryness. The pure tetra-peptide was obtained by trituration of the solid residue in diethyl ether. Yield (9 mg, 13 μmol, 99%).

YL_3-VS ^{1}H-NMR (500 MHz, CD_3OD) δ 7.05 (2H, d), 6.80 (1H, dd), 6.70 (2H, d), 6.64 (1H, t), 4.7 (1H, m), 4.45 (1H, m), 4.20 (1H, dd), 3.25 (1H, m), 2.95 (3H, s), 2.95 (2H, ddd), 1.50-1.78 (9H, M), 0.95 (18H, m).

FAB Mass Spectrum: [M+H] Calculated for $C_{29}H_{48}N_4O_6S$: 581.7 Found: 581.4

c. Synthesis of (D)-tyrosine-leucinyl-leucinyl-leucine vinyl sulfone ((D)YL_3-VS)

The tetra-peptide containing the D-isomer of tyrosine at the P4 position ((D)YL_3-VS) was synthesized as described above for the L-isomer. Yield (70%).

FAB Mass Spectrum: [M+H] Calculated for $C_{29}H_{48}N_4O_6S$: 581.7 Found: 581.4

d. Synthesis of Boc-glycine-leucinyl-leucinyl-leucine vinyl sulfone (Boc-GL_3-VS)

GL_3-VS was synthesized as described above for YL_3-VS except Boc-glycine was used instead of Boc-tyrosine. Boc-GL_3-VS was purified by flash column chromatography using ethyl acetate:hexane (2:1) as the mobile phase. Yield (71%).

Boc-GL_3-VS ^{1}H-NMR (300 MHz, Acetone-d_6) δ 7.85 (1H, d), 7.56 (1H, d), 7.23 (1H, d), 6.79 (1H, dd), 6.70 (1H, d), 6.62 (1H, d), 4.70 (1H, m), 4.37 (1H, t), 4.24 (1H, q), 3.75 (2H, dd), 2.96 (3H, s), 1.55- 1.82 (9H, m), 1.42 (9H, s), 0.88 (12H, m).

e. Synthesis of glycine-leucinyl-leucinyl-leucine vinyl sulfone (GL_3-VS)

GL_3-VS was synthesized from Boc-GL_3-VS by removal of the Boc protecting group using trifluoroacetic acid as described above for YL_3-VS. Yield (74%).

FAB Mass Spectrum: [M+H] Calculated for $C_{22}H_{42}N_4O_5S$: 475.6 Found: 475.3

f. Synthesis of p-Benzoyl-phenylalanine-leucinyl-leucinyl-leucine vinyl sulfone ($BpaL_3$-VS)

$BpaL_3$-VS was synthesized as described for YL_3-VS except Boc-p-benzoyl-phenylalanine was used instead of Boc-tyrosine. The Boc-$BpaL_3$-VS intermediate was purified by flash chromatography using ethyl acetate:hexane (3:2) as the mobile phase. Yield (Boc-Bpa-L_3-VS 92%, $BpaL_3$-VS, 96%).

$BpaL_3$-VS ^{1}H-NMR (500 MHz, CD_3OD) δ 7.78 (4H, dd), 7.65 (1H, t), 7.55 (2H, t), 7.45 (2H, d), 6.79 (1H, dd), 6.59 (1H, d), 4.65 (1H, m), 4.45 (1H, dt), 4.18 (1H, t), 3.18 (1H, m), 2.98 (3H, s), 1.30-1.75 (9H, m), 0.95 (18H, m).

FAB Mass Spectrum: [M+H] Calculated for $C_{36}H_{52}N_4O_6S$: 669.8 found: 669.5

3. Synthesis of AAF Containing Peptide Vinyl Sulfones

Peptide vinyl sulfones consisting of the core sequence AAF were synthesized essentially as described for the synthesis of L_3-containing vinyl sulfones described in chapter 2. The free amino AAF-VS compound was used as a building block for the synthesis of the tetra-peptide vinyl sulfone YAAF-VS. The synthetic procedures are described below.

a. Synthesis of Boc-phenylalanine-vinyl sulfone (Boc-Phe-VS)

Boc-phenylalanine-vinyl sulfone was synthesized exactly as described for the synthesis of Boc-leucine vinyl sulfone (compound V) in chapter 2 except Boc-phenylalanine was used in place of Boc-leucine. Similar yields were obtained at each step. The NMR data and typical yields obtained for each intermediate are listed below.

Boc-Phe-dimethyl hydroxyl amide: Yield (97%), ^{1}H-NMR (300 MHz, $CDCl_3$) δ 7.18-7.38 (5H, m), 5.19 (1H, d), 4.98 (1H, m), 3.69 (3H, s), 3.20 (3H, s), 3.0 (2H, ddd), 1.40 (3H, s).

Boc-Phenylalanal: Yield (73%), ^{1}H-NMR (300 MHz, $CDCl_3$) δ 9.65 (1H, s), 7.18-7.38 (5H, m), 5.05 (1H, d), 4.44 (1H, m), 3.15 (2H, d), 1.45 (9H, s).

Boc-Phe-VS : Yield (73%) ^{1}H-NMR (300 MHz, $CDCl_3$) δ 7.35 (3H, q), 7.19 (2H, d), 6.90 (1H, dd), 6 39 (1H, dd), 4.67 (1H, m), 4.58 (1H, d), 2.95 (2H, d), 2.93 (3H, s), 1.43 (9H, s).

b. Synthesis of phenylalanine-vinyl sulfone tosic acid salt (Phe-VS)

Phenylalanine-vinyl sulfone was synthesized from Boc-Phe-VS by removal of the Boc protecting group as described for Boc-Leucine-vinyl sulfone in chapter 2.

Phe-VS tosic acid salt ^{1}H-NMR (300 MHz, CD_3OD) δ 7.74 (2H, d), 7.35 (3H, q), 7.25 (4H, d), 6.74 (2H, m), 4.29 (1H, q), 2.99-3.23 (2H, ddd), 2.89 (3H, s), 2.39 (3H, s).

c. Synthesis of Boc-alanine-alanine methyl ester (Boc-AA-OMe)

To a flask was added Boc-alanine (2.0 g, 10.5 mmol), dicyclohexylcarbodiimide (DCC; 2.4g, 11.6 mmol), and HOBt (1.57g, 11.6 mmol) in 25 ml dimethyl formamide (DMF). The reaction was stirred for 30 min

at room temperature. A white precipitate formed upon standing. Alanine-methyl ester hydrochloride salt (2.2g, 15.8 mmol) was then added followed by diisopropylethyl amine (DIEA; 2.8 ml, 15.8 mmol). The reaction was stirred overnight at room temperature. Next, the crude reaction mixture was filtered to remove precipitated solids and then evaporated to a minimal volume by rotary evaporation. The resulting crude solution was diluted with 250 ml ethyl acetate and then extracted with three 75 ml portions of 0.1 N HCl and three 75 ml portions of saturated sodium bicarbonate. The remaining organic phase was dried over $MgSO_4$ and dried to a solid by rotary evaporation. The pure di-peptide methyl ester was isolated by re-crystallization from ethyl acetate/hexane. Yield (2.1g, 7.6 mmol, 72%).

Boc-AA-OMe ^{1}H-NMR (300 MHz, $CDCl_3$) δ 6.64 (1H, d), 5.01 (1H, d), 4.54 (1H, p), 4.15 (1H, q) 3.73 (3H, s), 1.42 (9H, s), 1.40 (3H, d). 1.38 (3H, d).

d. Synthesis of Boc-Alanine-alanine (Boc-AA-OH)

The methyl ester of Boc-AA was converted to the corresponding acid by hydrolysis using sodium carbonate and methanol as described for the synthesis of Boc-LL-OH (compound II) in chapter 2. This compound was used in subsequent steps without further purification.

e. Synthesis of Boc-alanine-alanine-phenylalanine vinyl sulfone (Boc-AAF-VS)

Boc-AAF-VS was synthesized by reaction of Boc-AA-OH with phenylalanine-vinyl sulfone tosic acid salt using the protocol described for the synthesis of Boc-L_3-VS described in chapter 2. The product was purified by flash column chromatography using ethyl acetate/hexane (4:1 v:v) as the mobile phase. Yield (57%).

Boc-AAF-VS ^{1}H-NMR (300 MHz, $CDCl_3$) δ 7.25-7.38 (4H, m), 7.20 (2H, d), 7.12 (1H, d), 6.88 (1H, dd), 6.60 (1H, d), 6.56 (1H, d), 4.98 (1H, m), 4.37 (1H, t), 4.05 (1H, q), 2.95 (2H, dd), 2.90 (3H, s), 1.48 (9H, s), 1.36 (3H, d), 1.34 (3H, d).

f. Synthesis of alanine-alanine-phenylalanine vinyl sulfone tosic acid salt (AAF-VS)

The Boc protecting group was removed from Boc-AAF-VS using the protocol described for removal of Boc from Boc-L_3-VS described in chapter 2. Yield (95%).

AAF-VS ^{1}H-NMR (300 MHz, CD_3OD) δ 7.74 (2H, d), 7.20-7.38 (7H, m), 6.85 (1H, dd), 6.56 (1H, d), 4.82 (1H, q), 4.32 (1H, q), 3.95 (1H, q), 2.99 (2H, dd), 2.90 (3H, s), 2.39 (3H, s), 1.50 (3H, d), 1.32 (3H, d).

FAB Mass Spectrum: [M+H] Calculated for $C_{17}H_{25}N_3O_4S$: 368.4 Found: 368.3

g. Synthesis of Boc-tyrosine-alanine-alanine-phenylalanine vinyl sulfone (YAAF-VS)

To a flask was added Boc-tyrosine (38 mg, 134 μmol) and PyBOP (74 mg, 134 μmol) in 1 ml of dichloromethane. Diisopropylethylamine (23 μl, 134 μmol) was then added and the reaction stirred at room temperature for 5 minutes. AAF-VS (66 mg, 122 μmol) was added to the reaction followed by diisopropylethylamine (22 μl, 122 μmol). The reaction was stirred for an additional 30 minutes. The crude reaction mixture was evaporated to dryness and applied directly to a silica gel column for purification. The pure Boc-YAAF-VS was isolated using 1% methanol in ethyl acetate as the mobile phase. Yield (65 mg, 100 μmol, 82%).

Boc-YAAF-VS ^{1}H-NMR (300 MHz, DMSO-d_6) δ 9.32 (1H, s), 8.24 (2H, d), 8.15 (1H, d), 7.31-7.52 (5H, m), 7.22 (2H, d), 7.05 (1H, d), 6.90 (1H, dd), 6.81 (2H, d), 6.75 (1H, d), 4.95 (1H, p), 4.42 (1H, dt), 4.23 (1H, t), 3.00 (3H, s), 2.85-2.95 (2H, m), 2.20 (2H, d), 1.40 (3H, s), 1.50 (3H, d), 1.32 (3H, d).

h. Synthesis of tyrosine-alanine-alanine-phenylalanine vinyl sulfone (YAAF-VS)

Boc-YAAF-VS (48 mg, 74 µmol) was dissolved in 2ml of dichloromethane. Trifluoroacetic acid (2.0 ml) was then added slowly to the stirring solution. The reaction was stirred for 1 hour at room temperature and then quenched by the addition of 10 ml of toluene. The resulting reaction mixture was evaporated to dryness by rotary evaporation and the residue re-dissolved in 10 ml toluene. Again the solution was dried to a clear oil. The pure salt of YAAF-VS was isolated by trituration of the crude oil with diethyl ether. Yield (48 mg, 73 µmol, 98%).

FAB Mass Spectrum: [M+H] Calculated for $C_{26}H_{34}N_4O_6S$: 531.6 Found: 531.2

4. Synthesis of LLG-Containing Vinyl Sulfones

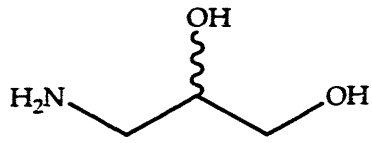
Vinyl sulfones containing glycine at position P1 were synthesized as described for the synthesis of the tri-leucine vinyl sulfones except for the method of preparation of the Boc-glycinal intermediate. Boc-glycinal was prepared by the oxidation of Boc-3-amino-1,2-propane diol (figure 3.1). The resulting aldehyde was converted to the corresponding vinyl sulfone as described for the synthesis of Boc-leucine vinyl sulfone.

a. Synthesis of Boc-3-amino-1,2-propane diol

3-amino-1,2-propane diol (5.0g, 55 mmol) was dissolved in 20 ml of 1,4-dioxane and 10 ml of 3M NaOH. Di-tert-butoxy cabonyl ($(Boc)_2O$; 12 g, 55 mmol) was then added and the reaction stirred for 4.5 hours until the evolution of gas ceases. The solvent was then removed by rotary evaporation and the resulting crude white solid dissolved in ethyl acetate. The ethyl acetate solution was then washed with three 50 ml portions of 5% potassium hydrogen sulfate ($KHSO_4$). The aqueous layers were then combined and back extracted with ethyl acetate. All organic layers were then combined, dried over $MgSO_4$, and

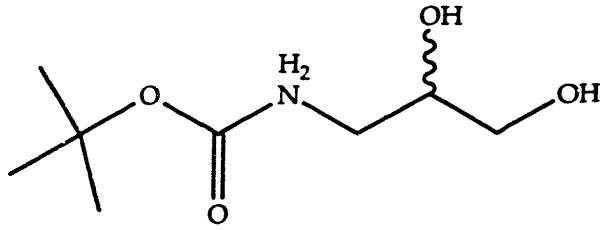
Figure 3.1. Synthesis of glycine-based vinyl sulfones by oxidation of 3-amino-1,2-propanediol derivatives.

(A) $(Boc)_2O$, 3N NaOH; (B) $NaIO_4$, pH 7.0 (C) $(EtO)_2P(O)CH_2S(O)$-PhOH, NaH, THF.



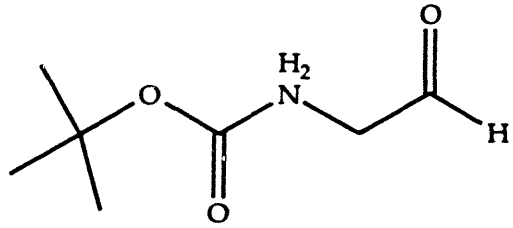
3-amino-1,2-propanediol

A



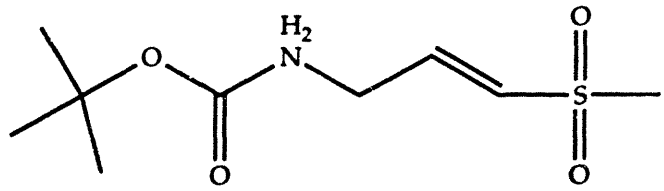
Boc-3-amino-1,2-propanediol

B



Boc-glycinal

C



Boc-Gly-vinyl sulfone

concentrated to an oil by rotary evaporation. The crude oil was used in subsequent steps without further purification.

b. Synthesis of Boc-glycinal (Boc-Gly-H)

Boc-3-amino-1,2-propane diol (3.8g , ~ 20 mmol) was dissolved as a crude oil in 30 ml of ethanol. Sodium periodate ($NaIO_4$; 12.8g, 60 mmol) was added as a solution in 90 ml of 0.01M phosphate buffer (pH 7). The reaction was then stirred for 1.5 hours. The reaction mix was washed with three 50 ml portions of chloroform with the addition of 50 ml of saturate sodium chloride (to separate organic and aqueous phases). The organic phases were then combined, dried over $MgSO_4$, and evaporated to dryness. The crude Boc-glycinal was used in subsequent steps without further purification.

c. Synthesis of Boc-glycine vinyl sulfone, cis and trans isomers (Boc-G-cVS and Boc-G-tVS)

The cis and trans isomers of Boc-glycine vinyl sulfone were synthesized from the crude Boc-glycinal as described for the synthesis of Boc-leucine vinyl sulfone from Boc-leucinal (chapter 2). This reaction resulted in two compounds which were separable by flash chromatography using hexane: ethyl acetate (2:1) as the mobile phase. Mass spectrum obtained for the two tri-peptides isolated were identical (NP-LLG-tVS and NP-LLGcVS; see below) indicating that they were likely to be isomers. Since enantiomers are not likely to be resolved by column chromatography, we concluded that the lack of a sidechain on glycine allowed for the formation of both the cis and trans isomers of the newly formed double bond. NMR analysis (shown below) confirmed this hypothesis. The two compounds had nearly identical spectra with the largest difference observed for the vinyl resonances. The alpha carbon was also found to be slightly different for the two isomers. These data were consistent with cis/trans geometric isomers. Finally the cis and trans isomers were assigned using Z-factor calculations (Pascual et al., 1966) to predict the chemical shift of the vinyl protons for the cis and trans isomers. The greatest difference in shifts between the two vinyl resonances was found for the cis isomer and was reflected in the NMR obtained

for the slightly less polar compound isolated. Yield (2:1 trans:cis, 43 % overall yield).

Boc-glycine vinyl sulfone cis isomer ^{1}H-NMR (300 MHz, $CDCl_3$) δ 6.45 (1H, dd), 6.32 (1H, d), 4.98 (1H, m), 4.28 (2H, t), 3.04 (3H, s), 1.45 (9H, s).

Boc-glycine vinyl sulfone trans isomer ^{1}H-NMR (300 MHz, $CDCl_3$) δ 6.94 (1H, dt), 6.52 (1H, dt), 4.78 (1H, m), 4.00 (2H, t), 2.96 (3H, s), 1.45 (9H, s).

d. Synthesis of glycine-vinyl sulfone tosic acid salt cis and trans isomers (G-cVS, G-tVS)

The Boc protecting group was removed from the glycine vinyl sulfones as described for the removal of Boc from Boc-leucine vinyl sulfone described in chapter 2. Yield (97%)

Glycine vinyl sulfone cis isomer ^{1}H-NMR (300 MHz, CD_3OD) δ 7.72 (2H, d), 7.24 (2H, d), 6.78 (1H, dt), 6.43 (1H, dt), 4.21 (2H, d), 3.08 (3H, s), 2.38 (3H, s).

Glycine vinyl sulfone trans isomer ^{1}H-NMR (300 MHz, CD_3OD) δ 7.72 (2H, d), 7.24 (2H, d), 6.95 (1H, d), 6.87 (1H, dt), 3.84 (2H, d), 3.02 (3H, s), 2.38 (3H, s).

e. Synthesis of Boc-Leucinyl-leucinyl-glycine vinyl sulfone cis and trans isomers (Boc-LLG-cVS, Boc-LLG-tVS)

Boc-LLG-VS cis and trans isomers were synthesized from the tosic acid salts of cis and trans glycine vinyl sulfone as described for the synthesis of Boc-L_3-VS (chapter 2). The compounds were purified by flash column chromatography using ethyl acetate as the mobile phase. Yield (95% cis, 97% trans).

Boc-LLG-cVS ^{1}H-NMR (300 MHz, $CDCl_3$) δ 7.52 (1H, m), 6.88 (1H, dq), 6.63 (1H, t), 6.54 (1H, d), 5.09 (1H, dd), 4.44 (1H, m), 3.94-4.09 (2H, m), 2.93 (3H, s), 1.40-1.82 (6H, m), 1.42 (9H, d), 0.95 (12H, m).

Boc-LLGtVS ^{1}H-NMR (300 MHz, $CDCl_3$) δ 7.52 (1H, m), 6.56 (1H, d), 6.30 (2H, m), 5.05 (1H, dd), 4.47 (1H, m), 4.34 (1H, t), 4.03 (1H, m), 3.06 (3H, s), 1.40-1.82 (6H, m), 1.42 (9H, d), 0.95 (12H, m).

f. Synthesis of leucinyl-leucinyl-glycine vinyl sulfone tosic acid salt cis and trans isomers (LLG-cVS, LLG-tVS)

The Boc protecting group was removed as described for the removal of Boc from Boc-leucine-vinyl sulfone in chapter 2. Yield (52% trans isomer; 45% cis isomer).

g. Synthesis of 4-hydroxy-3-nitrophenyl acetyl-leucinyl-leucinyl-glycine vinyl sulfone cis and trans isomers (NP-LLG-cVS, NP-LLG-tVS)

The hapten 4-hydroxy-3-nitrophenyl acetic acid was added to LLG-VS cis and trans isomers as described for the synthesis of NP-L_3-VS (chapter 2). the cis and trans products were purified by flash column chromatography using ethyl acetate as the mobile phase.

NP-LLG-cVS ^{1}H-NMR (300 MHz, DMSO-d_6) δ 8.29 (1H, d), 8.26 (1H, t), 7.96 (1H, d), 7.80 (1H, s), 7.44 (1H, d), 7.05 (1H, d), 6.53 (1H, dt), 6.18 (1H, dt), 4.13-4.36 (4H, m), 3.47 (2H, s), 3.10 (3H, s), 1.48-1.62 (6H, m), 0.75-0.90 (12H, m).

FAB Mass Spectrum: [M+H] Calculated for $C_{24}H_{36}N_4O_8S$: 541.6 Found: 541.3.

NP-LLG-tVS ^{1}H-NMR (300 MHz, DMSO-d_6) δ 8.29 (1H, d), 8.22 (1H, t), 7.79 (1H, s), 7.42 (1H, d), 7.05 (1H, d), 6.70 (1H, dt), 6.64 (1H, d), 4.28 (2H, m), 3.94 (1H, t), 3.89 (1H, t), 3.46 (2H, d), 2.98 (3H, s), 1.48-1.62 (6H, m), 0.75-0.90 (12H, m).

FAB Mass Spectrum: [M+H] Calculated for $C_{24}H_{36}N_4O_8S$: 541.6 Found: 541.3.

5. Synthesis of Phenolic Vinyl Sulfones

The phenolic vinyl sulfones were synthesized using the same protocol as described for the methyl vinyl sulfones except the phenolic version of the corresponding methyl phosphonate sulfone was used. Figure 3.2 shows the synthesis of a typical phenolic vinyl sulfone.

a. Synthesis of 3-Hydroxy-thiophenyl-methyl-diethylphosponate ($(EtO)_2P(O)CH_2S$-Ph-OH)

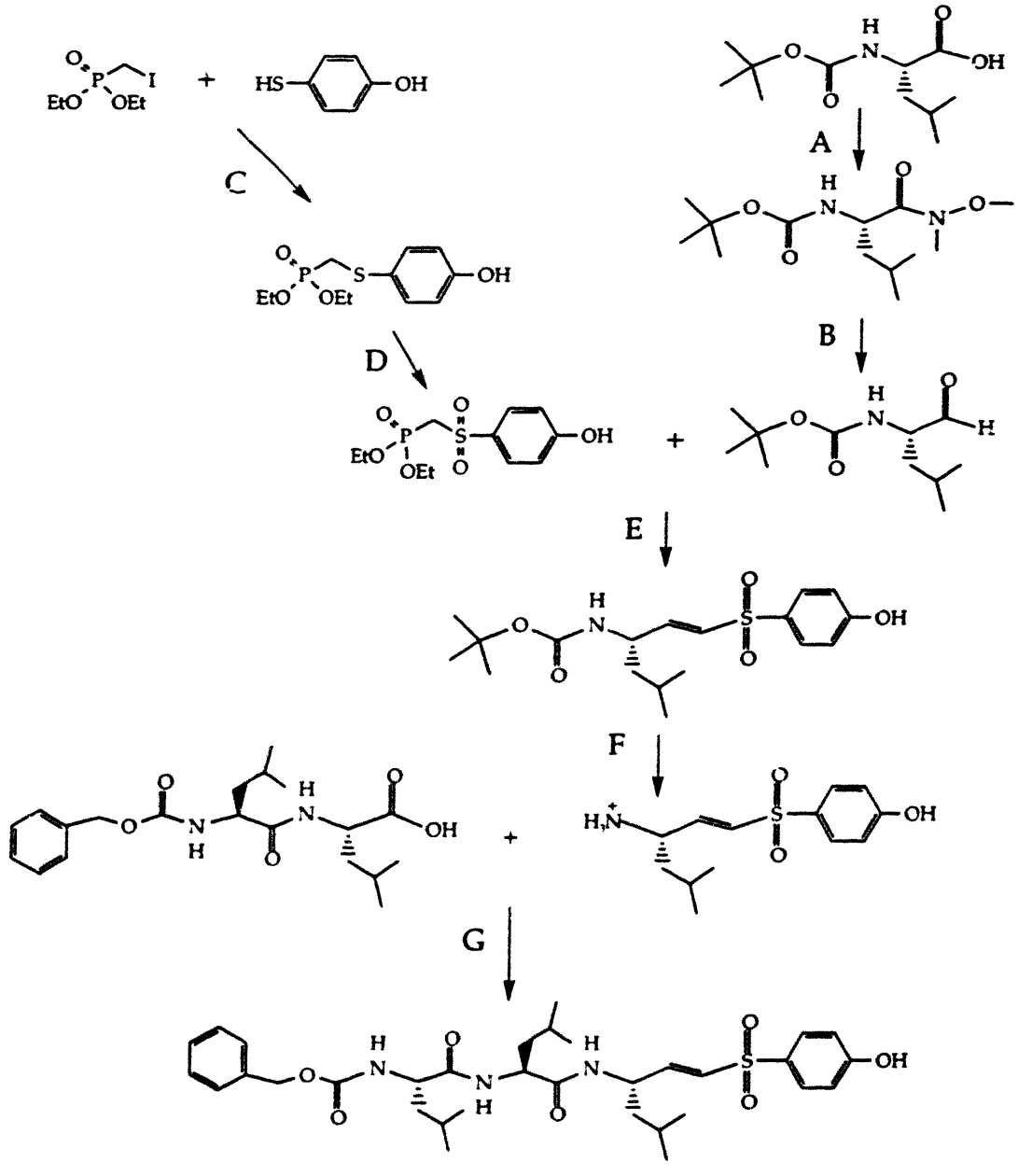
To a dried flask flushed with argon was added 4-hydroxy-thiophenol (90% stock, 4.44g, 31.7 mmol) in 20ml of diethyl ether. Sodium hydride (1.27 g, 31.7 mmol) was then slowly added and the reaction stirred at room temperature for 30 min. A solid mass formed upon standing. Diethyl iodomethyl phosphonate (8.11 g, 29.17 mmol) was then added as a solution in 15 ml of diethyl ether. The reaction was then stirred at room temperature for three hours. The reaction was quenched with 60 ml of 0.5 M citrate buffer (pH 4.0) and the resulting aqueous phase was extracted with three 40 ml volumes of diethyl ether. The combined organic layers were dried over $MgSO_4$ and concentrated to an oil. The crude oil was oxidized as described below without further purification.

b. Synthesis of 3-Hydroxy-thiophenyl-methyl-diethylphosponate sulfone ($(EtO)_2P(O)CH_2S(O)_2$-Ph-OH)

The crude oil of 3-Hydroxy-thiophenyl-methyl-diethylphosponate was dissolved in 30 ml of 1,4-dioxane and chilled on ice. Peracetic acid (30 ml, 32 % solution, 145 mmol) was then slowly added while stirring the reaction mixture. The reaction was maintained on ice for 20 minutes and then warmed to room temperature for 4 hours. Crushed platinum on carbon (2.8 g) was then added and the reaction stirred overnight at room temperature. Potassium iodide was used to insure that no peracetic acid remained. The platinum carbon solids were then removed by filtration and the remaining solution diluted with 250 ml of saturated sodium bicarbonate. The aqueous phase was extracted with 3X100 ml portions of ethyl acetate. The combined organic layers were then dried over

Figure 3.2. Synthesis of the phenolic peptide vinyl sulfone.

(A) PyBOP, DIEA, CH_3NHOCH_3, CH_2Cl_2; (B) LAH, Et_2O; (C) NaH, Et_2O; (D) $CH_3C(O)OOH$, 1,2-dioxane; (E) NaH, THF; (F) TsOH, Et_2O; (G) PyBOP, DIEA, CH_2Cl_2.



Z-L3-VS-PhOH

$MgSO_4$ and dried to a crude solid by rotary evaporation. The pure product was obtained by re-crystallization from hexane/ethyl acetate (1:2). Yield (7.1 g, 23.4 mmol, 80%).

4-hydroxy-thiophenyl-methyl diethylphosponate sulfone ^{1}H-NMR (300 MHz, $CDCl_3$) δ 9.25 (1H, s), 7.80 (2H, d), 6.75 (2H, d), 4.22 (4H, p), 3.81 (2H, d), 1.36 (6H, t).

c. Synthesis of Leucinyl-leucinyl-leucine-vinyl sulfone phenol tosic acid salt (L_3-VS-PhOH)

L_3-VS was synthesized as described in chapter 2 materials and methods section for the methyl vinyl sulfone counterpart (compound VIII) except that for the conversion of Boc-leucinal to Boc-leucine vinyl sulfone 4-hydroxy-thiophenyl-methyl diethylphosponate sulfone was used instead of methyl-thiomethyl diethylphosponate sulfone. The yields obtained in a typical reaction were similar to those reported for the methyl derivative.

L_3-VS-PhOH ^{1}H-NMR (300 MHz, CD_3OD) δ 7.68 (2H, d), 6.95 (2H, d), 6.78 (1H, dd), 6.45 (1H, d), 4.61 (1H, m), 4.42 (1H, t), 3.85 (1H, t), 1.40-1.78 (9H, m), 0.82-1.02 (18H, m).

FAB Mass Spectrum: [M+H] calculated for $C_{25}H_{41}N_3O_5S$: 496.6 Found: 496.3

d. Synthesis of 4-hydroxy-5-iodo-3-Nitrophenyl acetyl-leucinyl-leucinyl-leucine vinyl sulfone phenol (NIP-L_3-VS-PhOH)

NIP-L_3-VS-PhOH (IV) was synthesized by addition of 4-hydroxy-5-iodo-3-nitrophenyl acetic acid to L_3-VS-PhOH as described for the synthesis of NIP-L_3-VS in chapter 2. Similar yields were obtained.

NIP-L_3-VS-PhOH ^{1}H-NMR (300 MHz, DMSO-D_6) δ 8.30 (1H, d), 8.06 (2H, d), 8.00 (1H, s), 7.89 (1H, s), 7.61 (2H, d), 6.94 (2H, d), 6.60 (1H, dd), 6.50 (1H, d), 4.52 (1H, m), 4.22 (2H, m), 1.28-1.65 (9H, m), 0.73-0.98 (18H, m).

FAB Mass Spectrum: [M+H] Calculated for $C_{33}H_{45}N_4O_9SI$:801.7 Found: 801.4

e. Synthesis of Boc-leucinyl-leucinyl-leucinyl-leucine vinyl sulfone phenol (Boc-L_4-VS-PhOH)

Boc-L_4-VS-PhOH was synthesized from L_3-VS-PhOH (III) by the addition of Boc-leucine as described for the synthesis of Boc-YL_3-VS described above except that Boc-leucine was used instead of Boc-tyrosine and L_3-VS-PhOH was used instead of L_3-VS. Similar yields were obtained.

Boc-L_4-VS-PhOH ^{1}H-NMR (300 MHz, Acetone-d_6) δ 9.52 (1H, s), 7.72 (1h, d), 7.68 (2H, d), 7.39 (1H, d), 7.28 (1H, d), 6.98 (2H, d), 6.83 (1H, dd), 6.70 (1H, d), 4.71 (1H, m), 4.44 (1H, m), 4.15 (1H, m), 3.77 (1H, m), 1.30-1.89 (11H, m), 1.45 (9H, s), 0.74-0.97 (24H, m).

f. Synthesis of leucinyl-leucinyl-leucinyl-leucine vinyl sulfone phenol tosic acid salt (L_4-VS)

The Boc group was removed from Boc-L_4-VS-PhOH by treatment with trifluoroacetic acid as described above for the synthesis of YL_3-VS. Yield (98%).

L_4-VS-PhOH ^{1}H-NMR (300 MHz, CD_3OD) δ 7.72 (2H, d), 6.90 (2H, d), 6.75 (1H, dd), 6.65 (1H, d), 4.60 (1H, m), 4.44 (1H, t), 4.30 (1H, q), 3.85 (1H, m), 1.38-1.74 (12H, m), 0.80-1.00 (18H, m).

FAB Mass Spectrum: [M+H] Calculated for $C_{31}H_{52}N_4O_6S$: 609.8 Found: 609.3

6. Cells and Cell Culture

The mouse lyphoblastoid cell line EL-4 was maintained in Dubecco's Modified Eagle's Medium supplemented with 10% fetal calf serum. The human cell HOM-2 was maintained as described in chapter 2 Materials and Methods section.

7. Antibodies

The polyclonal rabbit anti-proteasome antibody was a kind gift of Dr. Migel Cruez and Dr. John Monaco (University of Cincinnati, Cincinnati, OH). The serum was prepared using purified 20S proteasomes as the immunogen.

8. Fluorogenic Substrate Hydrolysis Assay

Hydrolysis of the fluorogenic substrates: Suc-LLVY-MCA (chymotrypsin-like substrate), Boc-LRR-MCA (trypsin-like substrate), and Cbz-LLE-βNA (PGPH substrate) was measured. In a typical assay 5 μg of protein generated from the 5H 100,000 g centrifugation spin of lysates from the mouse lymphoblastoma cell line EL-4 (see below for preparation) was diluted to a final volume of 97μl. Inhibitors (1 μl of DMSO stocks) were added to final concentrations of 0, 1, 5, 10, 50, and 100 μM. Samples were pre-incubated for 45 min. at room temperature. Substrates were then added to a final concentration of 100 μM (by addition of 2 μl of a 5 mM DMSO stock). The hydrolysis reaction was allowed to proceed for 45 min. at 37°C. The reaction was quenched by addition of 1 ml of 1% SDS. Fluorescence was measured by excitation of samples at 380 nM (for MCA substrates) or 336 (for βNA substrates) and measurement of emission at 415 nM. IC_{50} values were calculated from plots of V_i/V_o vs. inhibitor concentration (where V_i is the fluorescence observed after inhibition and V_o is the amount of fluorescence observed for samples in which DMSO alone was added).

9. Partial Purification of Proteasomes from EL-4 Cells by Differential Centrifugation

Proteasomes were enriched by centrifugation of lysates generated from the mouse lymphoblastoma cells line EL-4 (for a description of preparation of lysates see the material and methods in chapter 2). Post nuclear supernatants were centrifuged at 100,000 g for 1 hour to remove microsomes and other cellular organelles. The supernatant from this centrifugation is called cytosoland was

centrifuged a second time at 100,000 g for 5 hours to pellet the proteasome. This pellet was re-suspended in homogenization buffer (50 mM Tris pH 7.4, 1 mM DTT, 5 mM MgCl2, 2 mM ATP, 20% glycerol). The 5H 100,000 g pellet is considered to be highly enriched for the proteasome and is referred to throughout this thesis as the 5H pellet. Protein concentration was determined using the BCA assay (Pharmacia, Uppsala Sweden) according to the manufacturer's instructions.

10. Labeling of Proteasomes with Peptide Vinyl Sulfones

Stock solutions of ^{125}I-labeled inhibitors were quantitated on a gamma counter so that equivalent amounts of radioactivity were used for all labeling experiments. It should be noted, however, that all inhibitors may not have been modified to the same extent during the iodination step described in chapter 2 and therefore, it is difficult to determine the amount of unlabeled inhibitor present in a given labeling stock. Since any unlabeled inhibitor may serve to compete for the proteasome active site and thus, decrease labeling, these reagents can not be used directly as quantitative measures of inhibitory activity. In all labeling experiments, the labeled inhibitors were added to a final concentration of 1.8 X 10^4 Bq/ml. All samples were labeled for 2 hours at 37°C unless otherwise noted. Samples were quenched by dilution of 4X SDS sample buffer to 1X (for 1D SDS-PAGE) or by dissolving urea to a final concentration of 9.5 M (for 2D SDS-PAGE).

11. Competition Experiments

Partially purified proteasome preparations (20 µg total protein in 100 µl homogenization buffer; see above) were incubated 0, 1, 5 and 10 µM of GL_3-VS or YL_3-VS for 45 min at room temperature. ^{125}I-YL_3-VS (1.8 x 10^4 Bq/ml) was then added and the samples incubated for 2 hours at 37°C. The labeling reaction was quenched by the addition of 4x SDS-sample buffer to a final concentration of 1x. Samples were then separated on a 12.5 % SDS-PAGE gel.

12. Gel Electrophoresis

One-dimensional SDS-PAGE, two dimensional, non-equilibrium SDS-PAGE, and fluorography were performed as described in chapter 2.

13. Immunoprecipitation

All immunoprecipitations were performed as described in chapter 2.

C. Results

1. Synthesis of Peptide Vinyl Sulfones

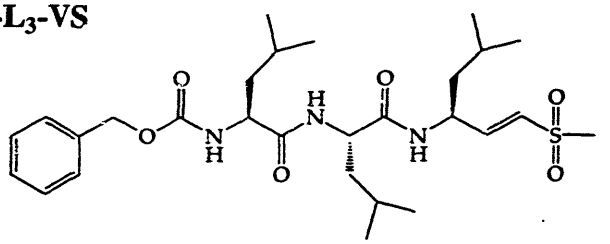
A series of tri and tetra-peptide methyl vinyl sulfones were synthesized based on the core sequence LLL, previously described as a potent inhibitor of the proteasome (chapter 2). The structures of several typical methyl vinyl sulfones are shown in figure 3.3. In addition to the methyl vinyl sulfones, we also synthesized a related derivative in which the methyl group adjacent to the sulfone moiety was replaced with a phenolic group (these vinyl sulfones will be referred to as phenolic vinyl sulfones while the methyl vinyl sulfones will be simply called vinyl sulfones). The structure of the phenolic vinyl sulfone equivalent of the previously described vinyl sulfone Z-L_3VS (Z-L_3-VSphOH) and NIP-L_3-VS (NIP-L_3-VS-PhOH) are shown in figure 3.3. This modification allows for the examination of the effect of steric bulk in the position likely to occupy a P-1 binding site (C-terminal to the site of hydrolysis). The P notation used to identify specific residue positions in a substrate or inhibitor relative to the site of hydrolysis is shown in figure 3.4. In addition, the phenolic group allowed for attachment of ^{125}I at the C-terminal end of the peptides and the presence of an iodinatable N-terminal cap was no longer required for labeling experiments.

The tri-peptide vinyl sulfone containing the L_3 core and possessing a free amino terminus (figure 3.5) was extended by addition of a fourth amino acid at the P4 position. To examine the effects of the P4 amino acid on binding we synthesized the sequences GLLL, YLLL, YAAF and LLLL as the corresponding peptide vinyl sulfones. To examine whether the binding of the P4 amino acid was sensitive to chiral geometry, the D-isomer of tyrosine was also added at P4. We introduced the amino acid Benzoyl-phenylalanine (Bpa) in the P4 position (Bpa-L_3-VS) to examine whether a bulky un-natural sidechain group could be accommodated by a secondary binding site. The Bpa group also offered the possibility for its use as a cross-liking reagent to examine interactions between P4 and the proteasome. The structures of the tetra-peptides vinyl sulfones are shown in figure 3.6.

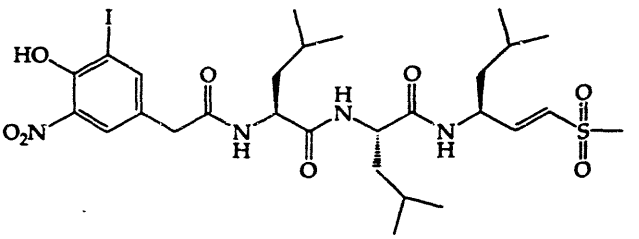
Figure 3.3. Structures of the methyl and phenolic N-terminally modified vinyl sulfones.

Methyl Vinyl Sulfones

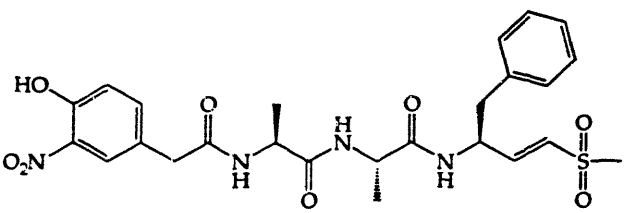
Z-L_3-VS



NIP-L_3-VS

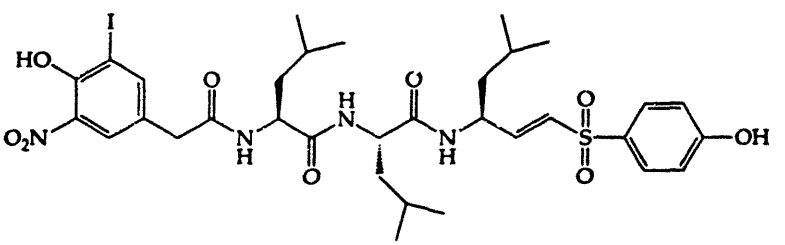


NP-AAF-VS



Phenolic Vinyl Sulfones

NIP-L_3-VS-PhOH



Z-L_3-VS-PhOH

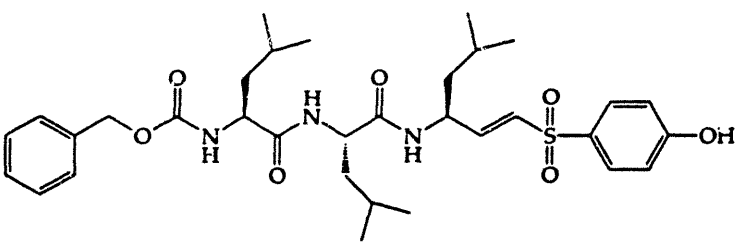


Figure 3.4. The P-notation used to identify amino acid positions relative to the site of hydrolysis.

The structures of a potential substrate as well as a tetra peptide inhibitor are shown.

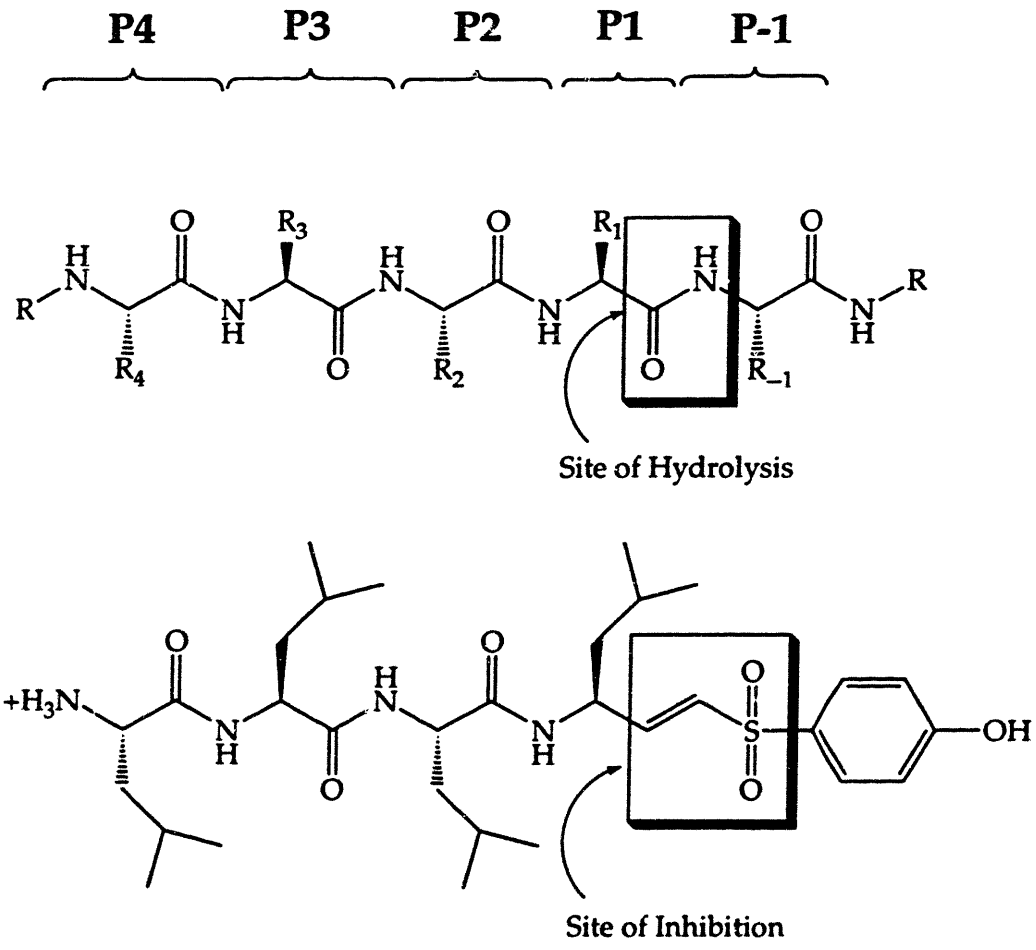
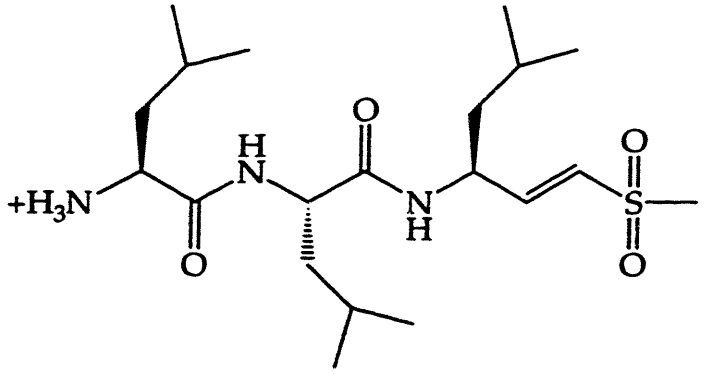
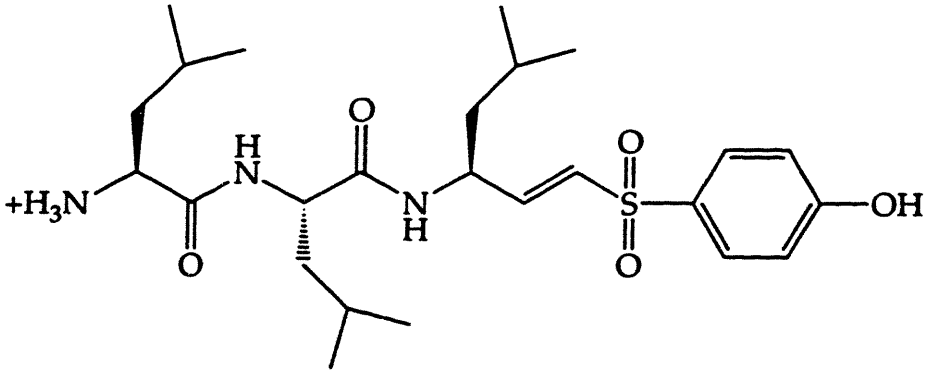

P4
P3
P2
P1
P-1
Site of Hydrolysis
Site of Inhibition
OH

Figure 3.5. Structures of the free-amino tri-peptide vinyl sulfones.

L_3-VS



L_3-VS-PhOH



AAF-VS

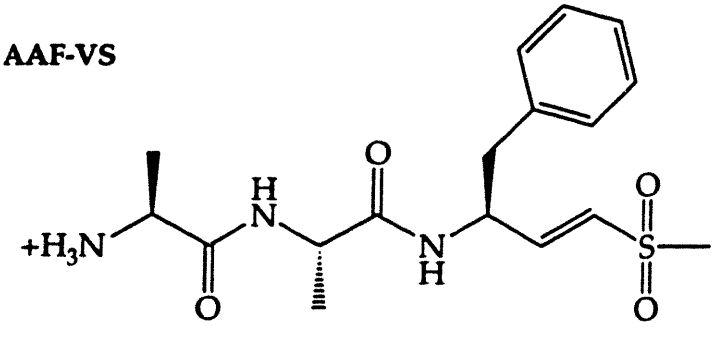
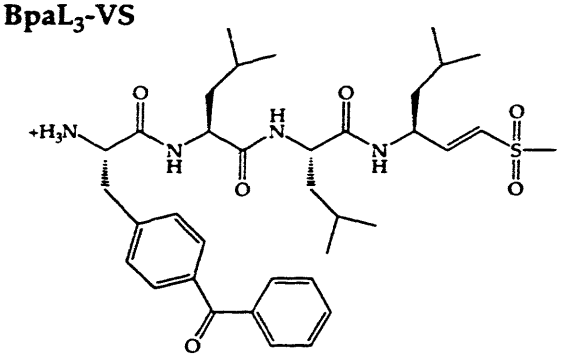
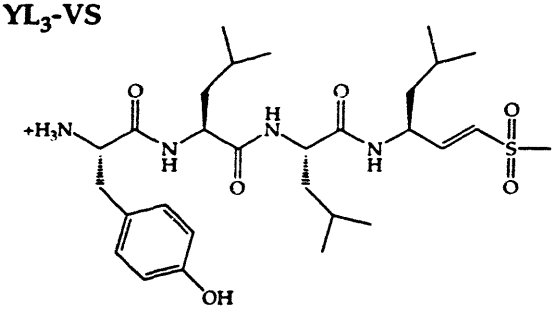


Figure 3.6. Structures of the free-amino tetra-peptide vinyl sulfones.

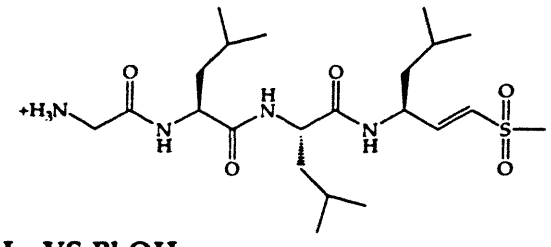
BpaL$_3$-VS



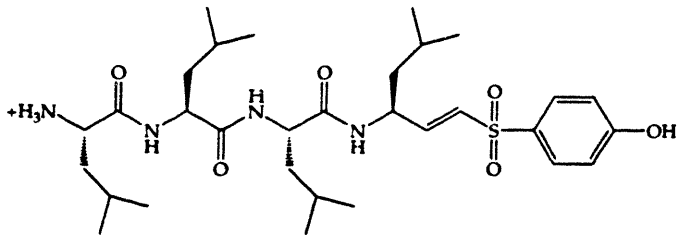
YL$_3$-VS



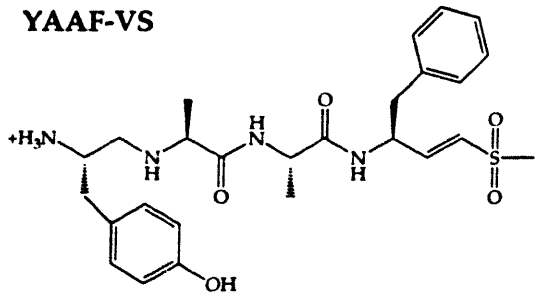
GL$_3$-VS



L$_4$-VS-PhOH



YAAF-VS



Finally, several other peptide vinyl sulfones were synthesized consisting of the sequences LLG and AAF. Interestingly, the lack of a sidechain at the P1 position in the LLG peptide resulted in the generation of a mixture of cis and trans geometric isomers of the vinyl sulfone group which could be separated by chromatography (figure 3.7). These isomers showed drastic differences in reactivity and will be discussed later in this chapter.

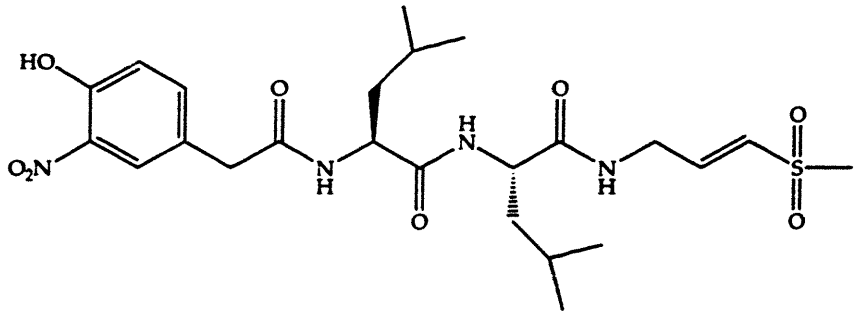
2. P4 amino acid influences inhibition of fluorogenic substrate hydrolysis

We examined the effects of the tetra-peptide vinyl sulfones on the ability of the proteasome to hydrolyze fluorogenic substrates. Hydrolysis of the proteasome substrates; Cbz-LLE-βNA (a substrate for the post-glutamyl peptidase activity), Boc-LRR-MCA (a substrate for the trypsin-like activity), and Suc-LLVY-MCA (a substrate for the chymotrypsin -like activity) was measured. As reported in chapter 2, the compounds Z-L_3-VS and NIP-L_3-VS potently inhibited multiple proteasome activities. However, the omission of the N-terminal carboxylbenzyl or nitro-iodo phenol yielded the free-amino compound which was much less inhibitory. Hydrolysis of the chymotrypsin-like substrate was dramatically reduced, with only 50% inhibition of activity at 100 μM (figure 3.8). Similar results were obtained for the LLE and LRR substrates. However, this loss in activity seen for the free-amino terminus was overcome by addition of a fourth amino acid. The free amino terminus-containing tetra-peptides YLLL and BpaLLL and LLLL displayed inhibition kinetics similar to or better than those observed for the amino-capped tri-peptide NLVS (Figure 3.9). Of the three proteolytic activities examined using fluorogenic substrates, the trypsin-like activity was most dramatically affected by the addition of an aromatic, aliphatic, or bulky P4 amino acid. This activity was blocked by all the tetra-peptide vinyl sulfones better than that observed for any of the tri-peptides examined, including NLVS .

The related compound in which the P4 tyrosine was replaced with the D-isomer ((D)YL_3-VS) was less active than the L-isomer equivalent but showed increased reactivity compared to free amino LLL-VS (figure 3.10). Interestingly, the inhibitory capacity of YL_3-VS against the trypsin-like activity of the proteasome was most affected by the change from D to L isomer (20-fold

Figure 3.7. Structures of the cis and trans isomers of NP-LLG-VS.

NP-LLG-tVS - Trans Isomer



NP-LLG-cVS - Cis Isomer

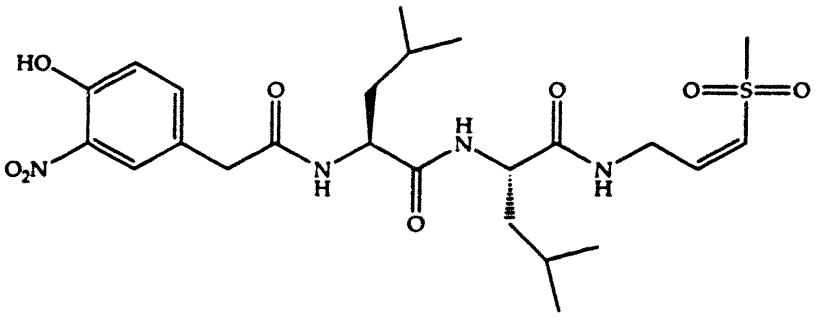


Figure 3.8. The free-amino tri peptide L_3-VS is a poor inhibitor of trypsin-like, chymotrypsin-like and PGPH activities of the proteasome when compared to NIP-L_3-VS and Z-L_3-VS.

(A) Partially purified proteasome preparations from EL-4 cells (see Material and Methods for preparation) were incubated with increasing concentrations of L_3-VS, NIP-L_3-VS, and Z-L_3-VS from 0-100 μM. Hydrolysis of the three fluorogenic proteasome substrates; Suc-LLVY-MCA, Z-LLE-βNA, and Boc-LRR-MCA was then measured. Plots are of V_i/V_0 versus inhibitor concentration where V_i is the inhibition observed at a given inhibitor concentration (i) and V_0 is the fluorescence observed for no addition of inhibitor (indicated as % residual activity).
(B) IC_{50} obtained from extrapolation of concentration where % residual activity equals 50% on the plots in (A). Values were obtained for all three fluorogenic substrates as indicated.

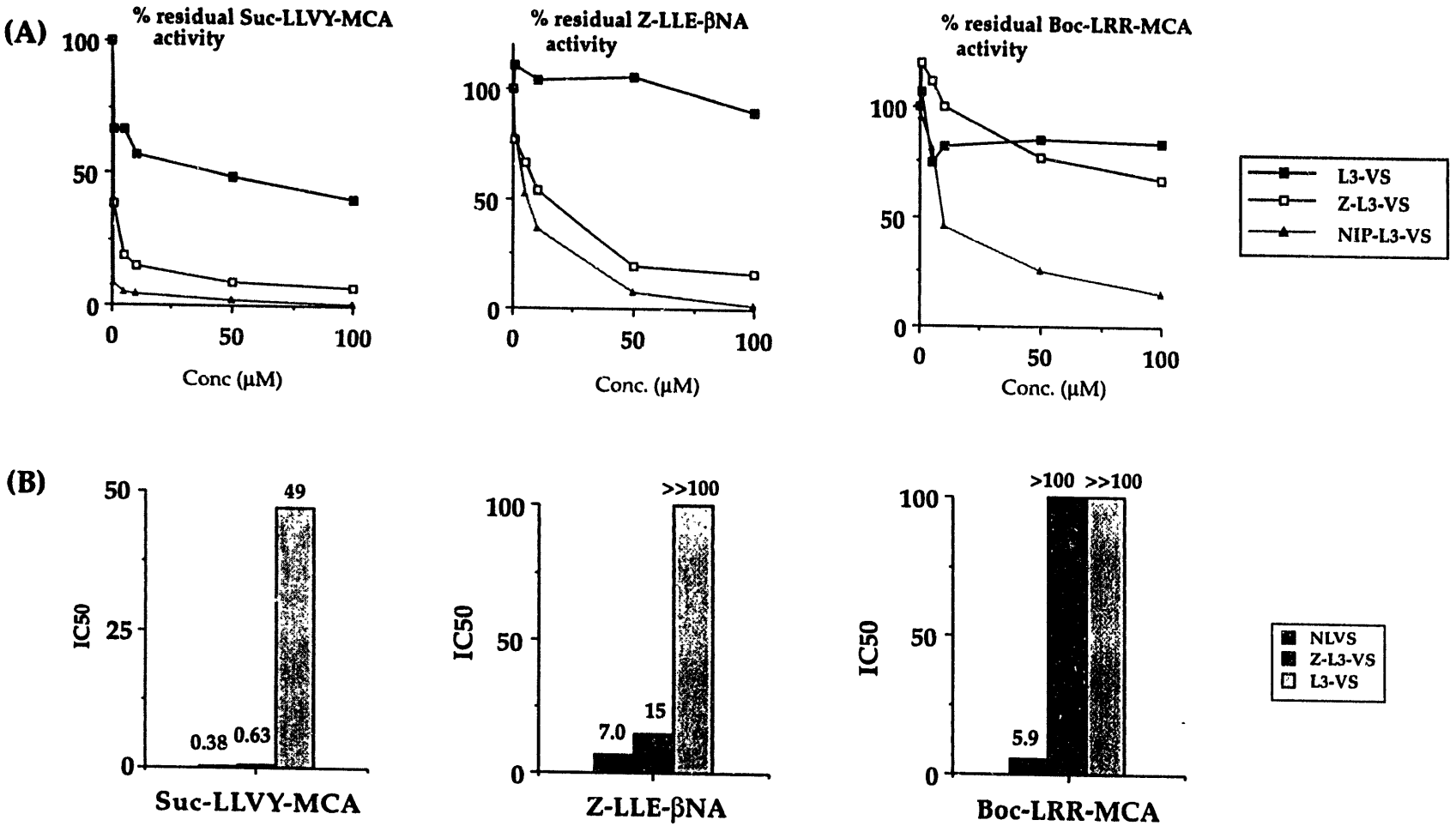
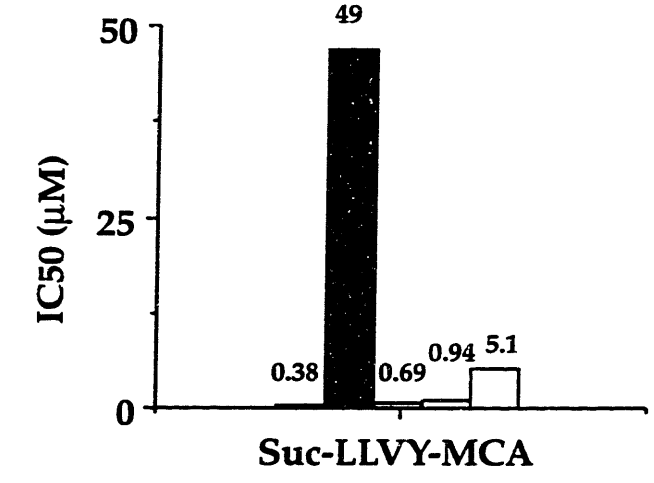
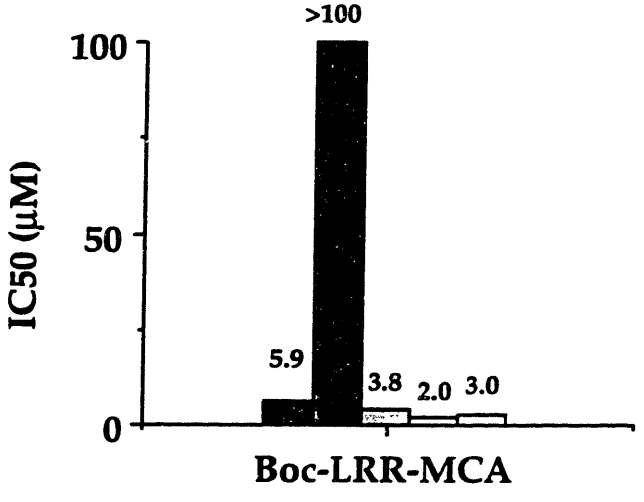
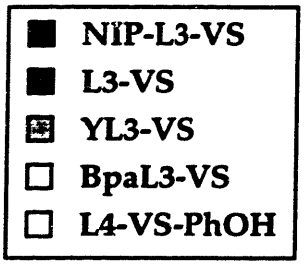

(A)
% residual Suc-LLVY-MCA activity
100
50
0
0
50
100
Conc (μM)
% residual Z-LLE-βNA activity
100
50
0
0
50
100
Conc. (μM)
% residual Boc-LRR-MCA activity
100
50
0
0
50
100
Conc. (μM)
L3-VS
Z-L3-VS
NIP-L3-VS
(B)
IC50
50
25
0
0.38
0.63
49
Suc-LLVY-MCA
IC50
100
50
0
7.0
15
>>100
Z-LLE-βNA
IC50
100
50
0
5.9
>100
>>100
Boc-LRR-MCA
NLVS
Z-L3-VS
L3-VS

Figure 3.9. Addition of an aromatic, aliphatic, or bulky P4 amino acid to the free amino tri-peptide L_3-VS results in potent inhibition of the trypsin-like, chymotrypsin-like and PGPH activities of the proteasome.

Inhibition of hydrolysis of fluorogenic peptide substrates by partially purified proteasome preparations was measured and plotted as described in figure 3.8A. IC_{50} values (generated by extrapolation of the concentration of inhibitor at which the % residual activity was found to be 50%) were obtained for inhibition of the chymotrypsin-like, trypsin-like, and PGPH activities by the tetra-peptide vinyl sulfones: YL_3-VS, $BpaL_3$-VS, and L_4-VS-PhOH compared to NIP-L_3-VS and the corresponding free-amino derivative L_3-VS.


50
25
0
IC50 (μM)
49
0.38
0.69
0.94
5.1
Suc-LLVY-MCA


100
50
0
IC50 (μM)
>100
5.9
3.8
2.0
3.0
Boc-LRR-MCA


NIP-L3-VS
L3-VS
YL3-VS
BpaL3-VS
L4-VS-PhOH

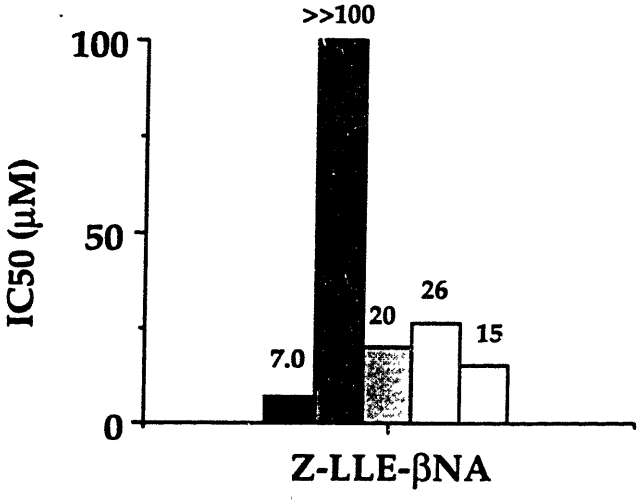
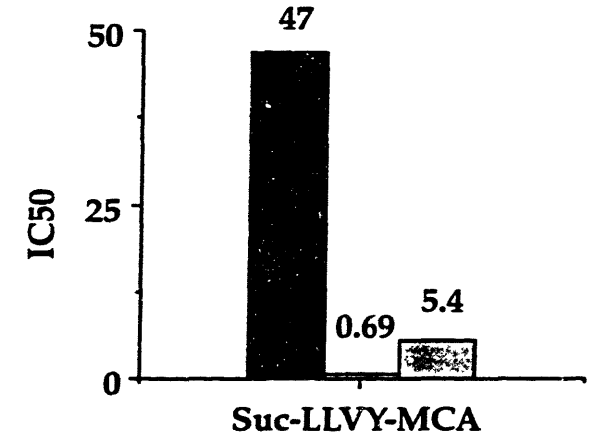
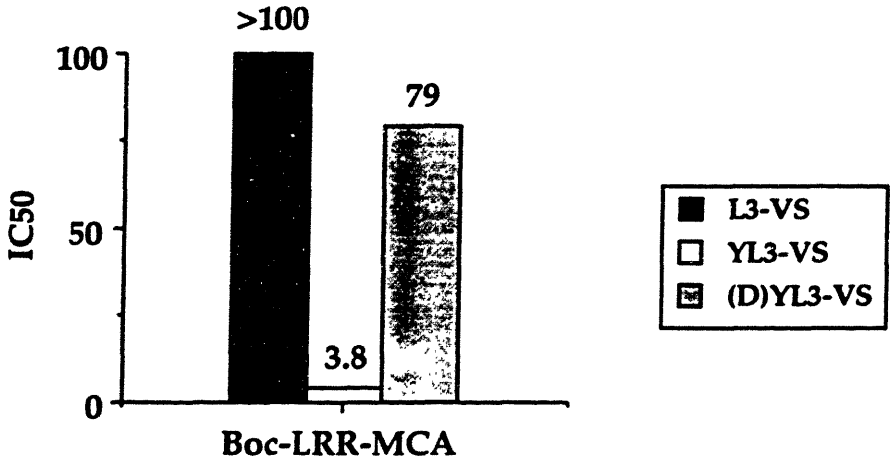
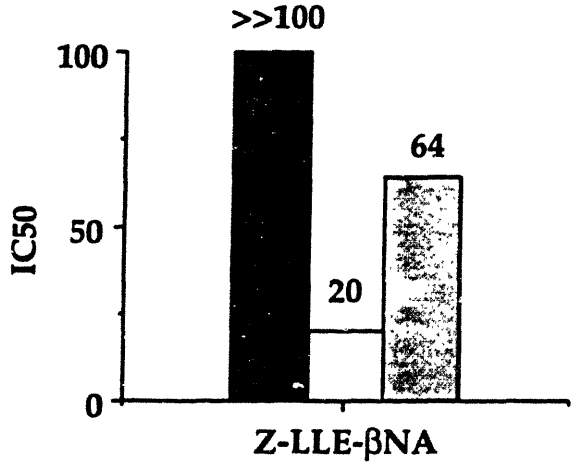

100
50
0
IC50 (μM)
>>100
7.0
20
26
15
Z-LLE-βNA

Figure 3.10. A D-amino acid at the P4 position results in a modest reduction of inhibitory potency of substrate hydrolysis by the proteasome.

Inhibition of hydrolysis of fluorogenic peptide substrates by partially purified proteasome preparations was measured and plotted as in figure 3.8A. IC_{50} values (generated by extrapolation of the concentration of inhibitor at which the % residual activity was found to be 50%) were obtained for inhibition of the chymotrypsin-like, trypsin-like, and PGPH activities by the tetra-peptide YL_3-VS containing the D and L isomers of tyrosine in the P4 position compared to the free amino tri-peptide L_3-VS. Note the relatively modest reduction in activity of the tetra-peptide containing the D-isomer.


47
50
25
IC50
0.69
5.4
0
Suc-LLVY-MCA


>100
100
79
IC50
50
3.8
0
Boc-LRR-MCA
L3-VS
YL3-VS
(D)YL3-VS


>>100
100
64
IC50
50
20
0
Z-LLE-βNA

reduction compared to an 8-fold reduction for the chymotrypsin-like activity and a 2.5-fold reduction for the PGPH activity). Thus, while the binding interaction of the P4 amino acid seems to display some chiral discrimination, it does not exclude binding of a D-isomer at this position.

The tetra-peptide GLLL, unlike the other tetra-peptides studied, has no sidechain at the P4 position. This compound showed dramatically reduced activity against hydrolysis of the Suc-LLVY-MCA and Boc-LRR-MCA substrates when compared to YL_3-VS (figure 3.11). Interestingly, the activity of GL_3-VS against the Z-LLE-βNA was nearly identical to the inhibition observed for YL_3-VS. These data indicate that the active sites for all three proteolytic activities prefer a P4 amino acid, but the requirements for the P4 amino acid sidechain may be different for different active sites.

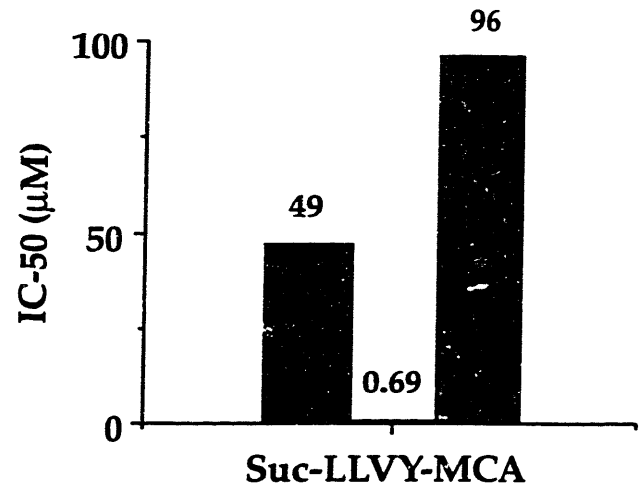
A similar increase in inhibitory activity was observed when tyrosine was placed in the P4 position by addition to the tri-peptide AAF-VS, indicating that P4 binding is not dependent on the sequences at P1-3 but rather is a general phenomenon which leads to increased potency of inhibitors (figure 3.12). Again the greatest enhancement of activity was observed for the trypsin-like activity.

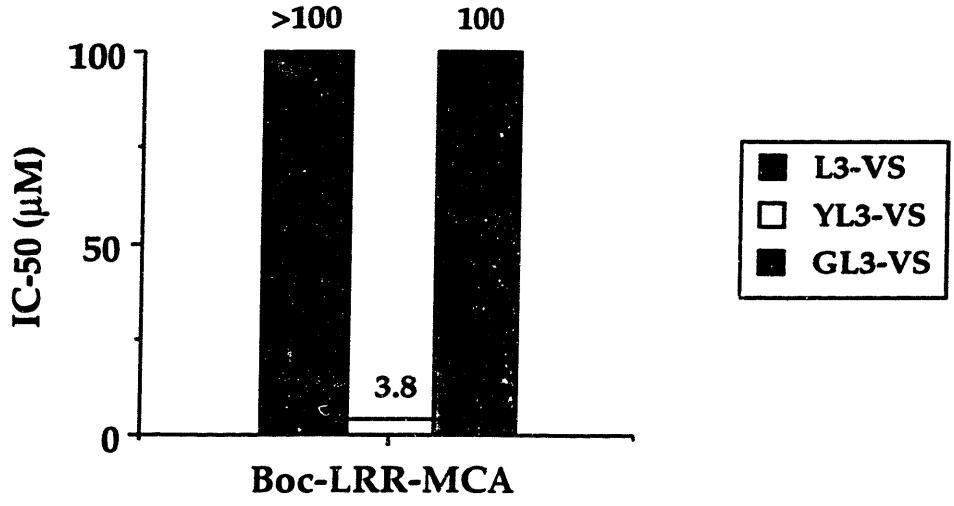
3. Labeling of proteasomes with tetra-peptide vinyl sulfones

The labeling pattern of proteasomes with the tri-peptide ^{125}I-NIP-L_3-VS has been described (chapter 2). This compound covalently labels multiple β-subunits including the IFN-γ inducible LMP-2 and LMP-7 subunits. Analysis of labeling of proteasomes with ^{125}I-NIP-L_3-VS by SDS-PAGE results in three subunits which overlap due to their nearly identical molecular weights but which are easily resolved by 2 dimensional IEF-SDS-PAGE. One-dimensional SDS-PAGE analysis of partially purified mixtures of 20S and 26S proteasomes, labeled with ^{125}I-NIP-L_3VS, ^{125}I-YL_3VS, ^{125}I-YAAF-VS, and L_4-VS-^{125}I-PhOH indicate difference in the relative intensities of individual polypeptides labeled (figure 3.13). ^{125}I- NIP-L_3-VS labels with greatest intensity a band of approximately 23 KDa in all three proteasome samples. This band was shown to be composed of the β-subunits LMP-7, X, and LMP-2 (in mouse the LMP-2 is resolved from LMP-7 and X and can be seen as a slightly faster migrating polypeptide). Two much less intensely labeled polypeptides are observed at around 28 and 30 KDa

Figure 3.11. Glycine in the P4 position increases potency of the free-amino tri-peptide L_3-VS against the PGPH activity but not the trypsin-like or chymotrypsin-like activities of the proteasome.

Inhibition of hydrolysis of fluorogenic peptide substrates by partially purified proteasome preparations was measured and plotted as in figure 3.8A. IC_{50} values (generated by extrapolation of the concentration of inhibitor at which the % residual activity was found to be 50%) were obtained for inhibition of the chymotrypsin-like, trypsin-like, and PGPH activities by the tetra-peptide GL_3-VS compared to the tetra-peptide YL_3-VS and the free amino tri-peptide L_3-VS. Note the loss of reactivity in the GL_3-VS inhibitor against the LLVY and LRR but not the LLE substrate hydrolysis when compared to YL_3-VS.


100
50
0
IC-50 (μM)
49
0.69
96
Suc-LLVY-MCA


100
50
0
IC-50 (μM)
>100
3.8
100
Boc-LRR-MCA
L3-VS
YL3-VS
GL3-VS

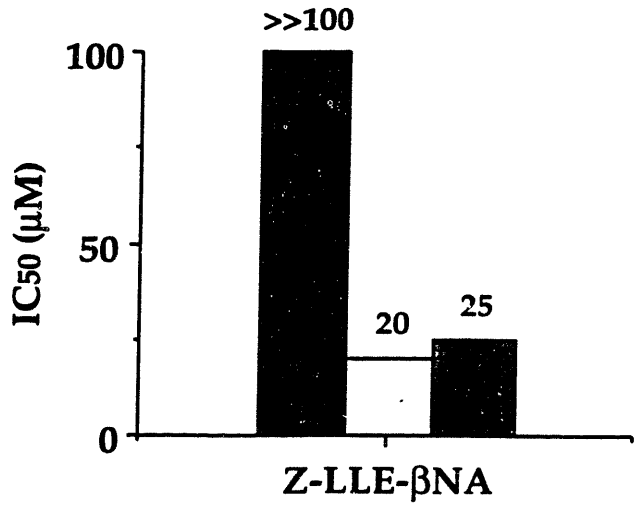

100
50
0
IC50 (μM)
>>100
20
25
Z-LLE-βNA

Figure 3.12. Addition of tyrosine to the free-amino tri-peptide AAF-VS increases potency against the chymotrypsin-like and trypsin-like activities of the proteasome.

Inhibition of hydrolysis of fluorogenic peptide substrates by partially purified proteasome preparations was measured and plotted as in figure 3.8A. IC_{50} values (generated by extrapolation of the concentration of inhibitor at which the % residual activity was found to be 50%) were obtained for inhibition of the chymotrypsin-like, trypsin-like, and PGPH activities by the tetra-peptide YAAF-VS compared to the tetra-peptide AAF-VS. Note the increase in potency against hydrolysis of the LLVY and LLR fluorogenic substrates by addition of the P4 tyrosine.

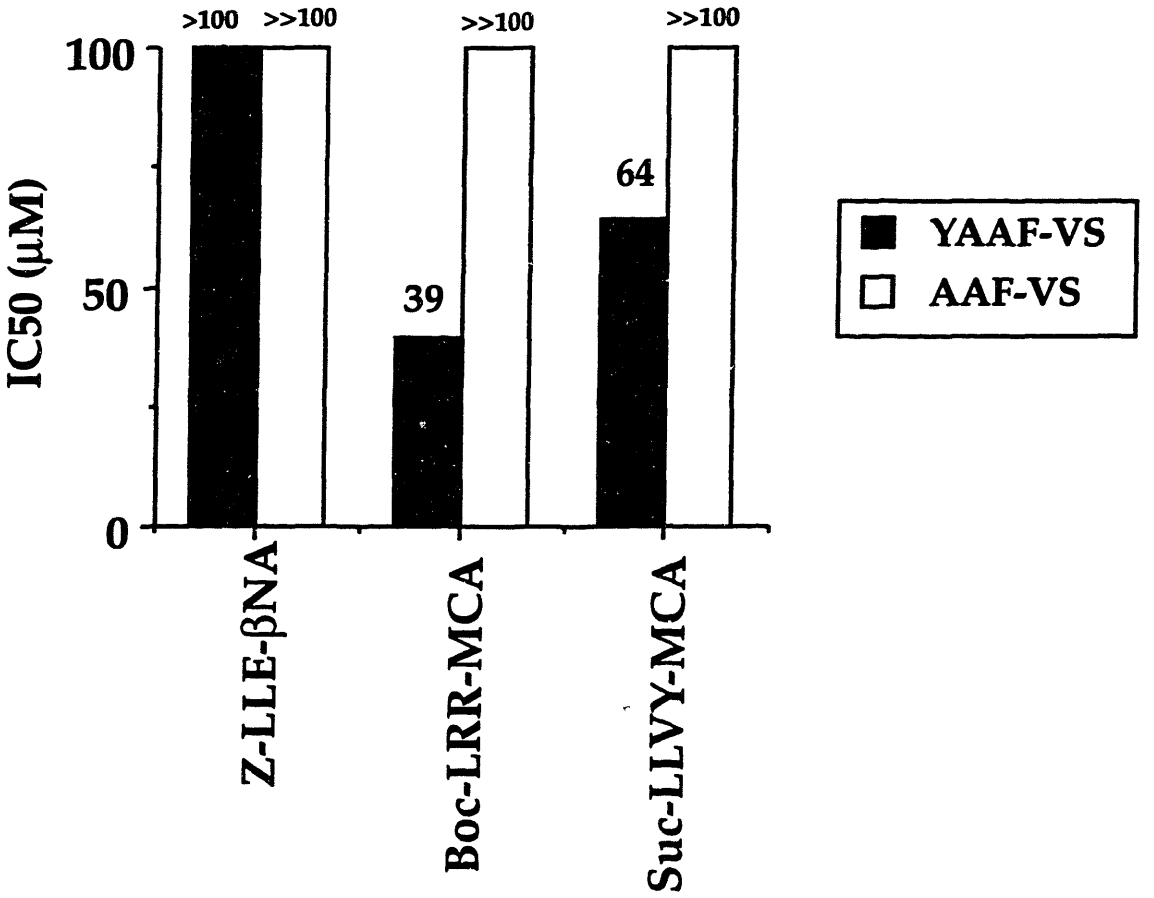
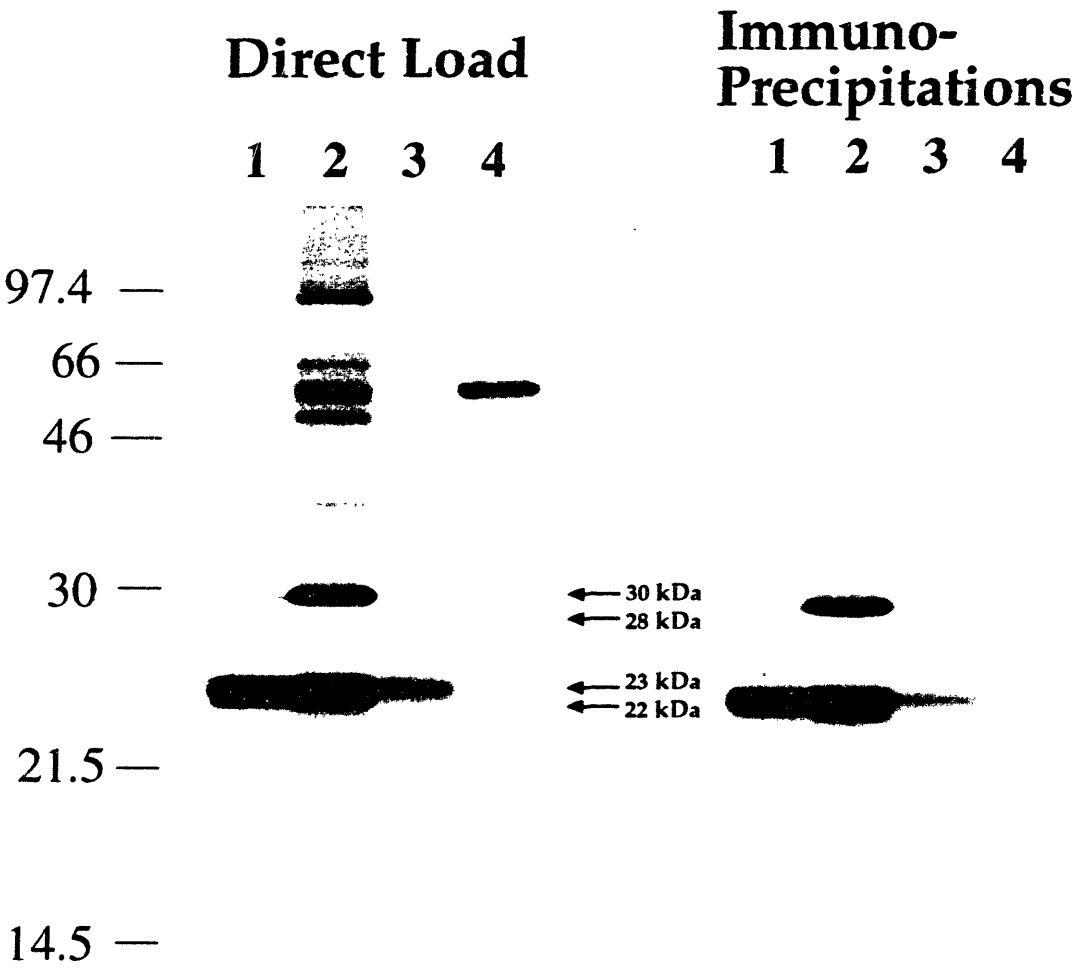

IC50 (μM)
100
50
0
>100
>>100
>>100
>>100
39
64
Z-LLE-βNA
Boc-LRR-MCA
Suc-LLVY-MCA
YAAF-VS
AAF-VS

Figure 3.13. The tetra-peptide vinyl sulfones label proteasomal β-subunits with different relative intensities.

Partially purified proteasome preparations were labeled with the N-terminally modified tri-peptide ^{125}I-NIP-L_3-VS as well as with the tetra-peptide vinyl sulfones; ^{125}I-YL_3-VS, ^{125}I-YAAF-VS, and L_4-VS-^{125}I-PhOH as described in the Material and Methods section. Samples were either separated directly by SDS-PAGE (Direct Load) or were first immunoprecipitated with the rabbit anti-proteasome antibody and then separated by SDS-PAGE (Immunoprecipitations). The relative molecular weights of the proteasomal polypeptides labeled are indicated.



Label: 1. NLVS
2. YL_3VS
3. YAAF-VS
4. L_4-VS-PhOH

and can be seen only upon long exposure of autoradiograms (see arrows). Interestingly, in samples labeled with ^{125}I-YL$_3$-VS the 30KDa polypeptide is labeled with nearly identical intensity to the 23 kDa polypeptides and with much greater intensity when compared to the labeling observed with ^{125}I-NIP-L$_3$-VS. ^{125}I-YAAF-VS, on the other hand, labels nearly exclusively the 23 kDa polypeptides and labels the 30 KDa species only weakly. L$_4$-VS-^{125}I-PhOH, unlike the other tetra-peptides labels with the greatest intensity the smallest polypeptide (likely to be LMP-2 or the Y subunit).This difference in labeling patterns observed for the tri-and tetra-peptides exists in both immunoprecipitated proteasomes as well as in partially purified proteasome preparations, indicating that the labeled polypeptides in question are in fact proteasome subunits and are not due to cross reactivity of the tetra-peptides with other proteolytic enzymes (figure 3.13).

4. GL$_3$-VS competes for binding of the 21 kDa proteasomal β-subunit

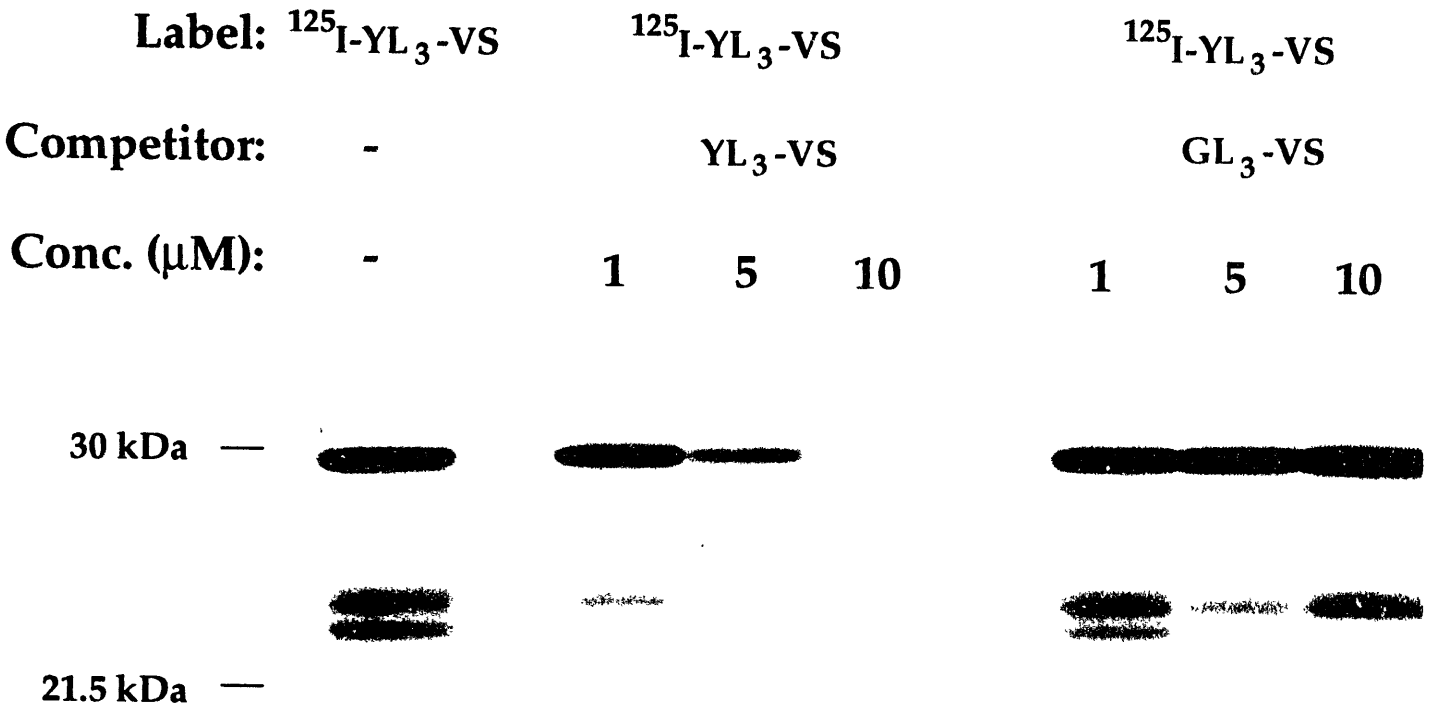
Based on the finding that GL$_3$-VS is a poor inhibitor of the trypsin-like and chymotrypsin-like activities of the proteasome but a fairly good inhibitor of the PGPH activity (see figure 3.11), we decided to examine whether this difference in activity could be correlated with subunit binding. Partially purified preparations of proteasomes were incubated with increasing concentrations of GL$_3$-VS or YL$_3$-VS and then labeled with ^{125}I-YL$_3$-VS (figure 3.14). The competition experiment indicates that while YL$_3$-VS is able to compete for binding to all polypeptides labeled with ^{125}I-YL$_3$-VS, GL$_3$-VS blocks binding of ^{125}I-YL$_3$-VS to only the 21 kDa polypeptide (likely to be LMP-2 or Y). This result combined with the data obtained for fluorogenic substrate hydrolysis, provides evidence that the 21 kDa polypeptide is responsible for the PGPH activity of the proteasome.

5. Modifications at the C-terminus of the peptide vinyl sulfones

Peptide vinyl sulfones containing a phenol moiety adjacent to the sulfone group were compared to the reactivity of peptide vinyl sulfones containing a methyl group. These compounds allow for the examination of binding

Figure 3.14. The tetra-peptide vinyl sulfone GL_3-VS binds predominantly the 22 kDa proteasomal β-subunit.

Partially purified preparations of proteasomes were incubated for 45 min. with either YL_3-VS or GL_3-VS or DMSO as a control (-) and then labeled with ^{125}I-YL_3-VS. Samples were separated on a 12.5% SDS-PAGE gel. YL_3-VS competes for all polypeptides labeled with ^{125}I-YL_3-VS while GL_3-VS competes only for the 22 kDa polypeptide.


Label: ^{125}I-YL$_3$-VS ^{125}I-YL$_3$-VS ^{125}I-YL$_3$-VS
Competitor: - YL$_3$-VS GL$_3$-VS
Conc. (μM): - 1 5 10 1 5 10
30 kDa
21.5 kDa

interactions at the C-terminal side of a predicted site of hydrolysis (likely to mimic the P-1 position). Interestingly, replacement of the methyl group in the compound NIP-L_3-VS with the phenol (NIP-L_3-VS-PhOH) resulted in very little change in the potency of the compound against hydrolysis of the LLVY, LRR, and LLE substrates (figure 3.15A). However, when the same comparison was made for the methyl and phenolic vinyl sulfones for the sequence Z-L_3-VS, a large loss in activity against the PGPH activity was observed when the methyl vinyl sulfone was replaced with the phenolic vinyl sulfone (figure 3.15B). These data suggests that the active sites in the proteasome responsible for the different hydrolytic activities are likely to have different substrate binding interactions which may include different preferences for the P-1 position.

The iodinated forms of several compounds containing the phenolic vinyl sulfone where then be used to examine the effects of the structural variation of the C-terminal vinyl sulfone on subunit modification. Partially purified proteasome preparations were labeled with the methyl vinyl sulfone containing NLVS, as well as with NLVS-PhOH, Z-L_3-VS-PhOH, and L_4-VS-PhOH, all of which contained the phenolic vinyl sulfone. Proteasome immuno-precipitations were separated by SDS-PAGE and are shown in figure 3.16. The labeling patterns obtained for these inhibitors indicated that binding was non-equivalent. The greatest difference in intensity of labeling was observed for the smallest polypeptide (~22 kDa). It therefore seems likely that this proteasome subunit has the greatest binding discrimination for regions of a substrate that are C-terminal to the site of hydrolysis.

Synthesis of the tri-peptide NP-LLG-VS allowed for the isolation of the cis and trans isomers of the C-terminal vinyl sulfone. Interestingly, the ability of these two compounds to inhibit hydrolysis of fluorogenic substrates by the proteasome was quite different. The cis isomer had little or no inhibitory capacity against any of the substrates, while the trans isomer was merely less active when compared to tri-peptides containing three leucines (figure 3.17). Even more surprising was the labeling pattern observed when these compounds were equipped with a ^{125}I moiety. The trans isomer, while a weaker inhibitor than the LLL equivalent, was still able to covalently tag the proteasomal β-subunits. The cis isomer, on the other hand failed to label any polypeptides in the 20-30 KDa size range expected for the proteasome, but efficiently labeled

Figure 3.15. The phenolic vinyl sulfones inhibit the trypsin-like, chymotrypsin-like, and PGPH activities of the proteasome with similar potency as the methyl vinyl sulfones.

(A) Inhibition of hydrolysis of fluorogenic peptide substrates by partially purified proteasome preparations was measured and plotted as in figure 3.8A. IC50 values (generated by extrapolation of the concentration of inhibitor at which the % residual activity was found to be 50%) were obtained for inhibition of the chymotrypsin-like, trypsin-like, and PGPH activities by the phenolic vinyl sulfones NLVS-PhOH compared to the methyl vinyl sulfone equivalent NLVS. The two compounds have nearly identical inhibitory potencies.
(B) IC_{50} values were obtained for Z-L_3-VS and Z-L_3-VS-PhOH as described for A. Note the loss of activity against hydrolysis of the LLE substrate observed for the phenolic vinyl sulfone compared to the methyl vinyl sulfone.

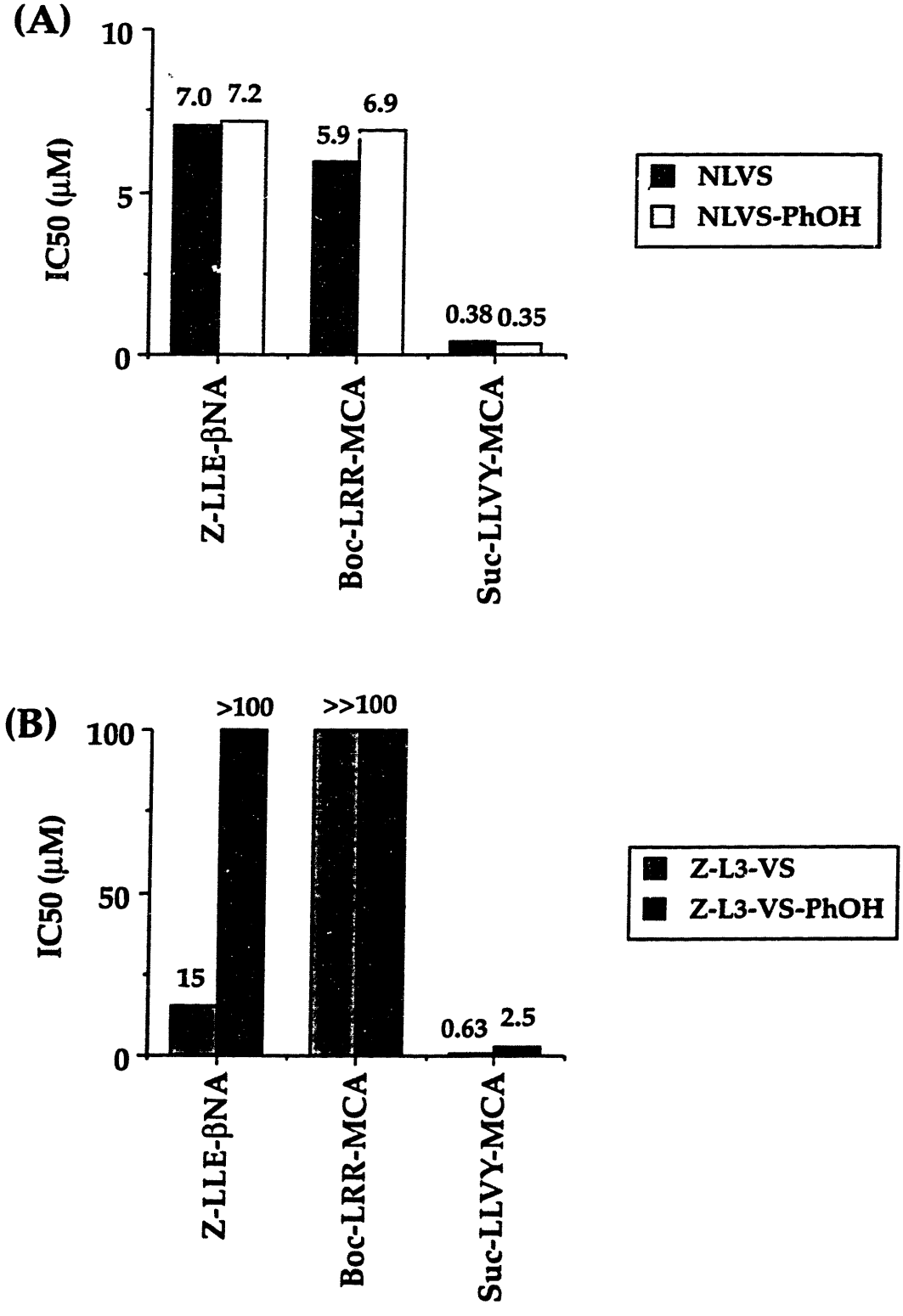

(A)
IC50 (μM)
10
5
0
7.0
7.2
5.9
6.9
0.38
0.35
NLVS
NLVS-PhOH
Z-LLE-βNA
Boc-LRR-MCA
Suc-LLVY-MCA
(B)
IC50 (μM)
100
50
0
15
>100
>>100
0.63
2.5
Z-L3-VS
Z-L3-VS-PhOH
Z-LLE-βNA
Boc-LRR-MCA
Suc-LLVY-MCA

Figure 3.16. The phenolic vinyl sulfones modify 22 kDa proteasomal β-subunit more effectively than the methyl vinyl sulfones.

Purified preparations of mouse proteasomes were labeled with NLVS-PhOH NLVS, L_3-PhOH, and L_4-PhOH and immunoprecipitated using the rabbit anti-proteasome antibody as described in the materials and methods section. The labeled polypeptides were separated by on a 12.5 % SDS-PAGE gel. Note the increased intensity of the 22 kDa polypeptide observed in samples labeled with phenolic vinyl sulfones compared to samples labeled with the methyl vinyl sulfone (NLVS).

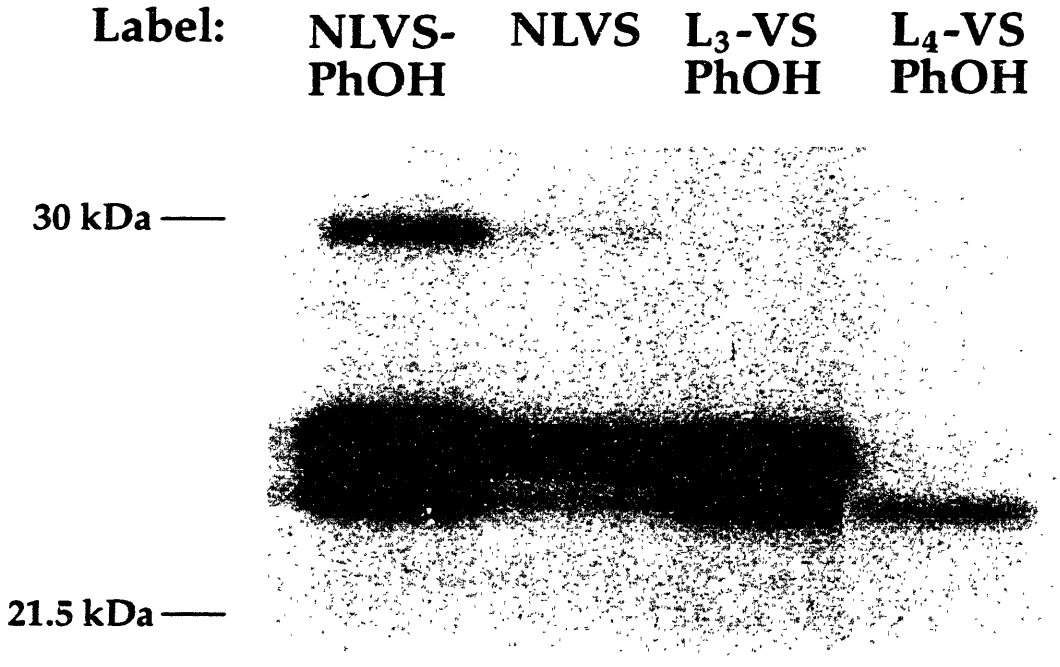

Label:
NLVS-PhOH
NLVS
L3-VS PhOH
L4-VS PhOH
30 kDa
21.5 kDa

Figure 3.17. A cis double bond abolishes the inhibitory potency of the peptide vinyl sulfones.

Inhibition of hydrolysis of fluorogenic peptide substrates by partially purified proteasome preparations was measured and plotted as in figure 3.8A. IC_{50} values (generated by extrapolation of the concentration of inhibitor at which the % residual activity was found to be 50%) were obtained for inhibition of the chymotrypsin-like, trypsin-like, and PGPH activities by the cis (cVS) and trans (tVS) isomers of NP-LLG-VS. Note the loss of activity by replacement of the trans double bond with the cis double bond.

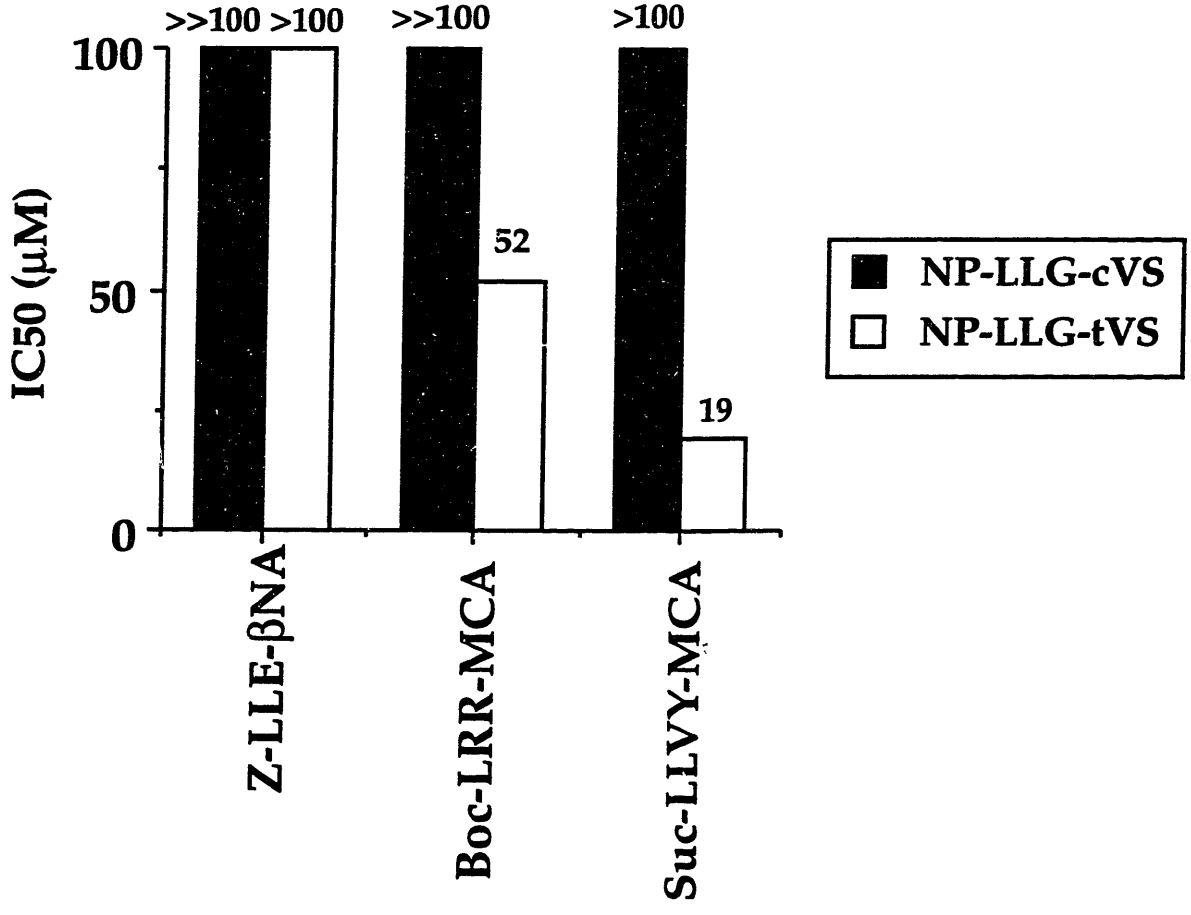
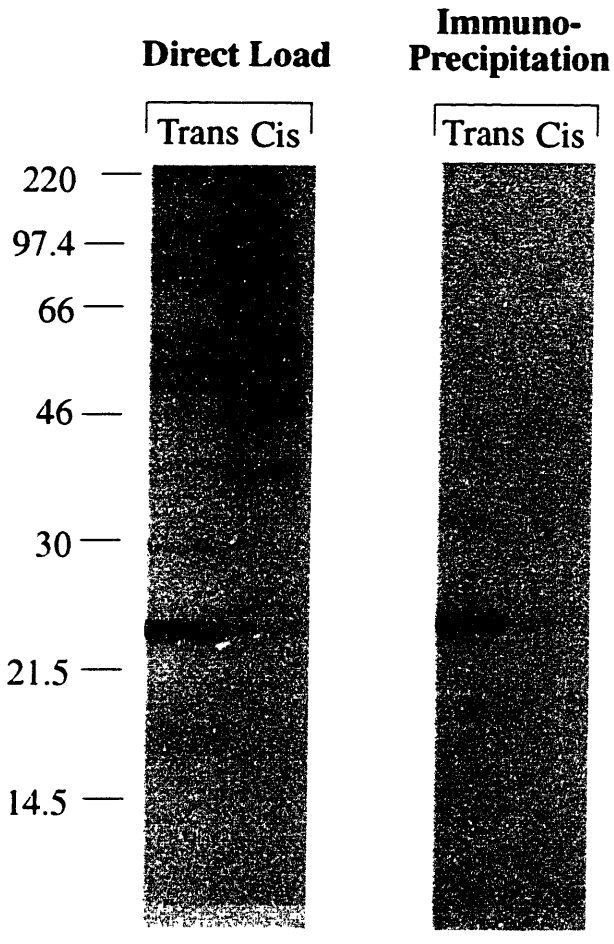

>>100
>100
>>100
>100
100
50
0
IC50 (μM)
52
19
NP-LLG-cVS
NP-LLG-tVS
Z-LLE-βNA
Boc-LRR-MCA
Suc-LLVY-MCA

Figure 3.18. The trans but not the cis isomer of NP-LLG-VS covalently modifies proteasomal β-subunits.

Partially purified proteasome preparations were labeled with the N-terminally modified tri-peptide ^{125}I-NP-LLG-cVS and ^{125}I-NP-LLG-tVS as described in the Material and Methods section. Samples were either directly separated by SDS-PAGE (Direct Load) or were first immunoprecipitated with the rabbit anti-proteasome antibody and then separated by SDS-PAGE (Immunoprecipitations).



Cis: ^{125}I-NIP-LLG-cVS
Trans: ^{125}I-NIP-LLG-tVS

several other species of higher molecular weight (figure 3.18). The identity of these additional polypeptides was not established. Thus, unlike replacement of a methyl with a phenol which resulted in only minor changes in activity, the sharp kink induced in the C-terminus by the cis double bond excludes binding to proteasomal β-subunits almost completely.

6. Inhibitor binding to β-subunits is sequence specific

To investigate the effects of amino acid sequences on the binding of inhibitors to specific subunits, we examined the ability of several tri-peptides to inhibit fluorogenic substrate hydrolysis and to label specific β-subunits of the proteasome when converted to ^{125}I-labeled form. Results from analysis of inhibition of the three proteasome substrates by the tri-peptide vinyl sulfones; NP-LLG-VS (trans isomer), NP-AAF-VS, and NIP-L_3-VS indicated that inhibition by these three compounds is non-equivalent (figure 3.19). Both NP-LLG-VS and NP-AAF-VS have little or no effect on the PGPH activity (IC_{50} = 100 μM or higher), while both are still able to inhibit the chymotrypsin activity , although NP-AAF-VS is at least 20 times more potent than NP-LLG-VS. NP-AAF-VS also has almost no activity against the trypsin-like activity, while NP-LLG is only 10-fold reduced compared to NLVS.

Labeling of semi-purified proteasomes with LLG(trans isomer), AAF, and LLL revealed differences in the relative intensity of polypeptides labeled. NIP-L_3-VS labeled predominantly the β-subunits X and LMP-7 each with similar intensity. Labeling of LMP-2, MECL-1(29 KDa PI 7.7) and Z (30 KDa PI 7.6) was observed, however with much less intensity (figure 3.20). NIP-AAF-VS showed labeling of predominantly LMP-7, with substantial reduction in the labeling of X. The next most abundant signal was observed for the subunit Z. Little if any labeling was observed for LMP-2 and MECL-1. NIP-LLG labeling resulted in a reduction in the amount of LMP-7 labeled with respect to X and a complete loss of labeling of LMP-2. The ratio of label intensity of Z to MECL-1 was similar to those observed for NIP-L_3-VS, but these subunits were modified to a greater extent when compared to LMP-7 and X.

Figure 3.19. Inhibition of proteasome by several N-terminally modified tri-peptide vinyl sulfones containing different amino acid sequences.

Inhibition of hydrolysis of fluorogenic peptide substrates by partially purified proteasome preparations was measured and plotted as in figure 3.8A. IC_{50} values (generated by extrapolation of the concentration of inhibitor at which the % residual activity was found to be 50%) were obtained for inhibition of the chymotrypsin-like, trypsin-like, and PGPH activities by the N-terminally modified tri-peptides, NLVS, NP-AAF-VS, and NP-LLG.

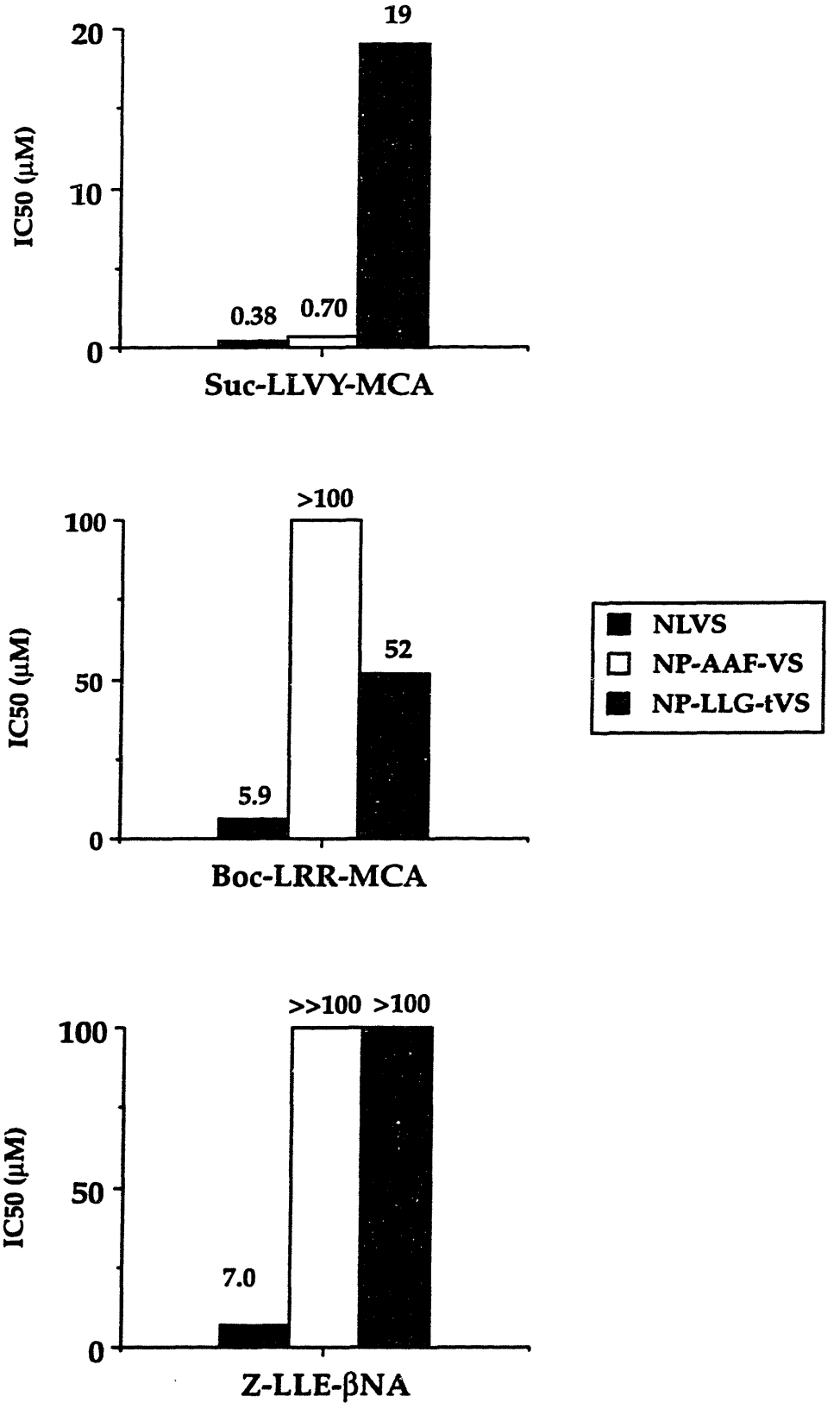
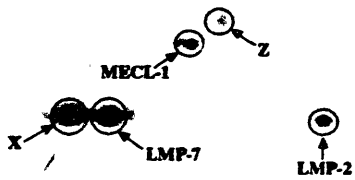

IC50 (μM)
20
10
0
19
0.38
0.70
Suc-LLVY-MCA
IC50 (μM)
100
50
0
>100
52
5.9
Boc-LRR-MCA
NLVS
NP-AAF-VS
NP-LLG-tVS
IC50 (μM)
100
50
0
>>100
>100
7.0
Z-LLE-βNA

Figure 3.20. Inhibitor binding to proteasomal β-subunits is sequence specific.

Lysates generated from the human B-lymphoblastoid line HOM-2 were labeled with ^{125}I-NIP-L_3-VS, ^{125}I-NIP-AAF-VS, and ^{125}I-NIP-LLG-VS and labeled polypeptides separated by NEPHGE using an 11% SDS-PAGE gel for the second dimension. The identities of the individual subunits are indicated. Note the change in relative intensity of spots labeled with LLG or AAF containing vinyl sulfones compared to the L_3 vinyl sulfone (NIP-L_3-VS).

Label: ⊖ ⊕

^{125}I-NIP-L_3-VS



^{125}I-NIP-AAF-VS

^{125}I-NIP-LLG-tVS

D. Discussion

The use of modified tri-peptides which contain an active site-directed inhibitory functional group at the C-terminus has proven to be an extremely valuable method for studying proteasome-substrate binding interactions. The synthesis of a series of tetra-peptide vinyl sulfones has uncovered the importance of the P4 amino acid position for binding and inhibition. Modifications at the C-terminal portion of the vinyl sulfone have also indicated some important differences in the way in which subunits bind substrates. In addition, the study of inhibitor binding by direct labeling experiments provide a link between substrate binding and inhibition of hydrolysis. Labeling of proteasomes with peptide vinyl sulfones containing different amino acid sequences has also provided evidence that binding and subsequent covalent modification is different for individual β-subunits. These data may help explain how the proteasome is able to control the multitude of proteolytic events which are crucial for cell survival.

The ability of a free-amino tetra-peptide vinyl sulfones to potently inhibit proteasomal proteolysis, while a slightly truncated free-amino tri-peptide remains a poor inhibitor, is quite remarkable. The tri-peptides capped with a bulky nitro phenol (NIP-L_3-VS) or benzyl (Z-L_3-VS) moiety are potent inhibitors, but removal of the cap results in severely diminished binding and inhibition. This loss of activity may be due in part to the exposure of a free amino group, which is likely to mimic a short peptide, the product of a hydrolysis reaction. However, this is not likely to be the only explanation for why the addition of a fourth amino acid increases potency, since the addition of the t-butyl blocking group (Boc-L_3-VS) results in a rather weak inhibitor (data not shown). Also, the placement of glycine at the P4 position results in a rather poor inhibitor, indicating that it is specific sidechain interactions which are likely to lead to increased binding.

The P4 binding site is unlikely to be as specific as that found for the P1 amino acid. Our data indicate that several different bulky residues including an amino acid containing a benzophenone sidechain are accepted in this position. In addition, unlike the P1 position in which use of a D-isomer results in complete loss of binding (See Chapter 2), placement of a D-isomer at P4 results in an active

inhibitor, albeit with reduced binding affinity. Thus, a binding site within the cavity of the proteasome is likely to exist which serves to stabilize longer peptide substrates and which does not possess a great deal of side-chain specificity. This would be expected for a protease such as the proteasome, responsible for the degradation of virtually all cytosolic proteins.

Our results also indicate that the structural requirements for binding of the P4 amino acid are different for different active sites. The trypsin-like and chymotrypsin-like activities of the proteasome seem to require a bulky, aromatic or hydrophobic group in the P4 position, while the PGPH activity is equally inhibited by glycine and tyrosine in the P4 position. Competition experiments using GL_3-VS indicate that it binds with highest affinity to the 21 kDa polypeptide (likely to be LMP-2 or Y) and indicated that it may be possible to correlate activity against substrate hydrolysis with subunit binding.

A rather surprising finding was the difference in labeling observed between a capped tri-peptide such as NIP-L_3-VS and a free-amino tetra-peptide such as YL_3-VS. Inhibition of substrate hydrolysis by the two compounds indicates that both have remarkably similar inhibition profiles for all three substrates tested. However, labeling experiments indicate that the two in fact bind with different relative affinities to different β-subunits of the proteasome. This difference in labeling may be explained by a scenario in which a protein substrate is processively degraded by the proteasome. In this case, a protein substrate binds to an active site which is designed to accommodate a large protein, perhaps near the opening of the inner cavity. The substrate is now cleaved and is able to proceed to a second active site designed to accommodate the small peptide fragments generated by the first active site. Thus, a slightly longer inhibitor may more accurately mimic a protein substrate while a tri-peptide mimics the products already acted upon by the proteasome. The difference in labeling may if fact reflect how the proteasome deals with these different substrates.

Labeling of proteasomes using a variety if tetra-peptide vinyl sulfones indicated that the increase in potency acquired by addition of the P4 amino acid is not likely to be the result of a single specific inhibitor-β-subunit interaction. In the case of YL_3-VS, the most pronounced difference in labeling compared to the tri-peptide NIP-L_3-VS was the abundance of the 30 kDa polypeptide. This increased modification of the 30 kDa species was not observed for YAAF-VS or

for L_4-VS. The binding site for a P4 amino acid may therefore contribute to binding at multiple active sites. The sequence of the peptide may dictate which active site binding is influenced the most by the presence of the P4 amino acid.

The differences between the labeling patterns and inhibition profiles against fluorogenic substrate hydrolysis observed for the tri- and tetra peptide inhibitors, as well as for the methyl and phenolic versions of NIP-L_3-VS, also indicate that β-subunits, at last to some extent must overlap in their hydrolysis activity. If each β-subunit were responsible for a single type of proteolytic activity (i.e. chymotrypsin-like, trypsin-like, or PGPH) a change in subunit labeling affinity would be directly reflected in the ability of a compound to inhibit hydrolysis of a specific substrate and this is not observed.

The functional group placed adjacent to the sulfone group at the C-terminal end of the inhibitor is likely to mimic the P-1 position of a protein substrate when bound in the active site. Our data support the fact that not all the active sites have the same degree of specificity, or lack there of, for the P-1 position. Hydrolysis of the Z-LLE-βNA substrate is inhibited quite effectively by the compound Z-L_3-VS but not by the equivalent compound containing a phenolic vinyl sulfone. Interestingly, the same difference is not observed when comparing the activity of the methyl and phenolic version of NIP-L_3-VS (figure 3.15).

While some active sites may not depend on interactions with the region of a substrate C-terminal to the site of hydrolysis, the inability of the cis double bond containing vinyl sulfone to bind to any subunits of the proteasome indicates that a dramatic kink in the peptide structure precludes binding.

Finally, labeling of proteasomes with inhibitors containing different amino acid sequences demonstrates that individual subunits have sequence specificity. It is not surprising that by changing the amino acid sequence of a tri-peptide inhibitor it is possible to change the relative inhibitory capacity of that compound. However, by comparing the changes in binding affinity of a compound for individual subunits relative to one another it is possible to examine how these inhibitors interact with individual subunits of the proteasome. Of interest is the ability of a NIP-AAF-VS to bind specifically to LMP-7 with greater affinity than to X. This is in direct contrast to NIP-L_3-VS, which binds both equally, and NIP-LLG-VS which bind preferentially to X. This result is surprising in light of the findings that replacement of X with LMP-7

results in the production of peptides with a hydrophobic C-terminal residues suitable for binding to class I molecules (Driscoll et al., 1993; Gaczynska et al., 1993; Gaczynska et al., 1994) and considering the fact leucine is a better C-terminal anchor residue for class I binding.

Fluorogenic substrates are often used to evaluate the ability of a compound to inhibit the proteolytic activity of an enzyme such as the proteasome. These tools, while useful, often fail to shed light on the underlying mechanisms by which a compound can cause inhibition. The data reported here serve to further support the usefulness of radiolabeled affinity reagents such as the vinyl sulfones to study substrate binding.

IV. THE HUMAN CYTOMEGALOVIRUS US2 AND US11 GENE PRODUCTS DISLOCATE MHC CLASS I HEAVY CHAINS FROM THE ENDOPLASMIC RETICULUM TO THE CYTOSOL FOR DEGRADATION BY THE PROTEASOME

Adapted from:

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Emmanuel J. H. J. Wiertz, Domenico Tortorella, Matthew Bogyo, Joyce Yu, Walter Mothes, Thomas R. Jones, Tom A. Rapoport, and Hidde L. Ploegh. (1996). *Nature* **384**, 432-438.

A. Introduction

The virus life cycle begins with infection of a host and hijacking of its cellular machinery to produce proteins necessary for the virus to survive and propagate. The host organism must therefore be able to recognize and kill infected cells if it is to eradicate the virus. This is accomplished by presentation of peptide epitopes derived from viral proteins, primarily on MHC class I molecules. These peptide -MHC complexes are displayed at the cell surface and are recognized by circulating cytotoxic T-lymphocytes, which then proceed to kill the infected cell . In order to escape this destruction by the immune response of the host, the virus must devise clever strategies to interfere with the process of antigen presentation. Over the course of tens of millions of years of evolution, nature has selected viruses equipped with a myriad of evasion strategies which include the synthesis of proteins that block the function of TAP to the ones that catalyze the complete destruction of MHC class I molecules.

The Herpes simplex viruses HSV 1 and HSV 2 are closely related and part of a family of viruses termed persistent viruses. Both undergo a period of latency after initial infection, during which time the virus produces only a select few proteins required for maintenance of the latent state. Over time these latent viruses become activated and begin to produce a large number of progeny viruses. It is at this time in the viral life cycle that it is most vulnerable to detection by the host's immune cells. HSV has apparently circumvented this dilemma by synthesizing a small cytosolic protein, ICP47 which inhibits the ATP-dependent peptide transporter, TAP (York et al., 1994; Hill et al., 1995; Früh et al., 1995). Thus, by plugging the hole by which peptides enter the ER and bind to newly synthesized MHC class I molecules, viral epitopes fail to be presented at the cell surface and the virus remains invisible to the host's immune system.

Similar to HSV, infection by both the human (HCMV) and murine (MCMV) cytomegalovirus results in a lifelong infection of the host (Reusser et al., 1991; Quinnan et al., 1982). In the case of MCMV, the virus eludes detection by the host immune response by multiple mechanisms; the details of some of these strategies remain unknown. Viral infection by MCMV results in the retention of the majority of class I molecules in ER and the few which escape to the cell

surface are accompanied by a viral glycoprotein termed gp34 (Kleijnen et al., 1997). The exact function of gp 34 is unknown but it has been suggested to lead to a reduction in the affinity of class I-peptide complexes for the T-cell receptor of CD 8+ cytotoxic T-cells (Kleijnen et al., 1997). The identity of the gene product responsible for the retention of class I molecules is currently nearing identification through the use of deletion studies of the viral genome. It appears that multiple genes may contribute to ER retention (Thäle et al., 1995).

HCMV infection, on the other hand results in the complete and rapid destruction of MHC class I molecules (Beersma et al., 1993; Warren et al., 1994). Systematic deletion of regions of the HCMV genome has uncovered two genes, US2 and US11 (for Unique Short region) responsible for the phenotype of loss of class I expression on the cell surface (Jones et al., 1995). These genes, when transfected into class I + cell lines result in rapid destruction of class I heavy chains. The mechanism by which US2 and US11 accomplish this destruction involves the dislocation of newly synthesized Class I heavy chains from the ER to the cytosol, at which point the single N-linked glycan is removed by a peptide N-glycanase activity, followed by destruction by the proteasome. This process of reverse translocation of a polypeptide from the ER to the cytosol is mediated by Sec61p protein complex, in what appears to be a reversal of the process by which proteins are inserted into the ER.

Thus the study of a viral evasion strategy has uncovered a novel route by which ER resident proteins may be destroyed in the cytosol by the proteasome. This method of protein destruction is not likely to be unique to cells infected with HCMV but rather a general process which a cell uses to remove unwanted proteins from the ER.

B. Materials and Methods

1. Cells and Cell Culture

U373-MG astrocytoma cells, US11 and US 2 transfectants prepared from this cell line have been described (Jones et al., 1994; Kim et al., 1995). The cells were cultured in Dulbecco's modified Eagle's medium supplemented with 5% fetal calf serum and Puromycin (Sigma, St. Louis, MO) at a final concentration of 0.375 μg/ml.

2. Antibodies

The following antibodies were used: mono-clonal antibody W6/32 (Parham et al., 1979), specific for assembled class I molecules; rabbit anti-heavy chain serum (Beersma et al., 1993), which recognizes unfolded class I heavy chains; anti-proteasome (Organon teknika, Oss, The Netherlands) which recognizes an α subunit as part of the mature 720-kDa proteasome complex; The mono-clonal antibody 66lg10 which recognizes the human transferrin receptor (Beersma et al., 1993); anti-Sec-61β described previously (Görlich and Rapoport, 1993); Rabbit anti-US2 serum (Jones and Sun, 1997); and polyclonal rabbit serum reactive with US11 (Jones et al., 1991).

3. Inhibitors

The proteasome inhibitor lactacystin was obtained from E.J. Corey (Harvard University) and used at a final concentration of 20 μM. Leucyl-leucyl-norleucinal (LLnL; Boehringer Mannheim, Germany) was dissolved in ethanol and used at a final concentration of 50 μM. Carboxylbenzyl-leucyl-leucyl-leucinal (Z-L_3-H or CbzLLL) was synthesized as described (Wiertz et al., 1996a), dissolved in ethanol, and used at a final concentration of 50 μM. Caboxylbenzyl-leucyl-leucyl-leucine vinyl sulfone (Z-L_3-VS) was synthesized as described in chapter 2, dissolved in DMSO, and used at a final concentration of 50 μM. Tunicamycin and Brefeldin A were from Sigma (Sigma Chemical Co., St. Louis). Treatment with endoglycosidase H (Endo H; New England Biolabs, Beverly) or

N-glycanase (Boehringer Mannheim, Germany) were performed as suggested by the manufacturers.

4. Pulse Chase Analysis

Cells were detached by trypsin treatment and incubated in methionine-free Dulbecco' modified Eagle's medium with or without added inhibitors for 1 hour. Two million cells (pulse time as indicated in figure legend; 250 μCi of ^{35}S-Methionine ((1200 Ci/mmol) NEN-Du Pont) or for short pulses (45-60 seconds) 5 x 10^6 cells and 500 μCi of label were used per sample. Incorporation was terminated by addition of non-radioactive methionine to a final concentration of 1 mM. Immediately following the chase, samples were placed on ice and lysed in 1 ml of ice-cold NP-40 lysis mix. Preparation of lysates and immunoprecipitations were performed as described (Beersma et al., 1993).

5. Gel electrophoresis

SDS-PAGE, one-dimensional IEF, and fluorography were performed as described (Ploegh, 1995).

6. Subcellular Fractionation

Cells (1x 10^8) were incubated in methionine-free medium with LLnL (20 μM; US11+ cells) or Z-L_3-H (20μM; US2+ cells) for 1 hour. Cells were then labeled with [^{35}S] methionine (250 μCi/ml) in a volume of 2 ml for 15 min. The cells were washed in homogenization buffer (0.25 M sucrose, 10 mM triethanolamine, 10 mM acetic acid, 1 mM EDTA [pH 7.4] and the protease inhibitors leupeptin [1 mM] iodoacetamide [50 μM], phenylmethylsulfonyl fluoride [1 mM] and LLnL or Z-L_3-H [20μM] and re suspended in 2 ml of homogenization buffer. Cells were homogenized on ice using a dounce homogenizer (30 or 50 strokes) with a tight fitting pestle (Thomas; Pestle type A). The homogenate was spun at 1,000 g for 10 min. which yielded the 1,000 g pellet. The supernatant was subjected to 10,000 g centrifugation for 30 min., which yielded the 10,000 g pellet. Finally, the 10,000 g supernatant was spun at 100,000

g for 1 hr., which resulted in the 100,000 g pellet and the 100,000 g supernatant (cytosol). Lysis mix was added to all pellets (but not the 100,000 g supernatant) and immunoprecipitations, SDS-PAGE, and fluorography were performed as described.

7. Re-immunoprecipitation of class I heavy chains or US2 from immuno complexes containing proteasomes, US2, or Sec61

US2+ cells were pulsed labeled as described above then chased for 0, 1, 2, 7, 20, and 60 minutes in the presence of Z-L_3-H (20 μM) at 37°C. Cells were lysed in digitonin lysis mix (1% Digitonin in 2.5 mM HEPES, pH 7.6, 10 mM $CaCl_2$). First round immunoprecipitations were performed as described (Beersma et al., 1993). Re-immunoprecipitations were performed by de-naturation of primary precipitates by treatment with 1% SDS containing 5% β-mercaptoethanol followed by boiling. Samples were then diluted 10X with NP-lysis mix described above and precipitations were carried out using the antibody specified. Proteins were then separated by SDS-PAGE.

C. Results

1. Class I heavy chains are destroyed in US11+ and US2+ cells

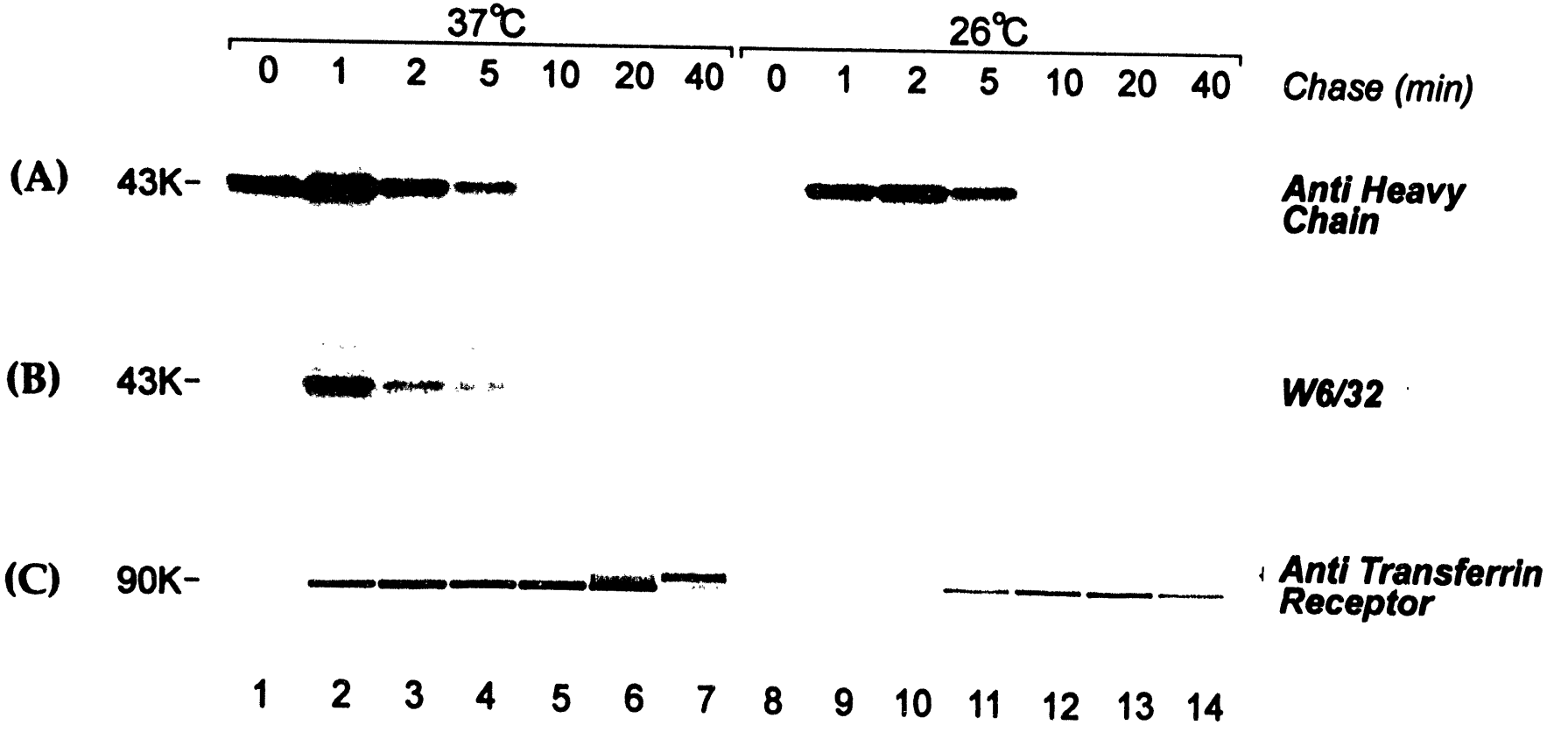
The effects of expression of the HCMV gene, US11, were examined by pulse-chase analysis in U373-MG cells transfected with the US11 gene. Cells were pulse labeled with ^{35}S-methionine for 60 seconds followed by a chase of 1,2 5, and 10 min. Class I heavy chains were precipitated using a conformation-specific antibody (W6/32) which recognizes only properly folded heavy chains complexed with light chain (β_2M) as well as with a rabbit serum that only recognizes free heavy chains (figure 4.1). Transferrin receptor was also precipitated to monitor synthesis of a control protein. The amount of recovered class I molecules increases from 0 to 1minute of chase, presumably because of the time required for the methionine pools to equilibrate within the cells. Greater than one half of the total recovered radioactivity disappears between the 1 and 2 minute chase points and complete destruction of heavy chains is observed by the 20 min time point in both W6/32 and anti-heavy chain precipitations. The kinetics of degradation seen here are very similar to those observed for HCMV infected cells (Wiertz et al., 1996b).

Due to the rapid time scale of this destruction process, degradation is likely to take place immediately after the nascent polypeptide is synthesized and before it can leave the ER for the Golgi apparatus. Transferrin receptor, on the other hand, is synthesized, glycosylated normally and fails to be destroyed, indicating that the observed break-down is specific for MHC class I molecules. In addition, when the same pulse chase experiment is performed at the reduced temperature of 26° C, there is little effect on the kinetics of the US 11 mediated breakdown of class I molecules.

In the same U373 cells described above transfected with the HCMV gene US2, class I heavy chains are much less stable than in non-transfected controls. In a pulse chase experiment, all heavy class I heavy chains are destroyed by the 30 min. chase point. Similar to the findings for US11+ cells, the US2 mediated destruction does not depend upon proper folding and assembly with β_2m (Wiertz et al., 1996b).

Figure 4.1 The half-life of class I heavy chains in US11+ cells is less than 1 minute.

(A) Free class I heavy chains were immunoprecipitated with rabbit serum raised against the lumenal domain of class I molecules (anti–heavy chain serum). US11+ cells were pulse labeled with [^{35}S] methionine for 60 s, and chased as indicated, at 37°C (lanes 1-7) or 26°C (lanes 8-14). The immunoprecipitations were separated on a 12.5 % SDS-polyacrylamide gel and visualized by fluorography.
(B) Class I heavy chain-β_2m complexes were immunoprecipitated with the monoclonal antibody W6/32; otherwise as A.
(C) Transferrin receptor was immunoprecipitated with the monoclonal antibody 66Ig10; otherwise as A.


37℃
26℃
0 1 2 5 10 20 40 0 1 2 5 10 20 40
Chase (min)
(A) 43K-
Anti Heavy Chain
(B) 43K-
W6/32
(C) 90K-
Anti Transferrin Receptor
1 2 3 4 5 6 7 8 9 10 11 12 13 14

2. A class I heavy chain break-down intermediate appears in US11+ and US2+ cells upon treatment with proteasome inhibitors.

In order to examine the mechanism by which US2 and US11 destroy newly synthesized class I heavy chains, transfectants were treated with protease inhibitors 30 minutes prior to pulse -chase analysis. Treatment of US11+ cells with Brefeldin A, a natural product shown to block anterograde transport from the ER to Golgi (Misumi et al., 1986) did not effect breakdown, indicating that transport of class I molecules to a site downstream of the ER is not required for efficient destruction (figure 4.2).

Treatment of US 11+ cells with The tri-peptide aldehyde leucyl-leucyl-norleucinal, LLnL (an inhibitor of the cysteine protease calpain I which has also been shown to inhibit the proteasome (Rock et al., 1994; Coux et al., 1996) resulted in the appearance of a new polypeptide with a slightly lower apparent molecular weight (40 kDa) than intact heavy chains (43kDa) in precipitations performed with anti heavy chain serum. This intermediate could not be seen in W6/32 precipitations and failed to appear when control (US11-) cells were treated with LLnL (figure 4.2).

The effect of several other classes of protease inhibitors on class I breakdown was examined next. In addition to LLnL, several more specific proteasome inhibitors have been reported. The natural product lactacystin has has exquisite specificity for the proteasome (Fenteany et al., 1995). Likewise, the tri-peptide vinyl sulfone carboxylbenzy-leucinyl-leucinyl-leucine vinyl sulfone (Z-L_3VS) has very little cross-reactivity with non-proteasomal proteases (Chapter 2). Finally, the tri-peptide aldehyde carboxylbenzyl-leucyl-leucyl-leucinal (Z-L_3-H or CbzLLL) is a potent inhibitor of the proteasome, although, like LLnL, it is not proteasome-specific (see chapter 2). Pre-treatment of both US11+ cells (figure 4.3A) and US2+ (figure 4.3 B)with these inhibitors resulted in the appearance of the lower molecular weight breakdown intermediate in all cases . These results provide strong evidence for the involvement of the proteasome in US2 and US11 mediated class I heavy chain breakdown.

Figure 4.2. LLnL, but not BFA, induces a class I breakdown intermediate in US11+ cells.

Class I molecules were immunoprecipitated from US11+ cells using the monoclonal antibody W6/32 (lanes 1-8), and rabbit heavy chain serum (lanes 9-16). The cells were pretreated with the appropriate inhibitors for 60 minutes, pulse labeled with [^{35}S] methionine for 15 min. and chased for 20 min. The proteins were separated on a 12.5 % acrylamide gel. The position of migration of core-glycosylated class I heavy chain (43-44 kDa; HC) and the breakdown intermediate (~40 kDa; HC with asterisk) are indicated.

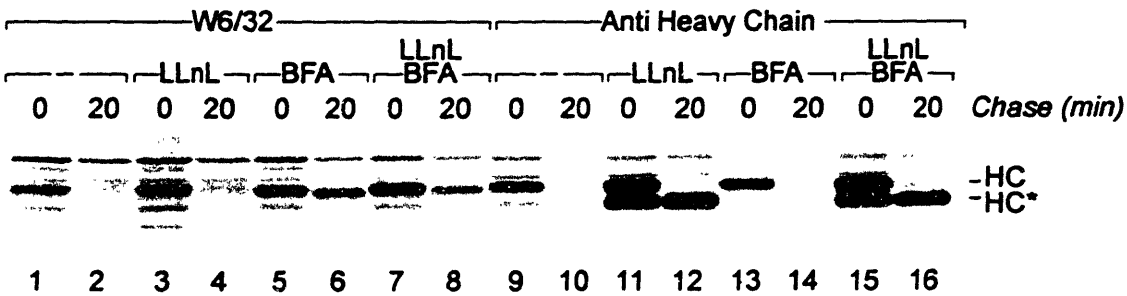
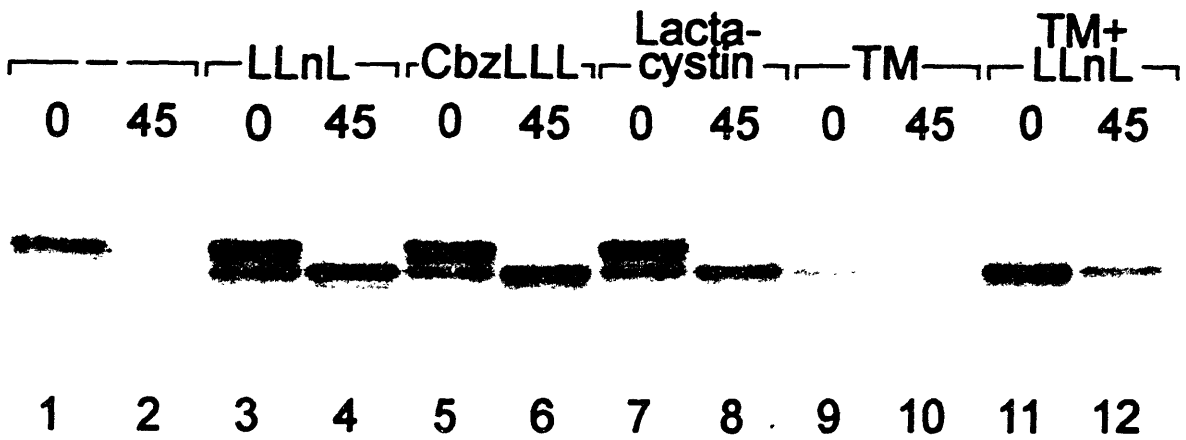

W6/32
Anti Heavy Chain
LLnL
BFA
LLnL
BFA
LLnL
BFA
LLnL
BFA
0 20 0 20 0 20 0 20 0 20 0 20 0 20 0 20
Chase (min)
-HC
-HC*
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16

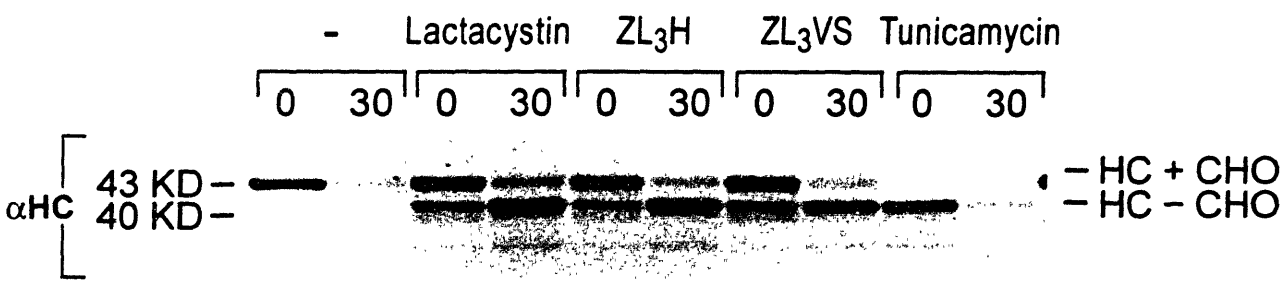
Figure 4.3. US11 and US2 mediated class I breakdown does not require N-linked glycans and is inhibited by inhibitors of the proteasome.

(A) US11+ cells were pretreated with LLnL, Z-L_3-H (CbzLLL), lactacystin, tunicamycin, or both LLnL and tunicamycin for 1 hour. Cells were labeled for 15 minutes and chased for 45 minutes in the continued presence of the inhibitors. the class I molecules were immunoprecipitated using the rabbit anti-heavy chain serum.
(B) US2+ cells were pre-treated with lactacystin, Z-L_3-H, Z-L_3-VS, and tunicamycin for 1 hour and then pulse labeled, chased, and immunoprecipitated as described in A. The fully glycosylated heavy chain (43 kDa) is indicated as HC + CHO and the de-glycosylated breakdown intermediate (40 kDa) is indicated as HC - CHO.

(A)


LLnL
CbzLLL
Lacta-
cystin
TM
TM+
LLnL
0 45 0 45 0 45 0 45 0 45 0 45
1 2 3 4 5 6 7 8 9 10 11 12

(B)


- Lactacystin ZL3H ZL3VS Tunicamycin
0 30 0 30 0 30 0 30 0 30
αHC
43 KD
40 KD
HC + CHO
HC - CHO

The identity of the lower molecular weight intermediate was further examined by treatment of cells with the glycosylation inhibitor, tunicamycin. The resultant non-glycosylated class I heavy chain co-migrated in SDS-Page gels with the class I heavy chain breakdown intermediate (figure 4.3). Thus, the heavy chain intermediate was likely to be the result of a deglycosylation reaction prior to destruction by the proteasome. Such deglycosylation is not inhibited by proteasome inhibitors.

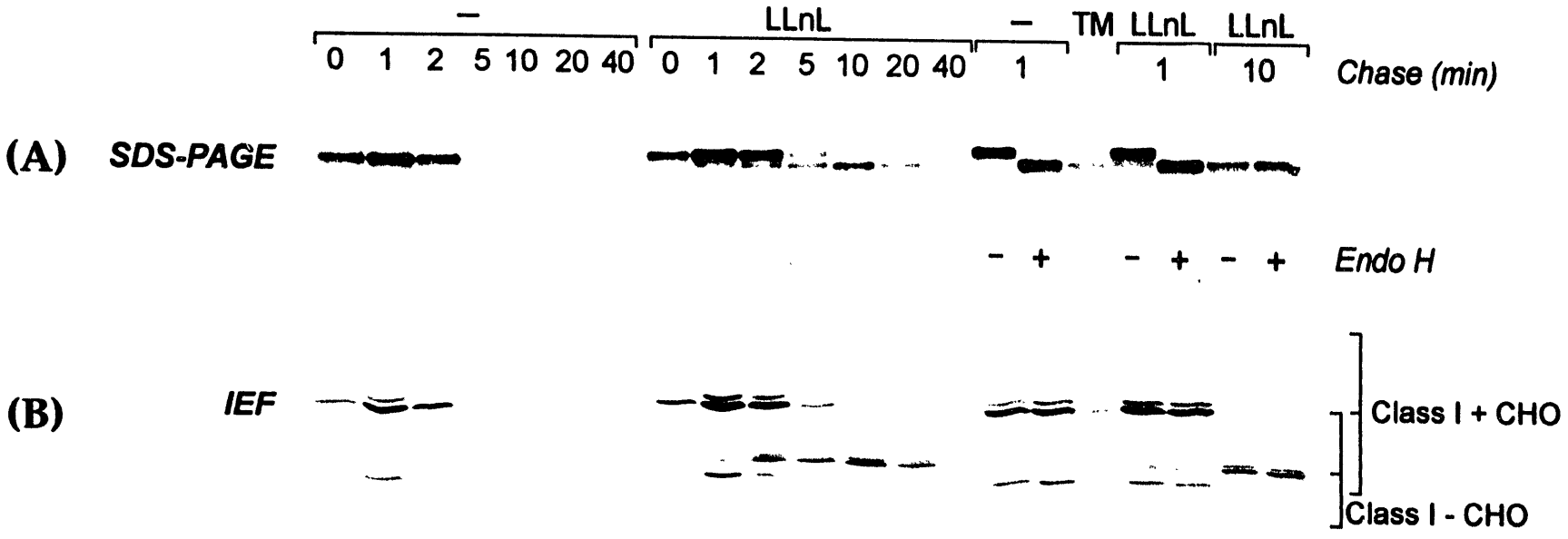
3. N-linked glycans are removed from class I molecules in US11 and US2 cells by a peptide N-glycanase activity.

Pulse chase experiments performed on US11+ cells in the presence of proteasome inhibitors indicate that a precursor-product relationship exists between intact heavy chain (43 KDa) and the breakdown intermediate (40 kDa). Since SDS-PAGE fails to distinguish between a non-glycosylated (tunicamycin treated sample) and a de-glycosylated (Endo H treated) class I heavy chain, (since the difference in migration caused by the presence of a single GlcNAc residue is too small to visualize), we could not exclude the possibility of the existence of truncated sugar residues on the 40 kDa intermediate. Further, involvement of an N-glycanase in conversion of intact heavy chain to the intermediate would result in conversion of a the asparagine residue at the site of glycan attachment to an aspartic acid upon hydrolysis (Tarentino and Plummer, 1994). This amino acid change would result in a change in the net charge of the polypeptide which could then be resolved by isoelectric focusing (IEF).

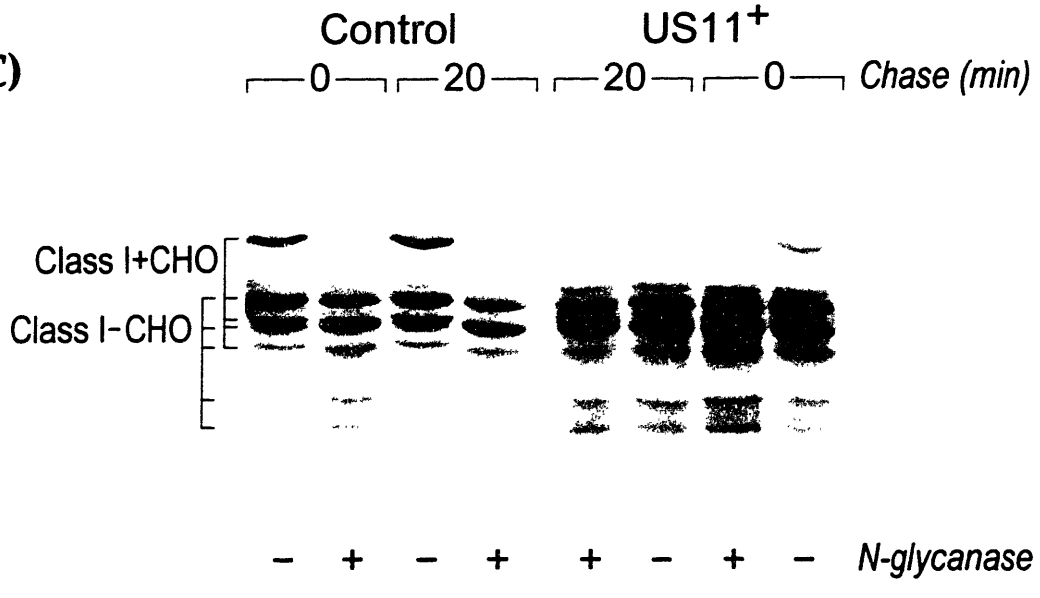
A pulse chase was performed on US11+ cells treated with LLnL as well as with tunicamycin. The class I heavy chain precipitates were subjected to SDS-PAGE and IEF (figure 4.4a). Isoelectric focusing of class I heavy chains from control cells results in the appearance of several bands due to the unique isoelectric points of the three products of the HLA loci (HLA-A, -B, and -C) that encode class I molecules. In US11+ cells treated with LLnL, the 40 kDa breakdown intermediate appears in the IEF gel at a more acidic pH than the intact glycosylated or non-glycosylated free heavy chains from tunicamycin treated cells. Based on previous observations (Eichholtz et al., 1992) the

Figure 4.4. N-linked glycans are removed from class I molecules in US11+ cells by an N-glycanase type activity.

(A) The precursor-product relationship of the 43 kDa breakdown intermediate was established in a 1 min. pulse chase experiment performed with US11+ cells in the absence (minus) or presence of LLnL. The cells were pulse labeled for 60 seconds and chased for the rimes indicated. Class I heavy chains were immunoprecipitated with rabbit anti-heavy chain serum and separated by SDS-PAGE. Endo H digestion was performed for three samples (untreated cells, 1 min. chase; LLnL treated cells, 1 and 10 min. chase). Samples before and after Endo H digestion are shown in lanes 15-21. The class I heavy chains obtained in the presence of tunicamycin (TM; lane 17) are devoid of carbohydrates.
(B) Isoelectric focusing (IEF) gel loaded in exactly the same order as the SDS-PAGE gel shown in (A). Note the shift in isoelectric point for the breakdown intermediate.
(C) US11+ and control cells were labeled for 20 min. and chased for 0 or 20 min. For US11+ cells, LLnL was included to the labeling and chase medium. Class I heavy chains were digested with N-glycanase where indicated. Following complete denaturation in SDS, heavy chains were re-immunoprecipitated with rabbit anti-heavy chain serum, and analyzed by 1D IEF. In control cells, digestion of class I heavy chains with N-glycanase shifts the banding pattern to a more acidic position for both the 0 and 20 min. time points (brackets; Class I-CHO). In US11+ cells, some of the core-glycosylated precursor is observed at the 0 min. time point and is still susceptible to N-glycanase digestion. Note that at the 20 min. time point, when only the breakdown intermediate is detectable, (see figure 3.3a), digestion with N-glycanase does not effect the IEF banding pattern. Moreover, the pattern observed in US11+ cells is indistinguishable from that seen for the N-glycanase-digested Class I heavy chains from control cells. The two panels shown are derived from the same electrophoretic separation, but different exposures were required to compensate for levels of incorporation. Cathode is at the top.


–
LLnL
–
TM
LLnL
LLnL
0 1 2 5 10 20 40
0 1 2 5 10 20 40
1
1
10
Chase (min)
(A)
SDS-PAGE
– + – + – +
Endo H
(B)
IEF
Class I + CHO
Class I - CHO

(C)


Control
US11+
0
20
20
0
Chase (min)
Class I+CHO
Class I-CHO
- + - + + - + - N-glycanase

difference in pI seen for the intermediate is consistent with a change in charge as a result of conversion of a single Asn to Asp.

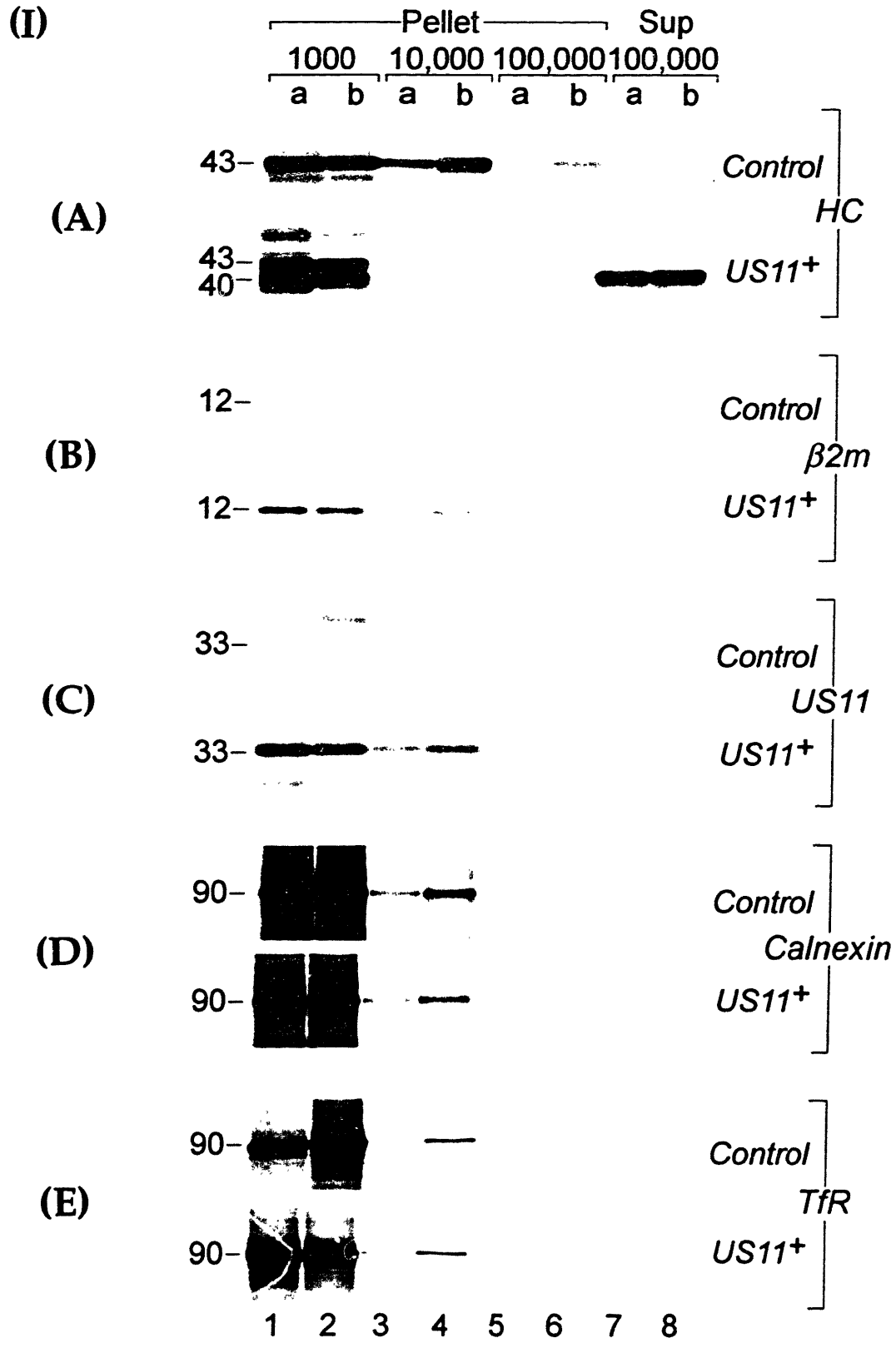
To confirm that the 40 KDa intermediate is the result of hydrolysis by peptide N-glycanase (PNGase), immunoprecipitations from a pulse chase reaction were digested in vitro with purified, recombinant PNGase and then subjected to IEF (figure 4.4B). Treatment of class I heavy chain from control cells with PNGase resulted in the appearance of several acidic polypeptides which co -migrated with the acidic polypeptide observed in US11+ cells without in vitro treatment with PNGase. Taken together, these results suggest that prior to degradation by the proteasome, class I heavy chains are de-glycosylated by a peptide N-glycanase activity which removes the single N-linked glycan by hydrolysis of the amide linkage to asparagine.

4. The de-glycosylated class I heavy chain is found in the cytosol.

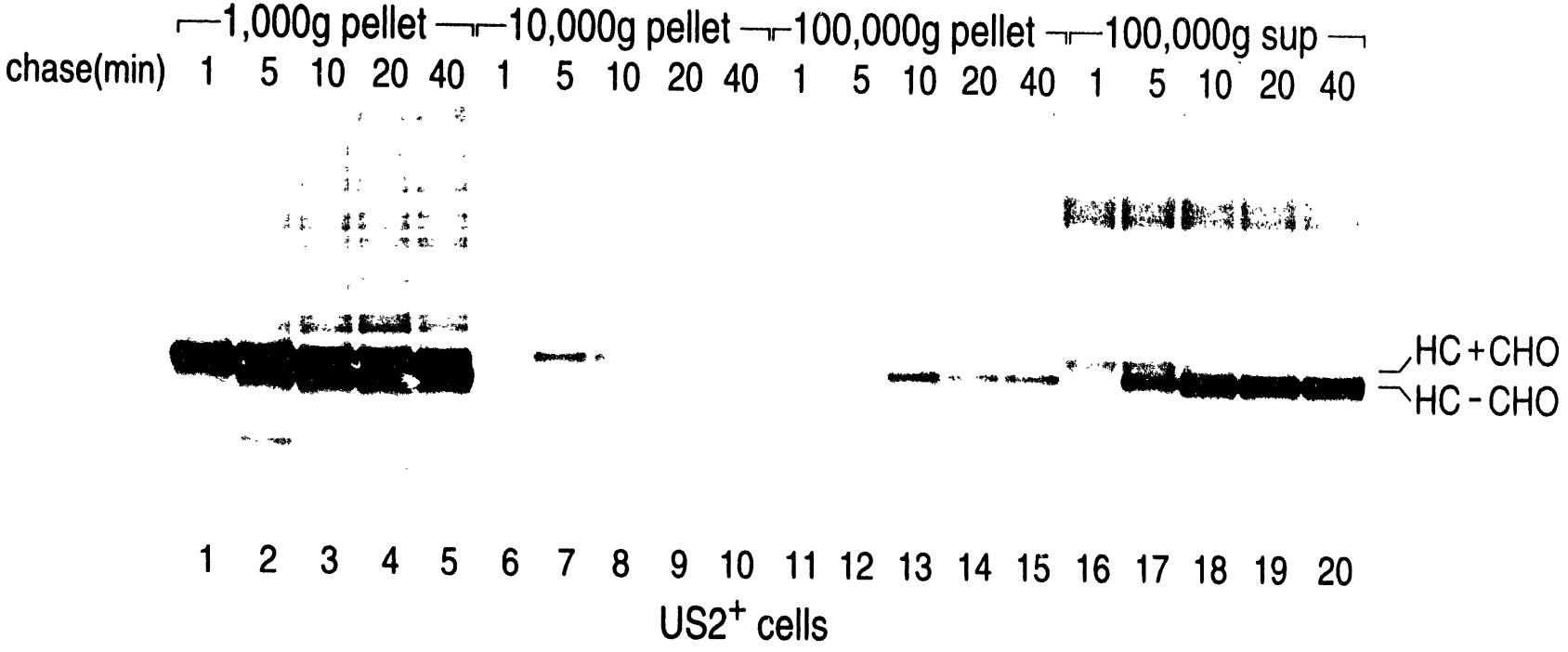
Since the proteasome and the peptide N-glycanase enzymes are thought to be predominantly cytosolic, we next examined whether the heavy chain intermediate could be found in the cytosol en route to destruction. Subcellular fractionation was performed on lysates from pulse-chased control or US11+ cells (figure 4.5I). The fraction obtained from the 1000 g spin is likely to represent unbroken cells and larger cellular debris that may trap soluble proteins. Therefore, the emphasis should be placed on the 10,000 and 100,000 g samples. The intact, glycosylated heavy chain could be precipitated from all particulate fractions (1000, 10,000, and 100,000 g) but not from the 100,000 g supernatant in either control or US11+ cells. The de-glycosylated intermediate from LLnL treated US11+ cells could also be precipitated from all three pellet fractions. However, the majority of this polypeptide was found in the 100,000 g supernatant. Further, to assure that the fractionation was successful, the lysate fractions were used to precipitate several control membrane associated proteins. In all cases, the membrane proteins were found exclusively in the pellet fractions, including US11 itself. The lack of detectable US11 in the 100,000 g supernatant fraction indicates that heavy chains must be escorted by US11 to the cytosol and at this point no association remains between the two molecules.

Figure 4.5. In US11+ cells treated with LLnL and US2+ cells treated with Z-L_3-H, most class I heavy chains are present in the cytosol.

(I) Subcellular fractionation on US11+ and control cells was performed as described in the Experimental Procedures. Class I heavy chains were immunoprecipitated from the subcellular fractions using the rabbit anti-heavy chain serum (A). Note the recovery of the breakdown intermediate from the 100,000 g supernatant. The β_2m molecules were immunoprecipitated using the monoclonal antibody BBM.1 (B). The US11 glycoprotein could be recovered from US11+ cells but not from control cells (C). Note that US11 sediments largely with the microsomal fractions and is absent from the 100,000 g supernatant, the compartment that contains the heavy chain breakdown intermediate. Calnexin (D) and transferrin receptor (TfR; E) were immunoprecipitated using the monoclonal antibodies AF8 and 66Ig10, respectively.
(II) Subcellular fractionation of US2+ cells performed as described for (I) except cells were treated with Z-L_3-H instead of LLnL. Class I heavy chains were immunoprecipitated using the rabbit anti-heavy chain serum. As found for US11+ cells, the breakdown intermediate was found largely in the cytosolic fractions (HC-CHO).


(I)
Pellet
Sup
1000
10,000
100,000
100,000
a b a b a b a b
(A)
43–
Control
HC
43–
40–
US11+
(B)
12–
Control
β2m
12–
US11+
(C)
33–
Control
US11
33–
US11+
(D)
90–
Control
Calnexin
90–
US11+
(E)
90–
Control
TfR
90–
US11+
1 2 3 4 5 6 7 8

(II)


1,000g pellet
10,000g pellet
100,000g pellet
100,000g sup
chase(min) 1 5 10 20 40 1 5 10 20 40 1 5 10 20 40 1 5 10 20 40
HC+CHO
HC-CHO
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
US2+ cells

A similar result is obtained from fractionation experiments performed on US2+ cells (figure 4.5II). The fully glycosylated class I heavy chains are found predominantly in the pellet fractions from the 1000, 10,000, and 100,000 g centrifugations, and the de-glycosylated heavy chains are found mainly in the 100,000 g supernatant. In contrast to US11, US2 exists as two forms that differ by the presence of a single N-linked glycan (as identified by treatment with Endo H). Both forms of US2 are detected in US2+ cells as well as in HCMV infected cells (Wiertz et al., 1996). Direct association with class I heavy chains can be demonstrated by co-immunoprecipitation of US2 using an antibody specific for properly folded heavy chains (figure 4.6). Fractionation experiments also indicate that the de-glycosylated form of US2 is found in the cytosol with the de-glycosylated heavy chain. Cytosolic US2 which has lost its glycan is also degraded by a process which is blocked by inhibitors of the proteasome (figure 3.6). These data, taken together, support a mechanism of dislocation of heavy chains by US2 in which US2 itself undergoes the same destructive fate as the heavy chains which it is escorting.

5. The de-glycosylated class I heavy chain directly associates with the proteasome.

In order to further establish the involvement of the proteasome in the US11 and US2 mediated breakdown of class I heavy chains, we examined whether breakdown intermediates could be detected in a physical complex with the proteasome when incubated with inhibitors which blocked its proteolytic activity. Precipitation of heavy chains from pulse chased control, US2+ , and US11+ cells followed by denaturaton of the primary precipiatations and re-immunoprecipitation with the same anti heavy chain serum resulted in the appearance of the characteristic 40 and 43 kDa bands for the glycosylated and de-glycosylated heavy chains. Immunoprecipitation from the same samples first using a proteasome specific antibody followed by denaturaton and re-precipitation with anti heavy chain serum indicated that only the 40 kDa polypeptide corresponding to the de-glycosylated class I heavy chain could be detected in the second immunoprecipitation and only when US2 or US11 was present (figure 4.7A).

Figure 4.6. US2 molecules form complexes with properly folded class I heavy chains, is deglycosylated and destroyed by the proteasome.

(A) US2+ cells were pre-treated with lactacystin, Z-L_3-H, Z-L_3-VS or tunicamycin for 1 hour then pulse labeled for 15 min. and chased for 0 and 30 min. Properly folded class I molecules were immunoprecipitated using the monoclonal antibody W6/32. Note the presence of the US2 glycoprotein (US2 + CHO; 24 kDa) in the W6/32 precipitates. The deglycosylated heavy chain is not detected in the W6/32 precipitations.
(B) The same samples from (A) were precipitated with anti-US2 polyclonal rabbit serum. US2 exists as a glycosylated (24 kDa, US2 + CHO) and as a non-glycosylated form (22 kDa; US2 - CHO). The non-glycosylated form is destroyed in a manner sensitive to inhibitors of the proteasome.

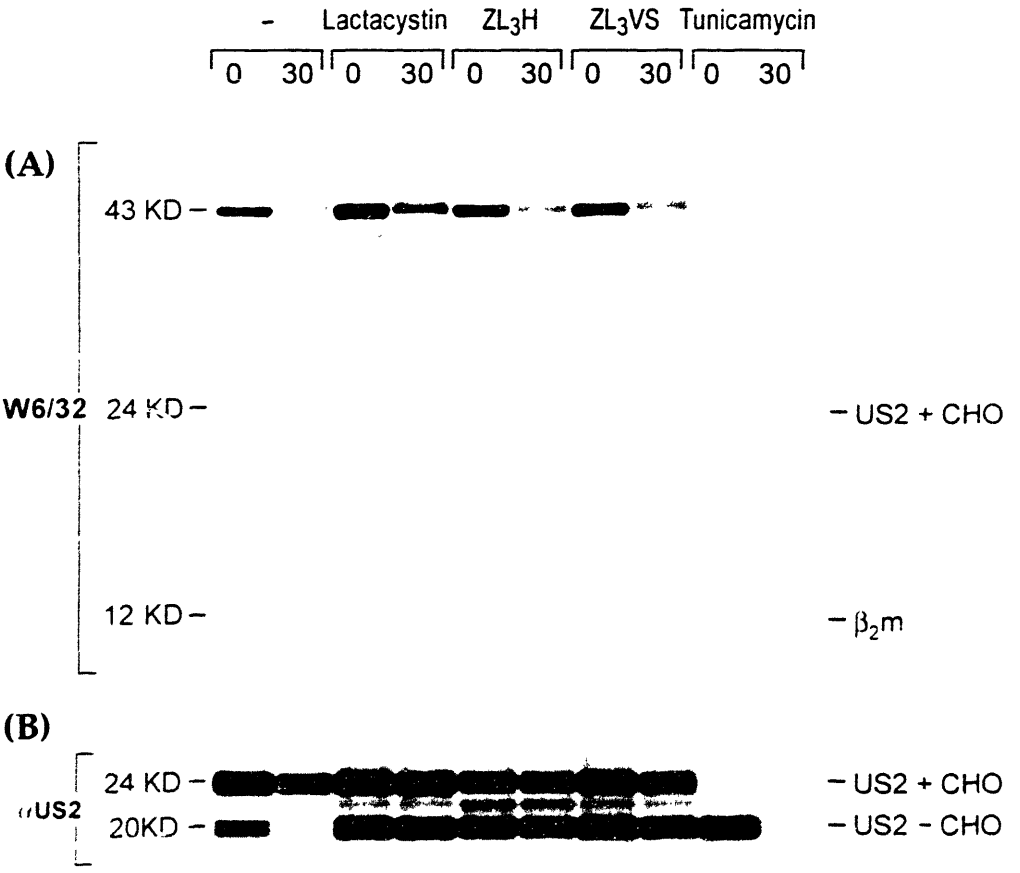
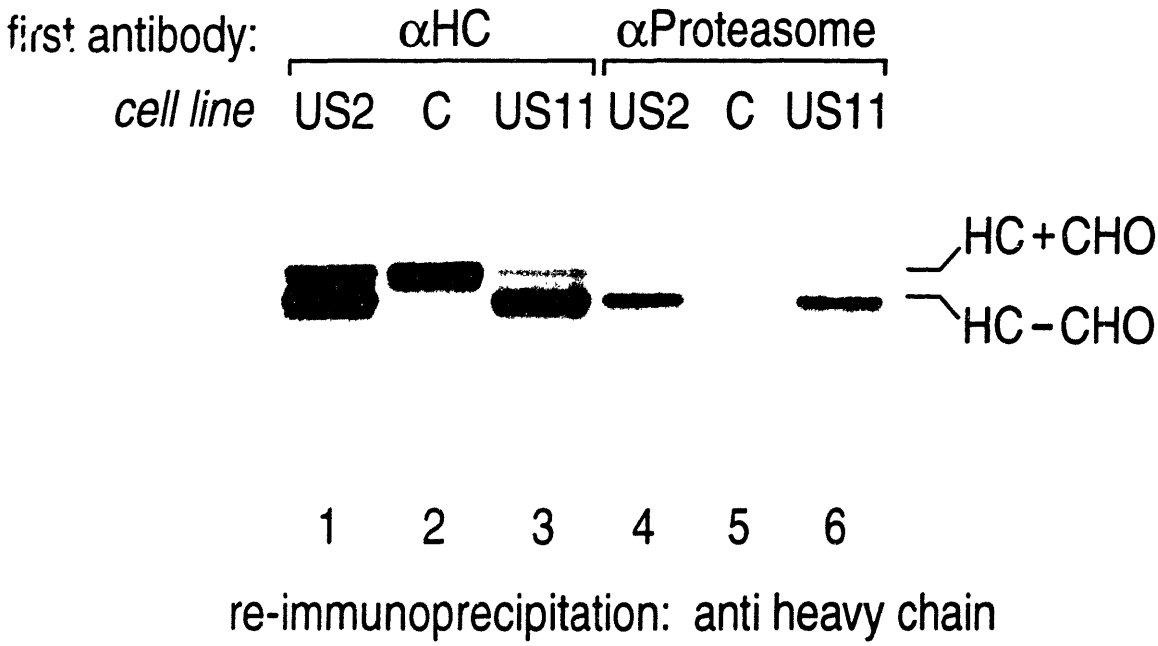
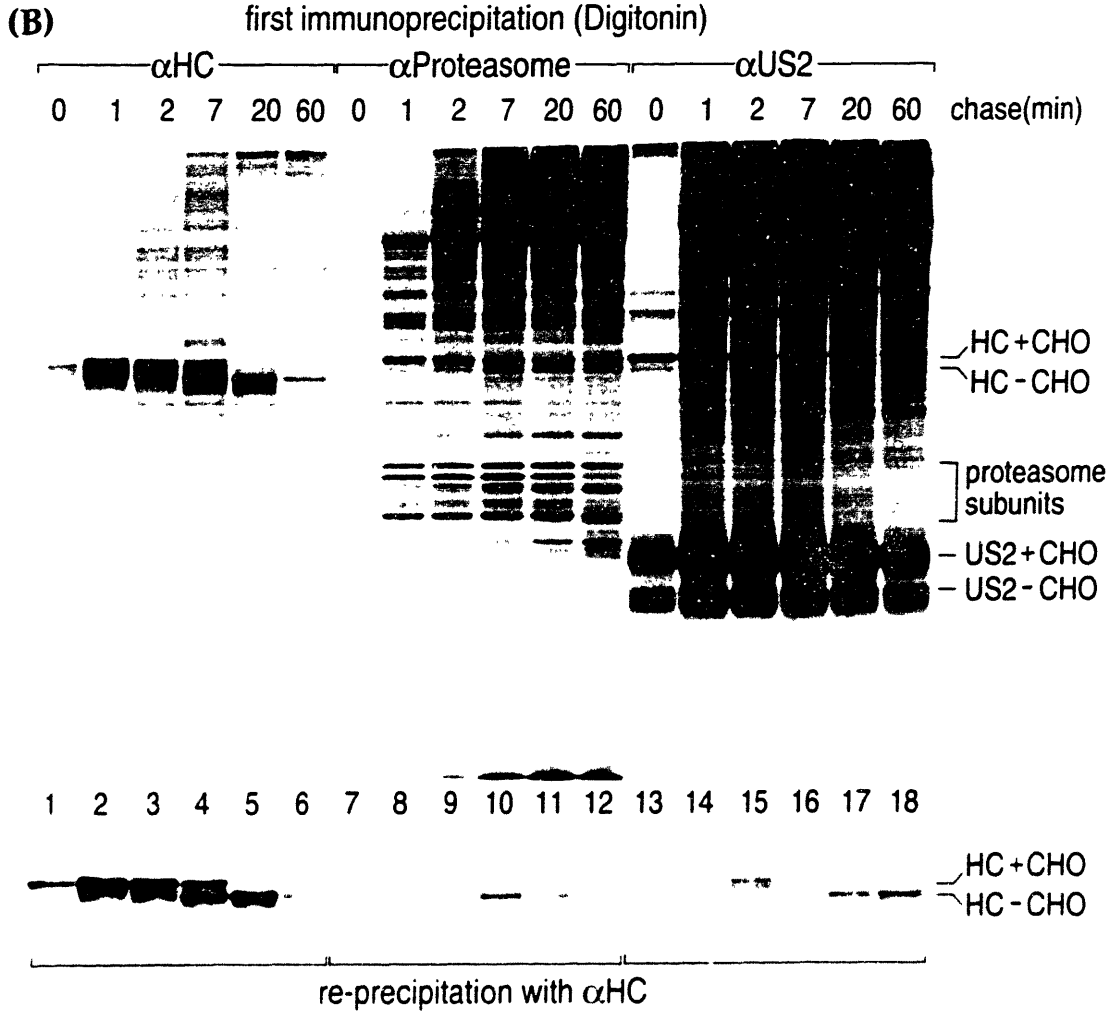

- Lactacystin ZL3H ZL3VS Tunicamycin
0 30 0 30 0 30 0 30 0 30
(A)
43 KD –
W6/32
24 KD –
– US2 + CHO
12 KD –
– β2m
(B)
24 KD –
αUS2
20KD –
– US2 + CHO
– US2 - CHO

Figure 4.7. The deglycosylated class I heavy chain breakdown intermediate can be found as a complex with the proteasome in US2+ and US11+ cells.

(A) US2+ and US11+ cells were pre-treated with Z-L_3-H, pulse labeled and chased as described in the Materials and Methods section. Digitonin lysates were immunoprecipitated with rabbit anti-heavy chain serum and an anti-proteasome antibody and the precipitates were completely dissociated and re-immunoprecipitated with rabbit anti-heavy chain serum. The position of migration of the heavy chains (HC) with or without N-linked glycan (+/- CHO) are indicated. Note the presence of the de-glycosylated heavy chain in proteasome precipitations from US2+ and US11+ transfectants only.
(B) A Pulse chase experiment was performed on cells treated with Z-L_3-H as described in (A) except several chase points were taken as indicated. Digitonin lysates were immunoprecipitated with the antibodies indicated at the top of the figure and the precipitates, following their complete dissociation in SDS, were re-immunoprecipitated with rabbit anti-heavy chain serum. The position of migration of US2 (US2) and heavy chain (HC) with and without N-linked glycan (+/- CHO) are indicated.

(A)


first antibody:
αHC
αProteasome
cell line
US2
C
US11
US2
C
US11
HC+CHO
HC-CHO
1
2
3
4
5
6
re-immunoprecipitation: anti heavy chain


(B)
first immunoprecipitation (Digitonin)
αHC
αProteasome
αUS2
0 1 2 7 20 60 0 1 2 7 20 60 0 1 2 7 20 60 chase(min)
HC+CHO
HC-CHO
proteasome subunits
US2+CHO
US2-CHO
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
HC+CHO
HC-CHO
re-precipitation with αHC

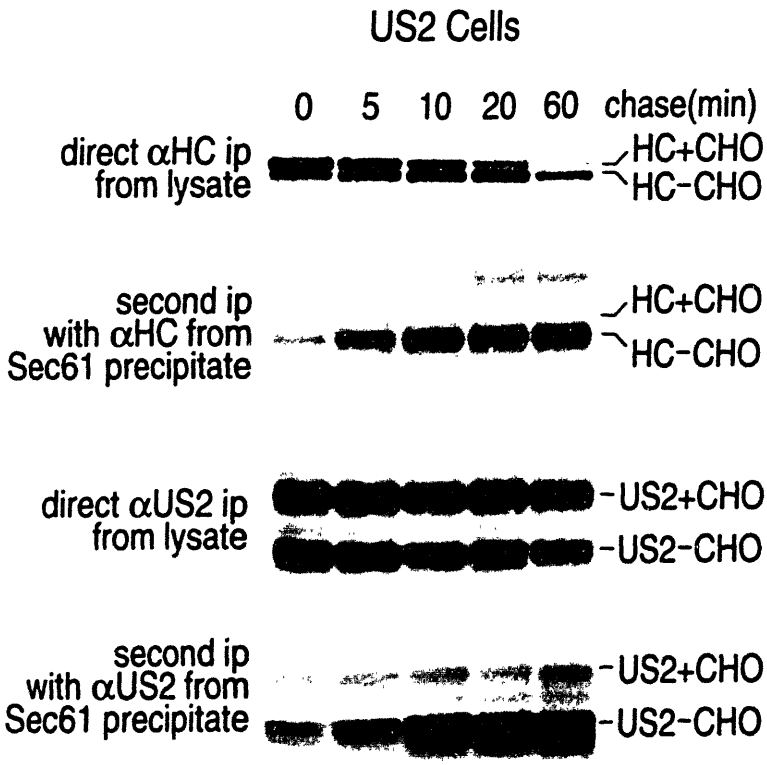
To further examine the interaction of the class I heavy chain breakdown intermediate with the proteasome, a pulse chase was performed on US2+ cells followed by immunoprecipitation, SDS denaturation, and re-immunoprecipitation (figure 4.7B). The presence of the deglycosylated class I heavy chain could be detected by pre treatment with the proteasome inhibitor Z-L_3-H. Again, only the de-glycosylated heavy chain could be re-precipitated from primary proteasome IPs. The amount of deglycosylated heavy chains recovered from proteasome precipitations was maximal between the 7 and 20 min chase point, consistent with the time scale for conversion of precursor glycosylated heavy chains to deglycosylated product. taken together these results prove a direct interaction of the proteasome with the deglycosylated heavy chain prior to its destruction.

6. US2 and the class I heavy chain intermediate associate with Sec61p.

We next examined whether it was possible to detect US2 and class I heavy chains in the process of being reverse translocated from the ER into the cytosol. Since the process of insertion in to the lumen of the ER is performed by the multi-subunit translocation complex Sec61p, we reasoned that perhaps the process of reverse translocation is accomplished by same membrane pore that is responsible for insertion of membrane bound glycoproteins. Due to the rapid kinetics of US2 and US11 catalyzed destruction of heavy chains, we examined whether we could detect breakdown intermediates in a complex with Sec61p in the process of undergoing reverse translocation. A pulse chase was performed on Z-L_3-H treated US2+ cells followed by primary immunoprecipitation with an antibody against the Sec61p β subunit and then re-immunoprecipitation with antibodies against either class I heavy chains or US2 (figure 4.8). Only the de-glycosylated form of class I heavy chains could be isolated from precipitations of Sec61. This data indicates that removal of the glycan from the intact heavy chain takes place while the class I heavy chain is still tethered to the translocation complex. Further, the heavy chain intermediate could not be isolated from Sec61 precipitations in the absence of the proteasome inhibitors, indicating that the process of reverse translocation and degradation by the proteasome are likely to be closely lcoupled. US2 could also be recovered from precipitations with the

Figure 4.8. Class I molecules destined for degradation associate with Sec61p.

Sec61 was immunoprecipitated from digitonin lysates of Z-L_3-H-treated cells and the precipitates were re-immunoprecipitated with either rabbit anti-heavy chain serum (αHC) and or rabbit anti-US2 serum (αUS2). Sec61 depleted lysates were also immunoprecipitated with αHC or αUS2. These samples are designated as direct immunoprecipitates. The positions of migration of heavy chains (HC) and US2 molecules (US2) with or without N-linked glycan (+/- CHO) are indicated. Exposure time of the autoradiogram for the re-precipitation experiments was 14 days; for direct precipitations, exposure was 2 days.


US2 Cells
0
5
10
20
60
chase(min)
direct αHC ip
from lysate
HC+CHO
HC-CHO
second ip
with αHC from
Sec61 precipitate
HC+CHO
HC-CHO
direct αUS2 ip
from lysate
US2+CHO
US2-CHO
second ip
with αUS2 from
Sec61 precipitate
US2+CHO
US2-CHO

Sec61β antibody. Similar to the results obtained for class I heavy chains, the deglycosylated form of US 2 was the predominant species found associated with Sec61p.

D. Discussion

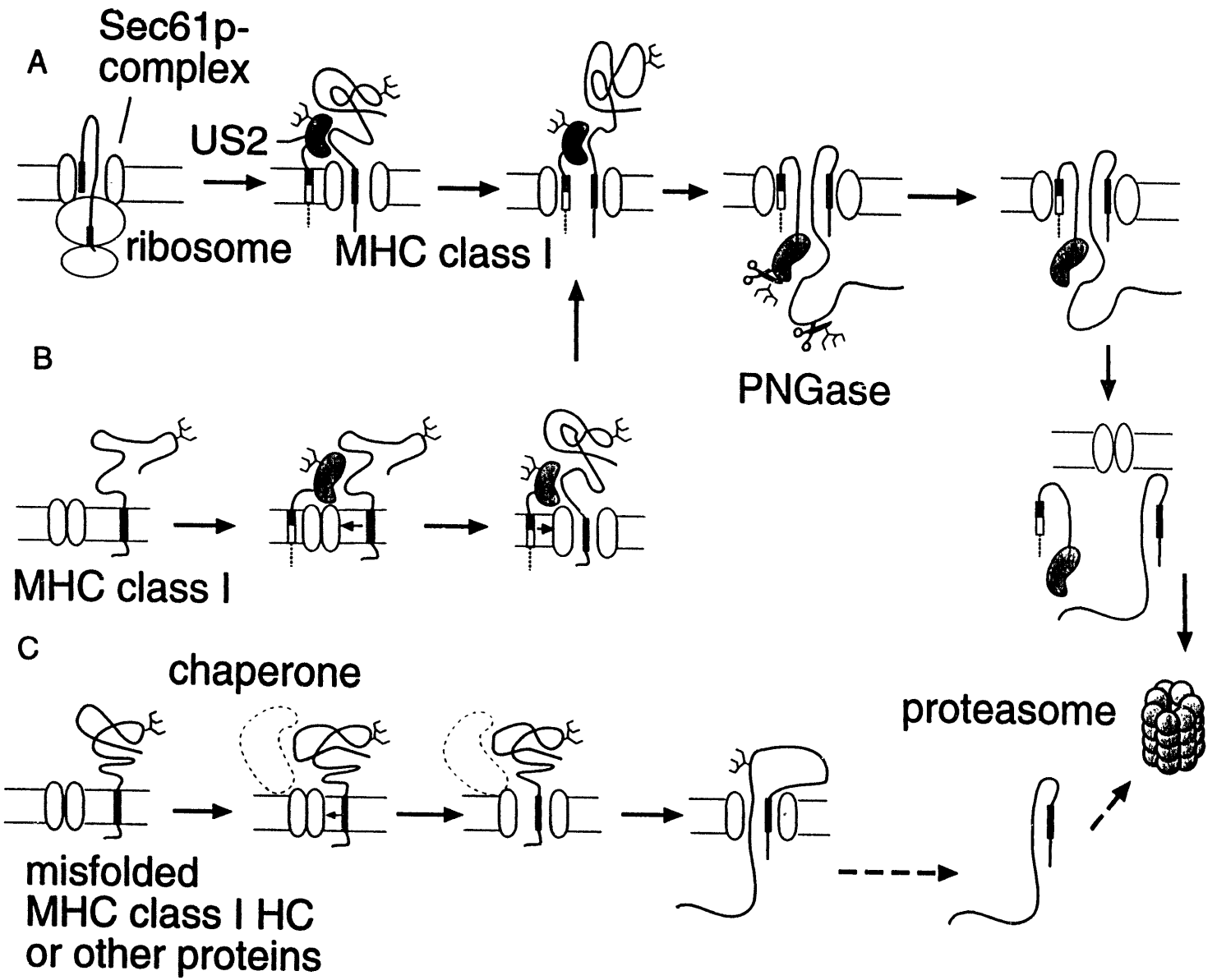
The process by which HCMV is able to elude the host immune response by down regulation of class I expression at the cell surface is yet another example of a clever viral evasion strategy. This down regulation of class I heavy chains is an extremely rapid process catalyzed by the viral gene products US2 and US11, involving the Sec61p translocation complex and the proteolytic activity of the proteasome. The process appears to be a reversal of the translocation process used to insert the newly synthesized heavy chain into the ER. Upon exposure to the cytosol, the fully glycosylated class I heavy chain is acted upon by a N-glycanase, rapidly removing its sugars while still associated with the Sec61 membrane channel. This deglycosylated heavy chain is then destroyed by the proteolytic action of the proteasome (figure 4.9).

Although transfection of cells with either US2 or US11 both result in the loss of class I expression, details of the mechanism of this down-regulation show that the two may accomplish their goal through different mechanisms. Fractionation experiments performed on US11+ cells showed that although the majority of the deglycosylated heavy chain was found in the cytosolic 100,000 g fraction, no US11 was found co-localized in that fraction. Thus, US11 may exert its effects distal to class I heavy chains and it may in fact be through other accessory proteins that it is able to induce dislocation of class I molecules. A possible scenario exists in which US11 interferes with proteins that have a chaperone-like function and are required for proper membrane translocation. In this case slowing or stopping of the translocation process may result in reversal of the insertion process.

US2, on the other hand, directly associates with the newly synthesized class I molecule and the de-glycosylated form, like the deglycosylated class I heavy chain, is found in the cytosol. Direct association of the deglycosylated US2 molecule with the Sec61p complex indicates that, in association with the class I heavy chains it is designed to destroy, it is dislocated through the Sec61 pore and is then deglycosylated by an N-glycanase and destroyed by the proteasome (figure 4.9). The fact that two separate gene products exist in the HCMV genome which are responsible for producing the same end result indicates the importance of the class I mediated cytotoxic T-cell response in viral

Figure 4.9. Model for Sec61-dependent dislocation.

(A) A nascent class I heavy chain inserts into the ER in a signal sequence-dependent fashion. US2 interacts with the class I heavy chain before the lateral escape from the Sec61 complex into the membrane. The lumenal, glycosylated domains of both class I heavy chains and US2 are subject to dislocation. A reaction that culminates in the action of N-glycanase and proteasomal proteolysis.
(B) An ER resident, properly folded class I heavy chain can be recruited by US2 to re-enter the Sec61 complex. US2 itself re-enters the Sec61 complex and both polypeptides are dislocated
(C) In normal cells, any misfolded or incompletely assembled protein could be recruited by the action of an ER-resident chaperone to re-engage the Sec61 complex for dislocation and cytosolic destruction. Whereas in (A) and (B), proteolysis may become rate-limiting owing to the high rate of dislocation, in (C) dislocation is the rate-limiting step.


A
Sec61p-
complex
US2
ribosome
MHC class I
B
PNGase
MHC class I
C
chaperone
proteasome
misfolded
MHC class I HC
or other proteins

clearance by the host. Further, the fact that US2 and US11 exert their effects by separate mechanisms suggest that there is a reason for duplication of tasks in this organism which may be explained by differential sensitivity of different hosts to HCMV infection.

The process by which the newly synthesized class I heavy chains are escorted by the viral gene products US2 or US11 into the cytosol through the Sec61p complex is extremely rapid and therefore most of the polypeptides undergoing this fate may yet have to be inserted into the lipid environment of the ER membrane. However, US2 and US11 are also capable of causing the destruction of properly folded class I molecules (Wiertz et al., 1996b). Since these class I molecules have already left the translocation machinery and are likely to have entered the membrane of the ER, it must also be possible for US2 and US11 to drag these molecules back into the Sec61 pore for reverse translocation (figure 4.9B). This process may be accomplished either directly or indirectly through other chaperone proteins.

The process of reverse translocation of an ER-resident protein into the cytosol for destruction by the proteasome is not likely to be unique for HCMV mediated destruction of class I heavy chains. There is mounting evidence that the process of retrograde transport from the ER into the cytosol is a general phenomenon used by normal cells to purge the ER of misfolded and abnormal proteins to be destroyed (figure 3.9C). In cells lacking the MHC class I light chain β_2M (known as Daudi cells), class I molecules are unable to assemble and must be destroyed. This destruction is sensitive to inhibitors of the proteasome, and a de-glycosylated form of the heavy chain can be detected (Hughes et al., 1997). Likewise, when the T-cell receptor α chain is expressed alone in TCR- cells, the monomeric subunit is rapidly degraded. This breakdown process is similar to the process described here, including the presence of a cytosolic de-glycosylated intermediate which can be observed only in the presence of proteasome inhibitors (Huppa and Ploegh, 1997). Interestingly, a similar breakdown process is observed when the T-cell receptor δ chain is expressed in the absence of other TCR subunits, however, no cytosolic intermediate is observed even thought the degradation can be inhibited by compounds such as lactacystin (A. M. Weissman communicated at the Cold Spring Harbor Symposium on the Biology of Proteolysis, 23-27 April, 1997). These findings

suggest that the process by which a protein is translocated from the Sec61 complex may be directly and physically coupled to the proteasome. Finally, there have been reports of integral membrane proteins which become ubiquitinated and degraded by the proteasome (Jensen et al., 1995). Although the process of reverse translocation is likely to be utilized in normal protein turnover, there is evidence to suggest at least some ER resident proteins are degraded within the ER in a proteasome-independent fashion (Mullins et al., 1995; Brodsky and McCracken, 1997).

The steps by which US2 and US11 are able to accomplish the controlled destruction of class I heavy chains were uncovered largely due to the ability to modulate the activity of the proteasome using pharmacological agents in living cells. The initial discovery that the commercially available compound, LLnL, resulted in the presence of an intermediate in the breakdown process of class I heavy chains was the first indication that the proteasome might be involved. Further studies using more specific inhibitors such as lactacystin and the peptide vinyl sulfone Z-L_3-VS, as well as ability to detect the deglycosylated class I heavy chain in a direct complex with the proteasome, proved that degradation was in fact being performed by the proteasome. Although proteasome inhibitors served to answer several important questions about the mode of action of these viral proteins, US2 and US11 transfectants may now also be used to answer fundamental questions about the reactivity and potency of different classes of proteasome inhibitors in living cells. The appearance of the 43 kDa deglycosylated polypeptide from class I heavy chain immunoprecipitations can be used as an indirect indication of proteasome inhibition. This system is likely to be extremely useful for assessing the activity of pharmacological agents against the proteasome, using a physiologically relevant target as a readout (see Chapter 2)

V. A NEW PROTEOLYTIC SYSTEM IN MAMMALIAN CELLS THAT CAN COMPENSATE FOR THE LOSS OF PROTEASOME FUNCTION

A. Introduction

Cytosolic proteolysis is controlled predominantly by the proteasome. This large, multi-catalytic protease complex is responsible for degradation of a wide range substrates required for cell survival (Coux et al., 1996). Not surprisingly, inhibition of proteasomes, either by genetic deletion or by inactivation by pharmacological agents results in death of the organism (Drexler, 1997; Gordon et al., 1993; Ghislain et al., 1993). We have found that prolonged exposure of cells to the peptide vinyl sulfone inhibitors leads to the outgrowth of distinct populations of cells whose survival is refractory to the action of these compounds. These cells, termed adapted, provide information about additional proteolytic systems that, in normal cells, may serve a complementary function to the proteasome.

The proteasome has been implicated in the proteolytic destruction of a large number of metabolically important proteins. The highly regulated timing of progression of cell division is controlled at several stages by proteasomal degradation of various cyclins and cyclin dependent kinase inhibitors (Glotzer et al., 1991; King et al., 1994). These proteins are first tagged with a poly-ubiquitin chain, known to be a signal for degradation by the 26S proteasome (Hershko and Ciechanover, 1992). Selective inhibitors have been used to confirm the proteasome's role in this degradation process. In addition, application of compounds such as peptide aldehydes and lactacystin results in a G2 arrest in the cell cycle (Drexler et al., 1997).

Proteasome subunit deletion mutants in yeast have shown the indispensability of the proteasome for cell survival. Deletion of a single catalytically active β-subunit results in a lethal phenotype (Hilt et al., 1993; Heinemeier et al., 1994; Hilt and Wolf, 1995; Orlowski, 1990). The lethality is presumably due to the inability of proteasomes to form viable complexes with the proper 7-fold symmetry, rather than elimination of proteolysis. However, replacement of a wild type β-subunit gene with a catalytically inactive mutant β-subunit results in viable yeast with impaired growth (Heinemeyer et al., 1993; Heinemeyer et al., 1991; Hilt et al., 1993; Enenkel et al., 1994). These mutants allow for the assembly of proteasome complexes which have impaired proteolysis but nonetheless retain enough activity to allow the organism to

survive. The extent to which proteasome mutants affect yeast cell growth depends on the number and types of subunits inactivated by mutation (Hilt et al., 1993; Hilt and Wolf, 1995). Mutations of this type have also helped to establish the role of individual subunits in the generation of specific proteolytic activities of the proteasome complex (Heinemeyer et al., 1993; Hilt et al., 1993; Heinemeyer et al., 1991; Enenkel et al., 1994).

Inhibition of the proteasome by compounds such as the tri-peptide vinyl sulfone, NIP-L3VS (NLVS), which are able to modify all catalytically active β-subunits (see chapter 2), results in cell death. This toxicity correlates with the ability of a compound to inhibit the proteasome's multiple active sites and therefore is likely to be the result of loss of proteasome function. We describe here that when cells are maintained in the continuous presence of NLVS at the lowest concentration that results in toxicity, a small population of cells (adapted cells) are able to survive. These cells are not the result of a genetic mutation nor are they caused by a multi-drug resistance mechanism which might have prevented the inhibitor from reaching its target. Purification of proteasomes from these adapted cells indicated that their ability to hydrolyze fluorogenic substrates is greatly impaired. Likewise, proteasomes from adapted cells treated with the radiolabeled form of NIP-L_3VS fail to label, indicating that the active sites have in fact been covalently inactivated.

The finding that adapted cells, unlike normal cells treated with proteasome inhibitors, appeared to have normal levels of ubiquitin conjugated proteins and are able to generate peptides for binding to MHC class I molecules prompted us to look for other proteolytic systems which could compensate for the absence of the active proteasome. Analysis of non-proteasomal proteolysis using the fluorogenic substrate AAF-MCA, indicates that cleavage of this substrate is enhanced in adapted cells. This AAF hydrolyzing activity was found to be a high molecular weight protein complex which is distinct from the proteasome and which behaves in gel filtration as a complex with an apparent molecular weight > 1,000 kDa. Blocking of this activity using a the tri-peptide chloromethyl ketone, AAF-cmk results in a block of cell growth of adapted but not normal cells. Thus, it appears that inhibition of proteasomal proteolysis results in activation of additional proteases which promote survival by performing crucial functions normally carried out by the proteasome.

B. Materials and Methods

1. Cells and Cell Culture

The mouse lyphobiastoma cell line EL-4 was used for all experiments reported in this chapter. Cells were maintained in Dulbecco's Modified Eagle's medium supplemented with 10 % (v/v) fetal calf serum. Adapted EL-4 cells were maintained in the presence of 50 μM of 5-iodo-4-hydroxy-nitrophenyl acetyl-leucyl-leucyl-leucine vinyl sulfone (NLVS; see chapter 2 and 3). NLVS was diluted from DMSO stocks such that the final concentration DMSO was less than or equal to 1%.

2. Inhibitors

^{125}I-NIP-L_3-VS, NIP-L_3-VS (NLVS), NP-AAF-VS, and NP-LLG-tVS were synthesized as described in chapters 2 and 3. All inhibitors were added to lysates and to cells by dilution of a DMSO stock solution such that the final concentration of DMSO was 1% or less. The chloromethyl ketone AAF-cmk was added to tissue culture media by dilution of a 5 mM water stock to a final concentration of 5 μM. The synthesis of the tri-peptide chloromethyl ketone AAF-cmk is shown below.

3. Synthesis of alanine-alanine-phenylalanine chloromethyl ketone (AAF-cmk)

a. Synthesis of Boc-Ala-Phe-OMe

Boc-Alanine (1.3 g, 7.0 mmol), HOBt (1.1, 8.3 mmol) and dicyclohexylcarbodiimide (DCC; 1.7 g, 8.3 mmol) were dissolved in 10 mls of DMF. the reaction was stirred at room temperature for 30 min. Phenylalanine-methyl ester hydrochloride salt (1.0 g, 4.6 mmol) was then added to the flask followed immediately by diisopropylethyl amine (0.88 ml, 5.1 mmol) and the reaction was stirred overnight at room temperature. The resulting reaction mix was filtered to remove the dicyclohexylurea by-product and the DMF was

removed by rotary evaporation. The crude oil was then diluted into 50 mls of ethyl acetate and washed 3 times with equal volumes of 0.1 N HCl and 3X with equal volumes of saturated sodium bicarbonate. The remaining organic phase was dried over magnesium sulfate and dried to a solid. The product was re-crystallized from ethyl acetate/hexane. Yield (1.25 g, 3.6 mmol, 77%).

Boc-ala-Phe-OMe- ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.10-7.35 (4H, m), 6.79 (1H, d), 5.10 (1H, t), 4.80 (1H, d), 4.20 (1H, q), 3.75 (3H, s), 3.05-3.25 (2H, dd), 1.40 (9H, m), 1.20 (3H, m).

b. Synthesis of Boc-Ala-Phe-OH

Boc-Ala-Phe-OMe (2.9 mmol, 1.0 g) was dissolved in 10 ml methanol. Potassium carbonate (10 mls of a 20% solution) was added to the flask and the reaction was stirred overnight. A white precipitate formed upon addition of the aqueous carbonate solution but disappears overnight. The methanol was then removed by rotary evaporation and the remaining water solution was diluted wit 50 ml of citrate buffer pH 4.0 and acidified to pH 4 by addition of 3N HCl. Upon neutralization the product precipitates as a white solid and is extracted with 3 volumes of ethyl acetate. The organic phase is then dried over magnesium sulfate and evaporated to dryness by rotary evaporation resulting in pure Boc-Ala-Phe-OH (0.96 g, 2.87 mmol, 99%).

Boc-Ala-Phe-OH ^{1}H-NMR (500 MHz, $CDCl_3$): same as for Boc-Ala-Phe-OMe except loss of methyl ester signal at δ 3.75.

c. Synthesis of Boc-Ala-Phe-diazomethyl ketone (Boc-AF-CHN_2)

Boc-Ala-Phe-OH (0.5g, 1.5 mmol) was converted to the mixed anhydride by reaction with isobutyl-chloroformate (IBCF; 200 µl, 1.5 mmol) and N-methyl-morpholine urea (NMM; 182 µL, 1.65 mmol) at -15°C (NaCl/ ice bath). The activated Boc-Ala-Phe-OH was then added to etherial diazomethane freshly distilled from diazald (3.55 g, 16.3 mmol) and potassium hydroxide (3.55 g, 61.4 mmol) as described by the manufacturer (Aldrich Chem. Co., Milwaukee, WI).

The reaction is stirred for 30 min at 0°C and then overnight at room temperature. The reaction was quenched by the addition of water until the formation of N_2 gas ceased. The resulting solution was then diluted with ether and washed with three volumes of water and three volumes of saturated sodium bicarbonate. The organic phase was then dried over magnesium sulfate and concentrated to an oil by rotary evaporation. The crude Boc-Ala-Phe-CHN_2 was purified by flash chromatography using a 1:1 ethyl acetate:hexane (v:v) as the mobile phase. Rf (1:1 EtOAc:Hex): 0.35. Yield (285 mg, 0.8 mmol, 53%).

Boc-Ala-Phe-CHN_2 ^{1}H-NMR (300 MHz, $CDCl_3$): δ 7.10-7.35 (4H, m), 6.62 (1H, d), 4.8-535 (1H, d), 4.65 (1H, t), 4.10 (1H, q), 3.05 (2H, d), 1.40 (9H, d), 1.25 (3H, d).

d. Synthesis of Ala-Phe-Chloromethyl Ketone (AF-cmk)

Boc-Ala-Phe-CHN_2 (250 mg, 0.69 mmol) was dissolved in 1 ml of methanol. HCl (1M solution in ether; 3.45 ml, 3.45 mmol) was then added dropwise. The reaction was stirred until the formation of N_2 gas ceased (20 min). The reaction solution was then evaporated to dryness and the product was obtained in pure form by trituration from ether. Yield (146 mg, 0.48 mmol, 69%).

Ala-Phe-cmk ^{1}H-NMR (300 MHz, CD_3OD): δ 7.21-7.38 (4H, m), 5.95 (1H, t), 4.22-4.50 (2H, dd), 3.90 (1H, q), 2.95-3.29 (2H, ddd), 1.48 (3H, d).

FAB Mass Spectrum: [M+H]Calculated for $C_{13}H_{17}N_2O_2Cl$: 269.5 Found: 269.2

e. Synthesis of Boc-Ala-Ala-Phe-Chloromethyl Ketone (Boc-AAF-cmk)

Ala-Phe-cmk (50 mg, 0.16 mmol), Boc-alanine (34 mg, 0.18 mmol), and PyBOP (99mg, 0.18 mmol) were dissolved in 1 ml of methylene chloride/dimethyl formamide (1:1; v/v). Triethylamine (50 µl, 0.34 mmol) was then added to the reaction mix. After 1 hour the reaction was quenched by removal of solvent followed by addition of 5 volumes of ethyl acetate. The resulting organic phase was washed three times with water then dried over

magnesium sulfate. The crude solid was purified by flash chromatography using 1:1 ethyl acetate:hexane (v:v) as the mobile phase. Rf (1:1 EtOAc:Hex); 0.2. Yield (26.2 mg, 0.06 mmol, 36%).

f. Synthesis of Ala-Ala-Phe-Chloromethyl Ketone (AAF-cmk)

The Boc group was removed from Boc-Ala-Ala-Phe-cmk (26.2 mg, 0.06 mmol) in 1ml of ethanol by the addition of 2 ml of a 1M solution of HCl in ether. The reaction was stirred for 2 hours at which time no starting Boc peptide could be seen by TLC. The reaction mix was then evaporated to dryness and the pure product was obtained by trituration from ether. Yield (22.2 mg, 0.059 mmol, 99%).

Ala-Ala-Phe-cmk ^{1}H-NMR (300 MHz, CD_3OD): δ 7.21-7.30 (4H, m), 4.60 (1H, dd), 4.25 (1H, q), 4.22-4.40 (2H, dd), 2.95-3.19 (2H, ddd), 1.45 (3H, d), 1.25 (3H, d).

Mass Spectrum: [M+H] Calculated for $C_{16}H_{22}N_3O_3Cl$: 340.8 found: 340.1

4. Antibodies

The following antibodies were used: anti-C9 (kind gift of Dr. John Monaco, University of Cincinnati) a polyclonal antiserum reactive against the C9 α-subunit of the proteasome; Y3 a mono-clonal antibody which recognizes the a1/a2 domain of properly folded mouse class I heavy chains (Hämmerling et al., 1982). P8 a mono-clonal antibody which recognizes an epitope in the cytoplasmic tail of H-2 K^b mouse MHC class I heavy chains (prepared in our laboratory as described; (Smith et al., 1986)); and anti-ubiquitin (Kind gift from Dr. Arthur L. Haas, Medical College of Wisconsin) a ploy clonal antiserum reactive with ubiquitin.

5. Cell Growth Analysis

Cells (100,000 in 1 ml of media) were grown in the presence of inhibitor or DMSO (1%) as a control in 24 well plates as described in the cell and cell culture section above. Daily, the cells were re-suspended, 20 µl aliquots were removed and mixed with trypan blue dye (0.1 %). Cells which did not take up the dye were considered to be alive and counted. Growth curves were generated by plotting number cells against time in days.

6. Limiting Dilution to Determine Frequency of Adaptation

EL-4 cells were plated in 96 well plates at increasing dilutions from 10,000 cells per well down to 39 cells per well by serial dilution by a factor of 4. Growth was monitored for 1 month. Positive wells were scored by visual inspection. The frequency of adaptation was determined by plotting the log of number of negative wells versus the number of cells per well seeded. The number of cells per well was extrapolated at which point 37 % of the wells were negative for cell growth. Based on the Poisson Distribution (Taswell, 1981), this is the frequency at which the number of adapted cells per well may be assumed to be 1.

7. Assembly of Proteasomes

Assembly of proteasome subunits was monitored by pulse-chase analysis using a polyclonal antiserum against the C9 α-subunit. This antiserum precipitates both immature (i.e. complexes which still contain unprocessed β-subunits) and mature 20S proteasome complexes. Cells (5 X 10^6) were treated with 50 µM NLVS or DMSO (1% as a control) for 1 hour and then labeled with [^{35}S]-methionine for 1 hour and chased with non-radioactive methionine for 24 hours. The same protocol was applied to adapted cells growing in the presence of 50 µM NLVS. Cells were then lysed with NP-40 lysis mix and immunoprecipitations were performed using the anti-C9 antiserum. Precipitations were analyzed by two-dimensional non-equilibrium isoelectric focusing SDS-PAGE, followed by fluorography as described in chapter 2.

8. Affinity Labeling of Intact Cells with ^{125}I-NIP-L_3-VS

Cells (1.5 x 10^6) or lysates (50 µg protein unless otherwise indicated) were labeled with ^{125}I-NIP-L_3-VS, diluted to a final concentration of 1.8 x 10^4 Bq/ml in tissue culture media (cells) or reaction buffer (lysates; 50 mM Tris pH 7.4, 2 mM DTT, 5 mM $MgCl_2$, 2 mM ATP). Samples were incubated at 37°C for 2 hours followed by addition of 1X (cells) or 4X (lysates) SDS sample buffer. Proteins were separated by SDS-PAGE as described.

9. Preparation of Lysates from Control EL-4, NLVS Treated or Adapted Cells

Cells were lysed using a bead smashing technique. Briefly, cells were washed 3X with cold PBS and then pelleted. A volume of glass beads (<10^6 microns, acid washed; Sigma Chem. Co., St. Louis, MO) equivalent to the volume of the pellet were added followed by a similar volume of homogenization buffer (50 mM Tris pH 7.4, 1 mM DTT, 5 mM $MgCl_2$, 2 mM ATP, 250 mM sucrose). Cells were vortexed at high speed for 1 min. The beads and broken cell debris was then removed by centrifugation at 10,000 g for 5 min. The resultant homogenate was then centrifuged at 10,000 g for 20 min to remove unbroken cells and nuclei. Protein concentration was determined using the BCA protocol described by the manufacturer (Pierce Chem. Co., Rockford, IL).

10. Fluorogenic Peptide Substrate Assay

Proteolytic activity was measured using fluorescently tagged peptide substrates. The substrates used were: Suc-LLVY-MCA and Z-LLG-MCA for analysis of the chymotrypsin-like activity of the proteasome; Boc-LRR-MCA for analysis of the trypsin-like activity of the proteasome; Z-LLE-βNA for analysis of the PGPH activity of the proteasome; and AAF-MCA for analysis of non-proteasomal proteolysis. In a typical assay, partially purified proteasomes from 5h pellets (described above; 5 µg total protein), or total lysates (10 µg) were diluted to a final volume of 100 µl in reaction buffer (50 mM Tris pH 7.4, 5 mM $MgCl_2$, 1 mM DTT). Inhibitors were added to samples as a DMSO stock as described above. Fluorogenic substrates were added by dilution of a 5 mM

DMSO stock to a final concentration of 100 μM. Samples were incubated at 37°C for 45 min. and the reaction quenched by addition of 1ml of 1% SDS. Fluorescence was then measured for each sample as described in chapter 3.

11. FACS Analysis of Cell Cycle

Cells (1 x10^6) were permeablized by treatment with ethanol (70%) overnight at 4°C then treated with RNAse A (100 units/ml) and the DNA stained with propidium iodide (50 μg/ml) for 1 hour at room temperature. Cells were then washed once in PBS and re-suspended in 1ml of PBS. Samples were analyzed using a fluorocytometer (Becton-Dickenson, FACScan).

12. Class I Assembly

To monitor class I assembly, a pulse chase experiment was performed on EL-4 cells, EL-4 cells treated with 50 μM NLVS, and EL-4 adapted cells. NLVS treated cells were pretreated with 50 μM NLVS for 16 hours prior to performing the pulse-chase. Cells were pulse labeled with [^{35}S]-methionine for 10 min and then chased for 0, 30 and 90 minutes by addition of media containing non-labeled methionine as described in chapters 2 and 4. Cells from each chase point were placed on ice and split into three equal portions. Each portion of cells was lysed by the addition of 1 ml of ice-cold lysis buffer (50 mM Tris pH 7.4, 0.5 % NP-40, 5 mM $MgCl_2$). The class I binding peptide Ova8 (SIINFEKL) was added to one portion of lysate from each chase point to a final concentration of 10 μM. Samples with and without peptide added were incubated at 37 °C for 20 min and returned to ice. One portion of lysate from each chase point was never exposed to peptide and remained on ice for the duration of the experiment. Class I molecules were isolated using the Y3 monoclonal antibody as described in chapters 2 and 4. Proteins were separated on a 12.5 % SDS-PAGE gel and a analyzed by fluorography as described above.

13. Gel Filtration of 5h Pellets

Lysates were prepared from control EL-4 and EL-4 adapted cells (1 X 10^8 cells) and fractionated by differential centrifugation as described above. The 5 hour 100,000 g pellets were re-suspended in 0.5 ml of homogenization buffer (50 mM tris pH 7.0, 20 % glycerol) and injected onto a superose 6 column (1.5 cm x 30 cm) at room temperature. The sample was eluted with a flow rate of 0.2 ml per min. Fractions of 0.5 ml were collected and placed immediately on ice. Aliquots of each fraction (20 µl) were used to measure hydrolysis of the Suc-LLVY-MCA and AAF-MCA using the protocol outlined above and as described in chapter 3. Mean fluorescence was plotted against fraction number.

C. Results

1. EL-4 cells can adapt to prolonged exposure of NLVS

The mouse T-lymphoblastoid cell line, EL-4 was treated with 0, 1, 10 and 50 μM NLVS. Control cells (no addition) and cells grown in the presence of 1 μM NLVS showed normal growth curves (figure 5.1A). However, growth of EL-4 cells was impaired upon addition of 10 or 50 μM of the proteasome inhibitor, and the majority of cells died within 3 days. Cells maintained at 10μM NLVS began to grow at a slow rate after nearly two weeks, while cells maintained in 50μM failed to overcome the inhibitor's toxicity. When the population of cells that survived in 10 μM NLVS were washed and re-exposed to increasing concentrations of NLVS ranging from 0-50 μM, growth proceeded normally, even in the presence of 50 μM NLVS (figure 5.1B). These cells will be referred to throughout this chapter as adapted and are continuously maintained in the presence of 50 μM of NLVS.

2. Adaptation is not the result of mutation

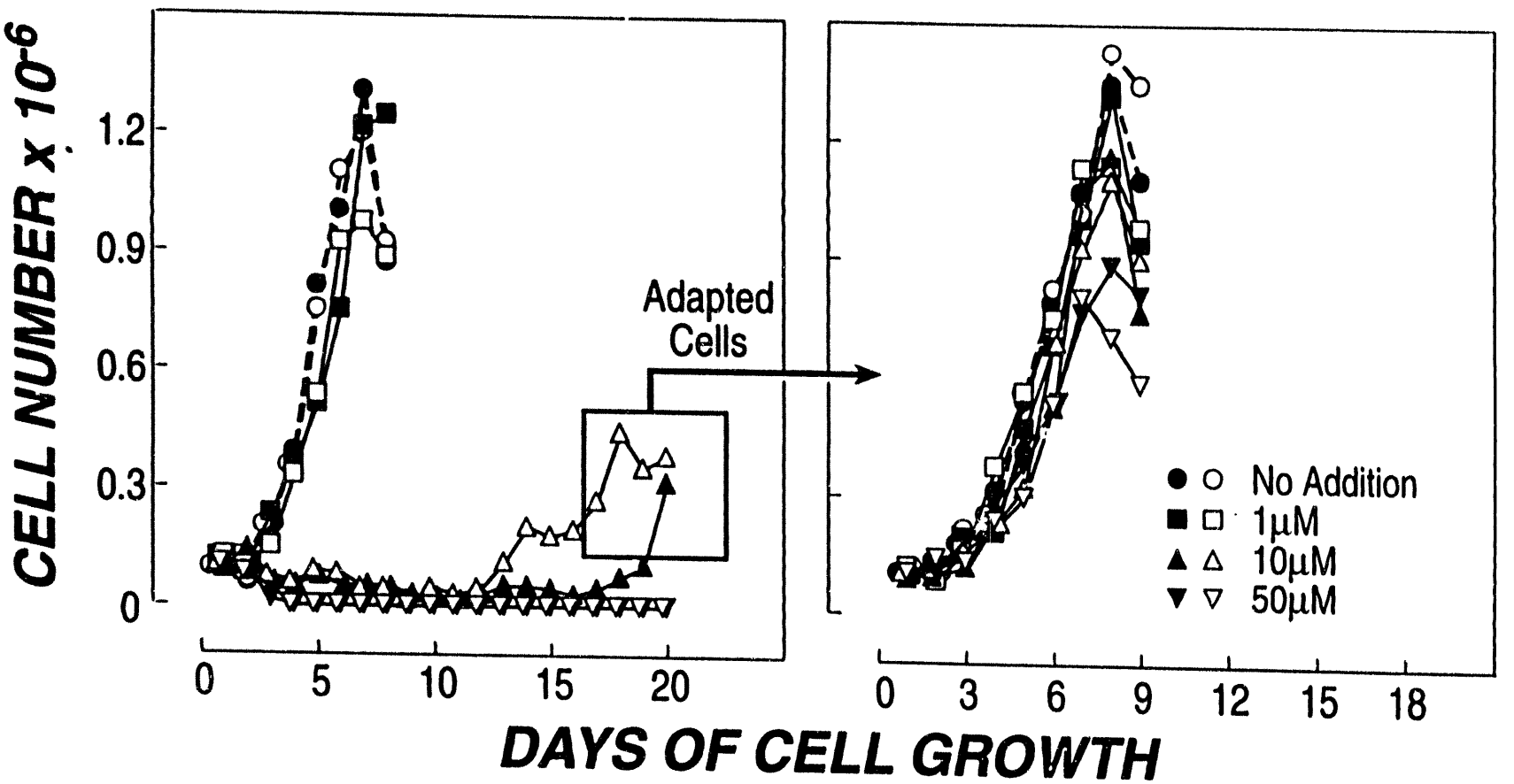
The frequency with which EL-4 cells became adapted to NLVS treatment was examined to determine if this phenomenon was the result of a mutation resulting in proteasomes that are refractory to inhibition by NLVS. Limiting dilution experiments indicated that 1 in approximately 1000 EL-4 cells was able to adapt and grow in the presence of NLVS (data not shown). This frequency of adaptation effectively rules out the possibility of a genetic mutation whose frequency is likely to be in the range of 1 in 1,000,000. In addition, when NLVS is washed away from adapted cells, over time, they regain sensitivity to inhibition by NLVS, again inconsistent with a permanent genetic mutation (see figure 5.7).

3. The proteasome is synthesized and assembled normally in adapted cells

A pulse chase experiment was performed on normal, NLVS treated, and adapted cells to monitor the synthesis and assembly of proteasome complexes.

Figure 5.1. EL-4 cells adapt and grow in the presence of otherwise toxic levels of NLVS.

(A) EL-4 cells (1×10^5) were maintained in the presence of 0, 1, 5, 10 and 50 μM NLVS for three weeks. Cells were sampled daily and number of live cells, as judged by trypan blue exclusion, were counted. Cell number is plotted against time. Populations of adapted cells are indicted with a box.
(B) The adapted cells from A (box) were washed and re-plated in fresh medium containing 0, 1, 5, 10 and 50 μM NLVS.


CELL NUMBER x 10⁻⁶
1.2
0.9
0.6
0.3
0
0
5
10
15
20
Adapted Cells
0
3
6
9
12
15
18
No Addition
1μM
10μM
50μM
DAYS OF CELL GROWTH

Cells were pulsed for 1 hour followed by a chase of 24 hours and precipitation with antiserum that recognizes the C9 α subunit and can immunoprecipitate the entire proteasome complex via this interaction. Samples were analyzed by two-dimensional NEPHGE/SDS-PAGE to resolve the multiple proteasomal subunits in the 20-30 kDa size range (figure 5.2). The relative amount of proteasome subunits were comparable in normal, NLVS treated, and adapted cells. The only notable difference was the absence of the LMP-7 subunit in immunoprecipitates from adapted cells. This loss of LMP-7 is not likely to substantially affect proteasomal proteolysis, due to the fact that it normally replaces the pre-existing X subunit upon IFN-γ treatment and cells deficient in LMP-7 and LMP-2 grow normally (Arnold et al., 1992; Driscoll et al., 1993; Gaczynska et al., 1993).

4. Toxicity of NLVS correlates with modification of proteasomal β-subunits

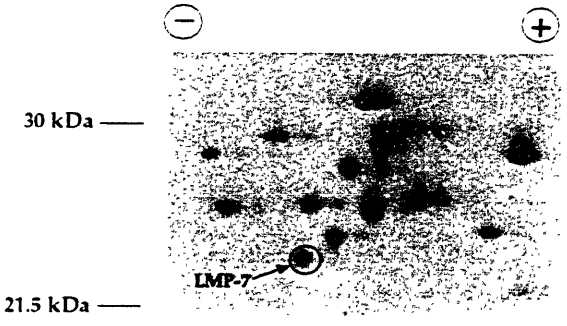
We examined whether the toxic effects observed from treatment of cells with NLVS was the result of proteasomal inhibition using several related analogs. The tri-peptide vinyl sulfones NP-LLG-VS (cis and trans) and NP-AAF-VS are described in chapter 3 and are structurally similar to NLVS. These compounds are less potent, however, and show reduced reactivity with some of the β-subunits modified by NLVS (see chapter 3). To examine the ability of these tri-peptide vinyl sulfones to modify mouse proteasomes cells were pre-treated for 16 hours with 50 μM of each of the four inhibitors, lysed and the lysates labeled with ^{125}I-NIP-L$_3$-VS (chapter 2) (figure 5.3A). The characteristic band at 23 kDa representing the LMP-2, LMP-7 and X proteasome subunits was visible for untreated control cells and for cells treated with NP-LLG-VS or NP-AAF-VS, but not for cells pre-treated with NLVS. The inability of the LLG and AAF vinyl sulfones to block labeling by ^{125}I-NIP-L$_3$-VS indicated that there are subunits which are modified by NLVS but not by the related AAF and LLG derivatives under the conditions used.

We next examined whether competition for NLVS labeling correlated with toxicity of the LLG and AAF vinyl sulfones. Figure 5.3B shows the growth curves for EL-4 cells treated with 0, 10, and 50 μM of NLVS (NIP-LLL-VS), NP-LLG-VS, and NP-AAF-VS. As expected, NLVS was toxic to growth at 10 and

Figure 5.2. Synthesis and assembly of proteasome subunits is normal in adapted cells.

Adapted and control EL-4 cells were pulse labeled for 1 hour, chased for 24 hours and proteasomes immunoprecipitated using an anti-C9 polyclonal antiserum as described in the materials and methods section. Samples were separated by two-dimensional NEPHGE and subunits visualized by fluorography. Note that the relative intensities of spots is the same for control and adapted cells except for LMP-7 (indicated with a circle). The anode (-) and cathode (+) are indicated.

Control EL-4 Cells



EL-4 Adapted Cells

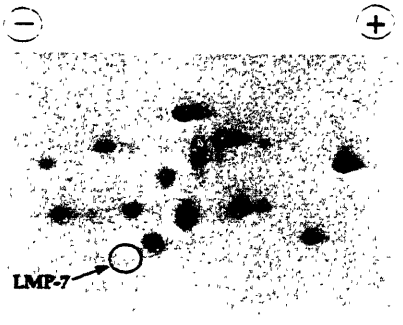
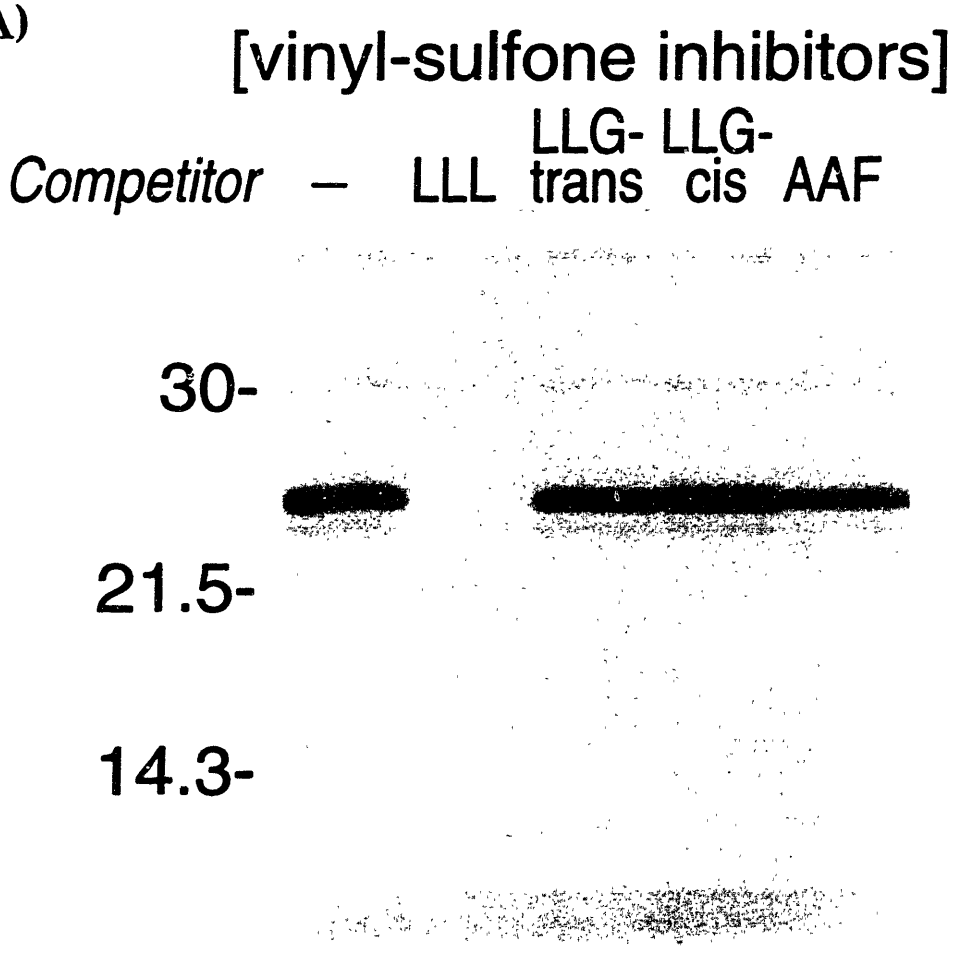
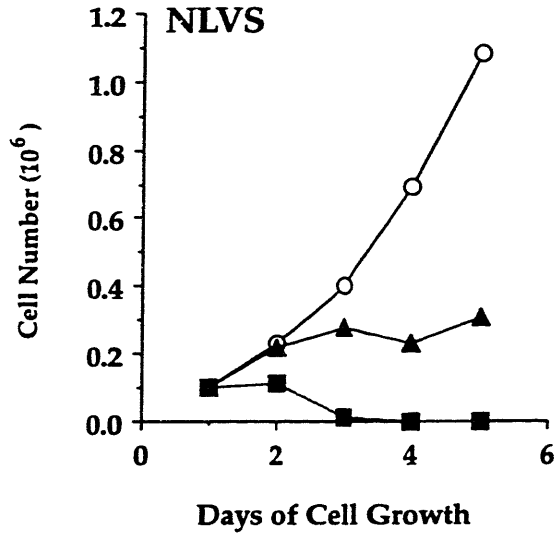


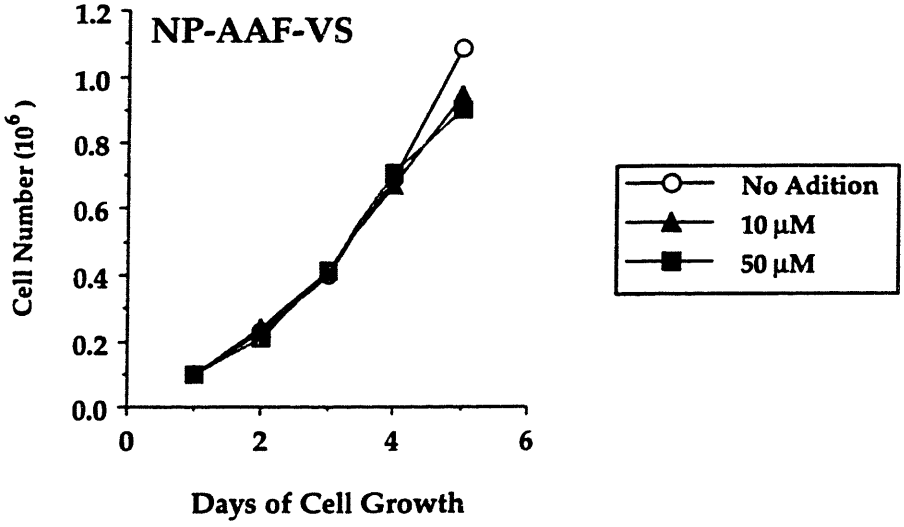
Figure 5.3. Acute toxicity of the peptide vinyl sulfones is sequence-specific.

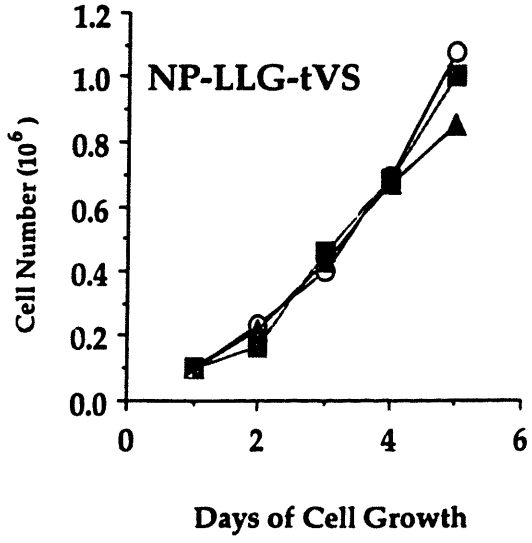
(A) EL-4 cells (1 x10^6) were treated for 16 hours with DMSO (-), NLVS (LLL), NP-LLG-tVS (Trans LLG), NP-LLG-cVS (Cis LLG), or NP-AAF-VS (AAF) and then labeled with ^{125}I-NIP-L_3-VS for 2 hours at 37°C. Proteasome subunits were then resolved using a 12.5 % SDS-PAGE gel and visualized by autoradiography. Note the loss of labeling of proteasome subunits only when cells were treated with NLVS.
(B) EL-4 cells (1 x 10^5) were maintained in the presence of 0, 10 and 50 μM of NLVS, NP-LLG-tVS, or NP-AAF-VS as indicated. The number of live cells (as judged by trypan blue exclusion) were counted and plotted against time in culture. Note that only NLVS treatment results in acute toxicity.

(A)


[vinyl-sulfone inhibitors]
Competitor
–
LLL
LLG- trans
LLG- cis
AAF
30-
21.5-
14.3-


NLVS
Cell Number (10^6)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0
2
4
6
Days of Cell Growth


NP-AAF-VS
Cell Number (10^6)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0
2
4
6
Days of Cell Growth
No Adition
10 μM
50 μM


NP-LLG-tVS
Cell Number (10^6)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0
2
4
6
Days of Cell Growth

50 μM. Neither of the related AAF or LLG derivatives were toxic to growth at either concentration. This direct correlation between toxicity and proteasome modification suggests that the toxicity observed for NLVS is due to its ability to block the active sites of the proteasome. Thus, compounds that fail to bind strongly to all the active β-subunits may leave some of the activity of the proteasome intact, allowing the cells to survive.

5. Labeling of proteasomal β-subunits by ^{125}I-NIP-L$_3$-VS is blocked in adapted cells

We next examined whether the β-subunits of the proteasome were modified and thus inhibited in adapted cells. Figure 5.4 shows labeling of mouse liver proteasomes, normal EL-4 and EL-4 adapted cells with ^{125}I-NIP-L$_3$-VS. Labeling of 1, 10, 100, and 500 μg of mouse liver protein resulted in the characteristic 23 kDa band (composed of LMP-2, LMP-7, and X) as well as the 29 and 30 kDa bands (MECL-1 and Z). At lower concentrations of protein, only the single major 23 kDa band was observed. The same 23 kDa band was observed in the labeling of normal EL-4 cells but was completely absent from labeling of adapted cells. To rule out the possibility of a change in permeability of adapted cells which could prevent the inhibitor from entering the cytosol and modifying the proteasome, lysates obtained from normal and adapted cells were labeled with 125I-NIP-L3-VS . Again we observed labeling of proteasomal β-subunits in normal but not in adapted cells, nor in cells treated with 50 μM NLVS, indicating that the active sites of the proteasome are modified in adapted cells (Figure 5.5).

6. Proteasomal hydrolysis of fluorogenic substrates is impaired in adapted cells

The ability of proteasomes to hydrolyze a short fluorogenic peptide substrate is often used as a way to assess their proteolytic activity. The substrates, Suc-LLVY-MCA and Z-GGL-MCA are used to monitor the chymotrypsin-like activity; Cbz-LLE-βNA is used to monitor the post-glutamyl peptidase activity (PGPH activity); and Boc-LRR-MCA is used to monitor the trypsin-like activity (see chapter 2). The hydrolysis of each of these substrates

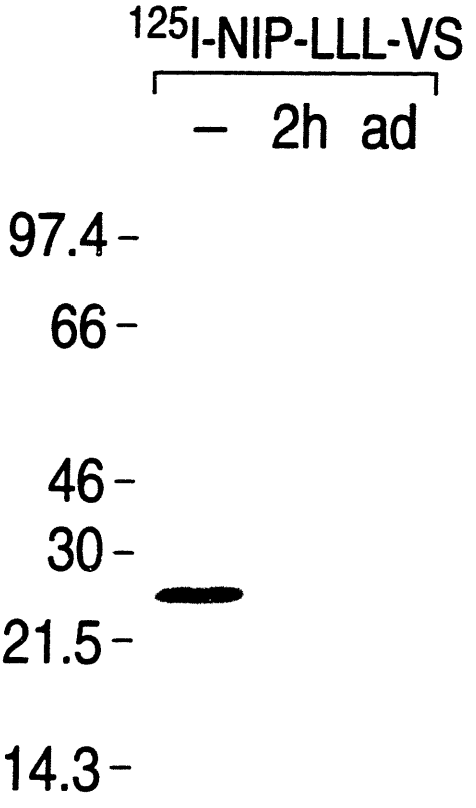
Figure 5.4. Labeling of proteasomal β-subunits is blocked in adapted cells.

Control (EL-4) and adapted EL-4 (EL-4ad) cells were incubated with ^{125}I-NIP-L_3-VS for 2 hours at 37°C. Cells were then lysed in SDS-containing buffer and proteins separated by SDS-PAGE on a 12.5 % gel. Labeled polypeptides were visualized by autoradiography. Increasing amounts of protein from mouse liver tissue lysates (μg protein) were also labeled as described above and were included to show the labeling pattern of proteasomal β-subunits at different loads of protein.

ad=adapted

Figure 5.5. Labeling of proteasomal β-subunits is blocked in adapted cell lysates.

Control EL-4 cells, EL-4 cells treated with 50 μM NLVS for 2 hours, and adapted EL-4 cells were lysed. The resulting lysates were labeled with ^{125}I-NIP-L_3-VS and the labeled proteasomal β-subunits visualized by SDS-PAGE followed by autoradiography (12.5% gel). Note the loss of labeling of both NLVS treated and adapted cell lysates.



ad=adapted

was measured for lysates from normal EL-4 cells, for EL-4 cells treated with 50μM NLVS and for adapted cells. As expected, the amount of hydrolysis of all three substrates was substantially reduced upon treatment of cells with NLVS. Adapted cell lysates showed a similar reduction in hydrolysis of all three substrates. In both NLVS treated and adapted cells, the chymotrypsin-like activity was diminished most strongly, followed by the PGPH and the trypsin-like activity (figure 5.6).

We next examined whether the decrease in hydrolysis of fluorogenic substrates correlated with active site modification by the vinyl sulfone moiety. Normal EL-4 cells were treated for increasing times with 50μM NLVS. Cells were lysed and hydrolysis of the chymotrypsin-like substrates Z-GGL-MCA and Suc-LLVY-MCA were measured. The same lysates were subjected to labeling with ^{125}I-NIP-L_3-VS to determine the extent of active site modification. The same experiment was performed on adapted cells growing in the presence of 50 μM NLVS, except that the samples were taken at different times after removal of the inhibitor (figure 5.7). These results indicate that loss of labeling with ^{125}I-NIP-L_3-VS, as the result of prior active site modification, directly correlates with loss of hydrolytic activity as measured using fluorogenic substrates.

To rule out the possibility that a adapted cells were up-regulating a cytosolic factor which could inactivate NLVS before it had the opportunity to inhibit the proteasome, we assayed the hydrolytic activity of proteasomes isolated by a differential centrifugation protocol (see Materials and Methods). Centrifugation of lysates from adapted cells for 5 hours at 100,000 g results in a protein pellet which is highly enriched for the 20S and 26S proteasome. Hydrolysis of the LLVY and GGL proteasome substrates was inhibited to a similar extent in the cytosolic extracts, 5 hour spin supernatant, and 5 hour pellet from adapted cells (figure 5.8). Again, the labeling of these fractions correlated with hydrolysis data, with a near complete loss of labeling of proteasome in all samples generated from fractionation of adapted cells. Thus, the proteolytic activity of proteasomes isolated from adapted cells is greatly impaired.

The high speed spin pellet described above was fractionated by gel filtration on a superose 6 column to further resolve proteins by size. When the 5 hour pellet obtained from normal EL-4 cells was fractionated, hydrolysis of the Suc-LLVY-MCA substrate revealed the presence of two predominant peak

Figure 5.6. Hydrolysis of fluorogenic substrates is reduced in cells treated with proteasomal inhibitors as well as in adapted cells.

Lysates were prepared from control EL-4 cells (EL-4), from EL-4 cells treated for 16 hours with 50 μM NLVS (EL-4 + 50 μM NLVS), and from adapted cells (EL-4 Ad) as described in the materials and methods section. Hydrolysis of the fluorogenic substrates; Suc-LLVY, Z-GGL-MCA, Boc-LRR-MCA, and Z-LLE-βNA was then measured for each lysate. The fluorescence values obtained for the control EL-4 cells were set at 100%. All other values are expressed as a percentage of control hydrolysis.

% Control Peptidase Activity
100
50
0
EL-4
EL-4 + 50 μM NLVS
EL-4 Ad
Suc-LLVY-MCA
Z-GGL-MCA
Boc-LRR-MCA
Z-LLE-βNA

Figure 5.7. Proteasomal activity is rapidly restored upon removal of NLVS from adapted cells.

EL-4 cells were treated with 50 μM NLVS for 0-3 hours as indicated. Lysates were prepared and hydrolysis of the fluorogenic substrates Suc-LLVY (LLVY-MCA) and Z-GGL-MCA (GGL-MCA) was measured (top panel). The same lysates were labeled with ^{125}I-NIP-L_3-VS, separated by SDS-PAGE and visualized by auto-radiography (bottom panel). EL-4 adapted cells grown in the continuous presence of 50 μM NLVS were washed to remove NLVS and grown in the absence of NLVS for 3 weeks. Samples were taken at various times after washing and the same procedure was performed as described above . Note the correlation between labeling and hydrolysis of fluorogenic substrates.

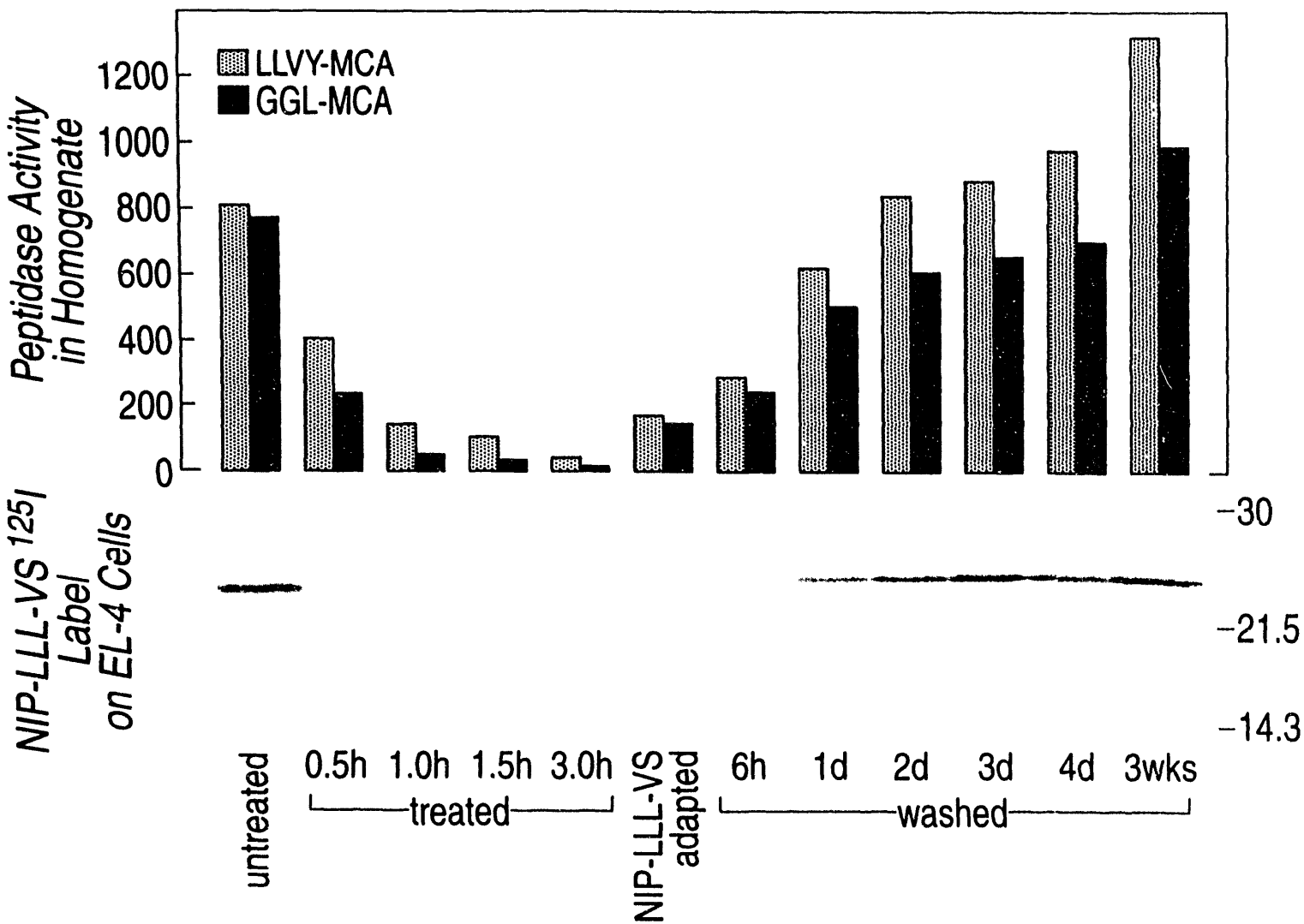
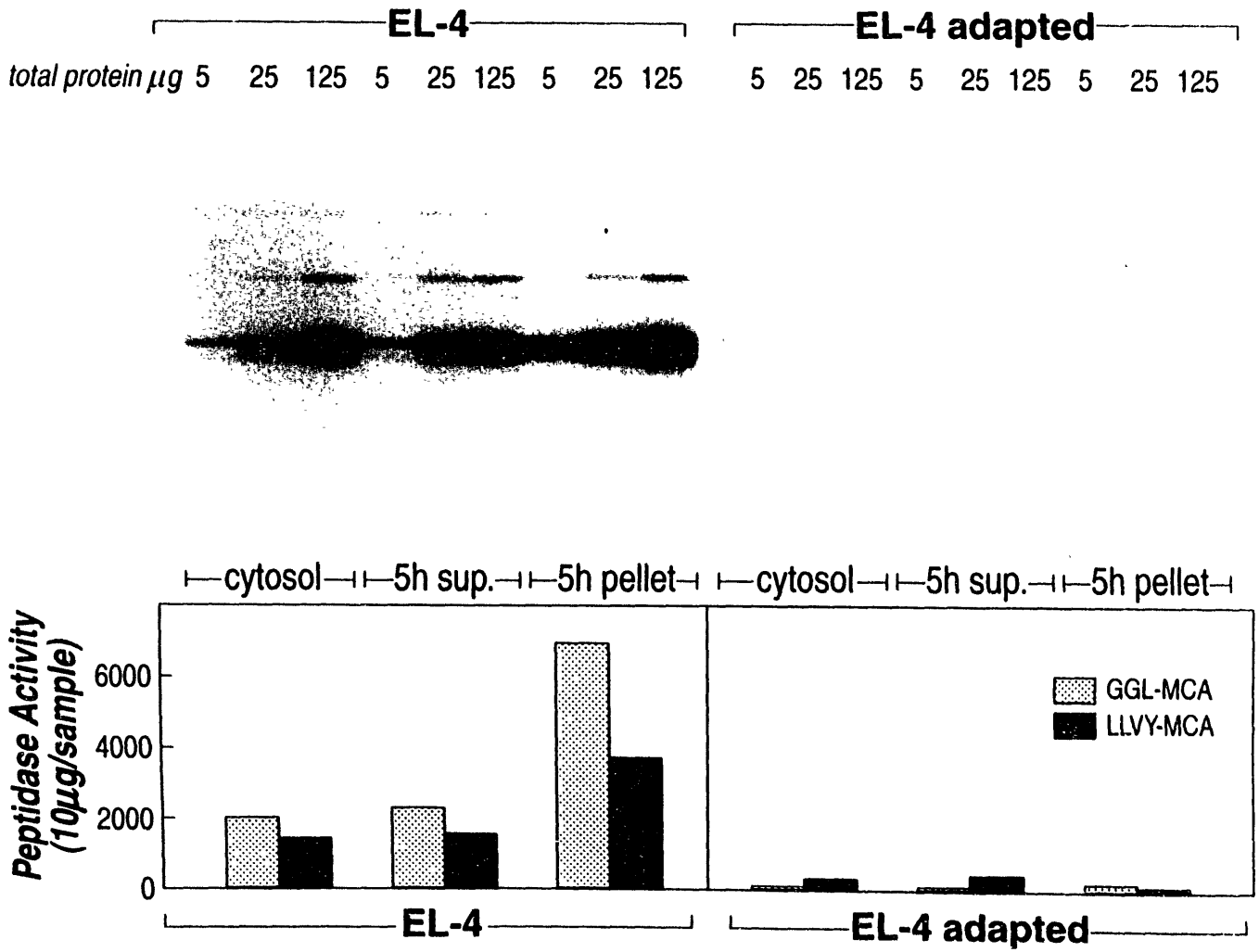

LLVY-MCA
GGL-MCA
Peptidase Activity in Homogenate
1200
1000
800
600
400
200
0
NIP-LLL-VS 125I Label on EL-4 Cells
-30
-21.5
-14.3
untreated
0.5h
1.0h
1.5h
3.0h
treated
NIP-LLL-VS adapted
6h
1d
2d
3d
4d
3wks
washed

Figure 5.8. Labeling of proteasomal β-subunits with ^{125}I-NIP-L_3-VS in adapted cells is blocked in cytosol fractions as well as in pellet fractions highly enriched for the proteasome.

Control and adapted EL-4 cells were fractionated by differential centrifugation and labeled with ^{125}I-NIP-L_3-VS. Hydrolysis of Suc-LLVY-MCA and Z-GGL-MCA were measured as described in the materials and methods section. The cytosol fraction represents the lysate after removal of nuclei and microsomes and prior to the 5 hour 100,000 g centrifugation. Increasing amounts of protein were labeled as indicated (total protein).


EL-4
EL-4 adapted
total protein μg 5 25 125 5 25 125 5 25 125 5 25 125 5 25 125 5 25 125
cytosol
5h sup.
5h pellet
cytosol
5h sup.
5h pellet
Peptidase Activity (10μg/sample)
6000
4000
2000
0
GGL-MCA
LLVY-MCA
EL-4
EL-4 adapted

activities, representing the 20S and the 26S proteasomes. When the same experiment was performed using the 5 hour pellet from adapted cells, 20S and 26S proteasome peaks were almost completely absent, again indicating that proteasomal activity in adapted cells is greatly diminished (figure 5.9).

7. Adapted cells are able to generate peptides for binding to MHC class I molecules

The proteasome has been shown to play an integral role in the generation of peptides for binding to MHC class I molecules and display to cytotoxic T-cells (see chapter I). Inhibition of proteasome function results in a loss of class I binding peptides (Harding et al., 1995; Rock et al., 1994). These peptides are required for class I molecules to fold properly and exit the ER en route to the cell surface. Thus, the ability of a MHC class I molecules to fold can be used as a method for examining a cell's ability to generate peptides and can also be used as an indirect measure of proteasome function. Class I maturation and assembly can be monitored using an antibody (P8) which recognizes an epitope of the cytoplasmic tail of the class I heavy chain. The light chain, β2m, is recovered by this antibody only if it is associated with the heavy chain.

Normal EL-4 cells, EL-4 cells treated with 50μM NLVS, and adapted cells were pulsed labeled and chased for 0, 30, and 90 minutes. The lysates obtained for each of the chase points were then incubates at 4°C to stabilize empty class I molecules or exposed to 37°C in the presence and absence of a class I binding peptide. Any class I-β_2m complex which does not contain peptide dissociates at 37°C. In normal cells, class I molecules were found associated with β_2m and were processed by addition of the single N-linked glycan. This processing is seen as a shift in the apparent molecular weight of the heavy chain and is complete within 90 min. of chase (figure 5.10). As expected, treatment of cells with 50μM NLVS for 16 hours resulted in class I heavy chains which showed a greatly reduced amount of associated β_2m. Further, the small amount of heavy chain-β_2m complexes obtained were completely lost upon incubation at 37°C in the absence of peptide. However, the amount of heavy chain-β_2m complexes recovered could be restored to the level of untreated cells by the addition of peptide to the lysate. Thus, the reason for loss of stable assembly of these

Figure 5.9. Partially purified proteasomes isolated from adapted cells have severely diminished proteolytic activity.

Lysates obtained from control and adapted EL-4 cells were fractionated by differential centrifugation as described. Pellets from the 100,000 g centrifugation were passed over a Superose 6 column and Suc-LLVY-MCA hydrolysis was measured for each fraction. Note the presence of a peak of activity for the 26S and 20S proteasomes in control cells which is substantially reduced for adapted cells.

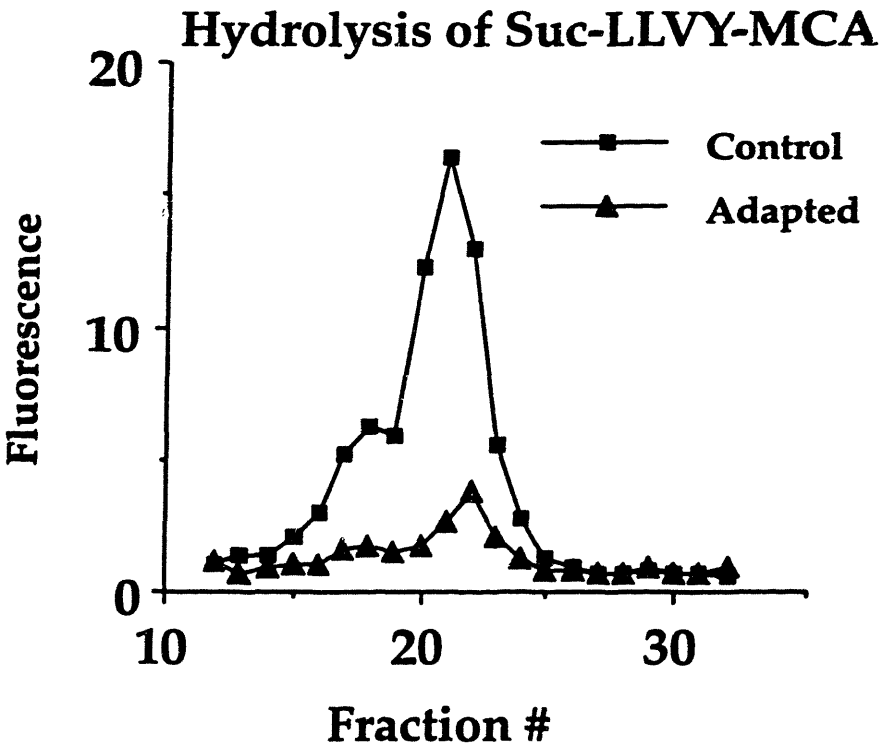
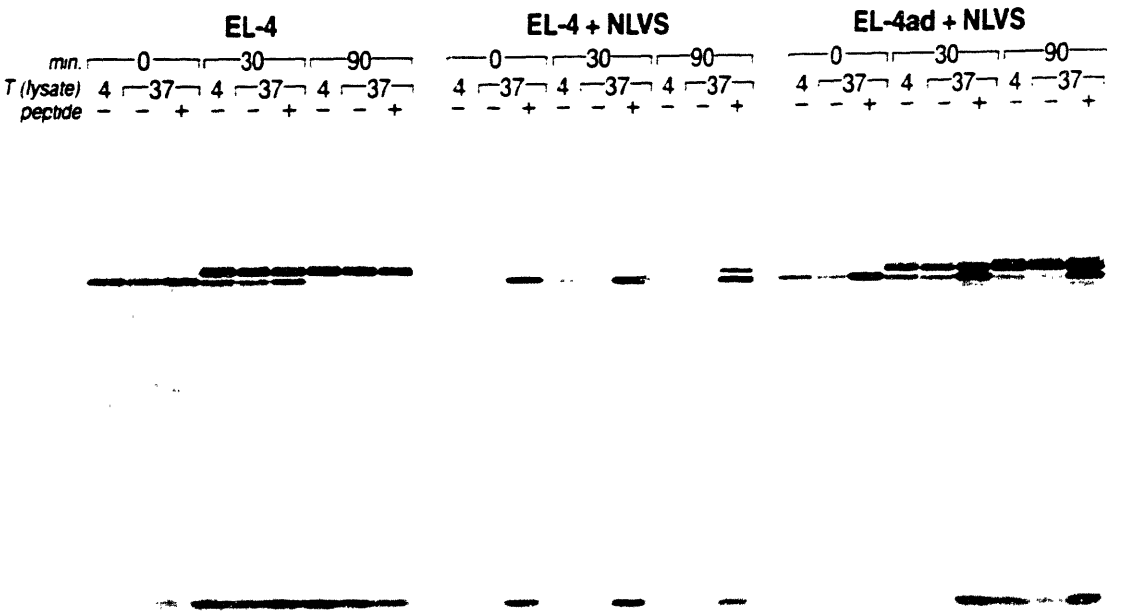

Hydrolysis of Suc-LLVY-MCA
20
10
0
Fluorescence
10
20
30
Fraction #
Control
Adapted

Figure 5.10. Assembly of MHC class I molecules is impaired in cells treated with NLVS but not in adapted cells.

Control, NLVS treated (50 μM for 16 hours), and adapted EL-4 cells were pulse labeled for 10 min. and chased for 0, 30, and 90 min. Properly conformed class I molecules were immunoprecipitated using the Y3 antibody in the presence (+) or absence (-) of class I binding peptide SIINFEKL. Lysates were also exposed to 37°C (37)to cause dissociation of class I molecules lacking bound peptide. Polypeptides were separated by SDS-PAGE (12.5% gel) and labeled proteins visualized by fluorography. The class I light chain (β_2m) is seen at the bottom of each panel. Note the loss of recovery of class I molecules in the lysates exposed to 37°C and the slow maturation to the fully glycosylated form of the heavy chain in NLVS treated cell lysates but not in adapted lysates.


EL-4
EL-4 + NLVS
EL-4ad + NLVS
min.
0
30
90
T (lysate)
4
37
peptide
−
+

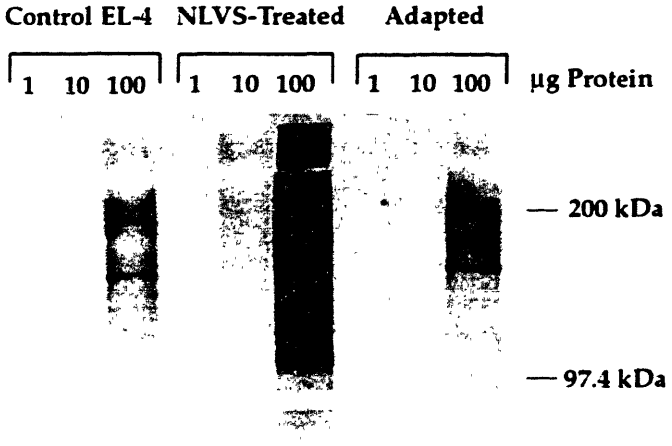
molecules was the absence of peptide. Treatment of normal cells with NLVS also led to a slower rate of processing of newly synthesized heavy chains (compare the 90 min. chase for treated and untreated cells). When the same experiment was performed on adapted cells, assembly and maturation of class I molecules was almost completely restored to the levels observed for control cells. Addition of peptide to lysates from adapted cells resulted in only a minor increase in the amount of properly folded class I molecules isolated. The time scale for maturation of class I molecules in adapted cells was quite similar to that observed for normal EL-4 cells. Thus, in adapted cells, although proteasome function is impaired, peptides are generated for binding to class I molecules.

8. Poly-ubiquinated proteins accumulate in cells treated with NLVS but not in adapted cells

One of the main functions of the 26S proteasome is the destruction of proteins which have been tagged with ubiquitin. If cells are treated with proteasome inhibitors, these ubiquitin-tagged proteins begin to accumulate. We therefore investigated whether such an accumulation occurred in adapted cells. EL-4 cells, EL-4 cells treated with 10μM NLVS for 24 hrs, and adapted cells were lysed and post-nuclear supernatants were analyzed by Western blotting using a polyclonal antiserum reactive with ubiquitin (figure 5.11). Poly-ubiquinated proteins are heterogeneous in size and therefore are seen mostly as high molecular weight aggregates near the top of the gel. As expected, a 24 hour treatment with NLVS resulted in the accumulation of ubiquitin-conjugated proteins in control cells. Only a slight increase in the amount of ubiquitin conjugated proteins was observed in adapted cells. This observation suggests that proteolytic activities other than the proteasome must be responsible, at least in part, for the degradation of proteins modified by attachment of poly-ubiquitin chains.

Figure 5.11. Polyubiquinated proteins accumulate in NLVS treated but not in adapted EL-4 cells.

Lysates were prepared from control, NLVS treated (25 μM for 24 hours), and adapted EL-4 cells. Lysates (1, 10 and 100 μg) were separated by SDS-PAGE (10% gel) and polyubiquinated proteins visualized by immunoblotting. Note the accumulation of a high molecular weight smear in NLVS treated lysates only.



Antibody: Mono-Clonal Anti-Ubiquitin

9. Adapted cells overcome the G2 cell cycle arrest induced by proteasomal inhibition

When normal cells are treated for prolonged periods of time with inhibitors of the proteasome, growth arrest occurs. Since adapted cells are able to grow normally in the presence of concentrations of NLVS which would otherwise result in growth arrest, we examined DNA content of adapted cells. The fluorescent DNA stain propidium iodide can be used to measure the total DNA content of cells by cytofluorimetry. Since cells in S and G2 phases of the cell cycle will have more DNA than cells in the Gl and M phases, this marker is often used as an indirect measure of G1/G2 ratio of asynchronously growing cells. Upon addition of 50 μM of NLVS, cells accumulate in the G2 phase due to the block in proteasomal proteolysis (figure 5.12, center panels). In adapted cells however, cells overcome this block and the DNA content of cells in the G2 phase of the cell cycle is restored to that observed for normal EL-4 cells (figure 5.12, right panels). Adapted cells therefore are able to progress normally through the cell cycle, even in the absence of functional proteasomes.

10. A non-proteasomal activity is up-regulated in adapted cells

In the process of examining the proteolytic activity found in lysates from adapted cells using different fluorogenic substrates we found that hydrolysis of the substrate AAF-MCA was increased. This tri-peptide free amine is a poor substrate for the proteasome, but is efficiently hydrolyzed when the amino terminus is capped with a succinyl moiety. Figure 2.13 shows hydrolysis of the proteasome substrate Suc-LLVY-MCA and the non-proteasome substrate AAF-MCA by lysates from normal as well as several independently isolated lines of adapted cells. In all adapted cell lines examined, a reduction in LLVY hydrolysis and an increase in AAF hydrolysis was observed.

Figure 5.12. The G2 cell cycle arrest caused by NLVS treatment of EL-4 cells is overcome in adapted cells.

(A) Control, NLVS treated (25 μM for 16 hours), and adapted EL-4 cells were permeablized and DNA was stained with propidium iodide. The content of DNA was quantitated using fluorocytometry. Cell number was plotted versus total fluorescence (FL3-H). Cells with 2X the fluorescence observed for a peak of G1 cells were considered to be in the G2 phase of cell cycle. The G1 and G2 populations of cells are indicated.
(B) Plot of the % of total cells injected into the fluorocytometer in A which are in the G1 and G2 phase of the cell cycle for control, NLVS treated, and adapted EL-4 cells. Note the increase in the % of cells in G2 for NLVS treated compared to control and adapted cells.

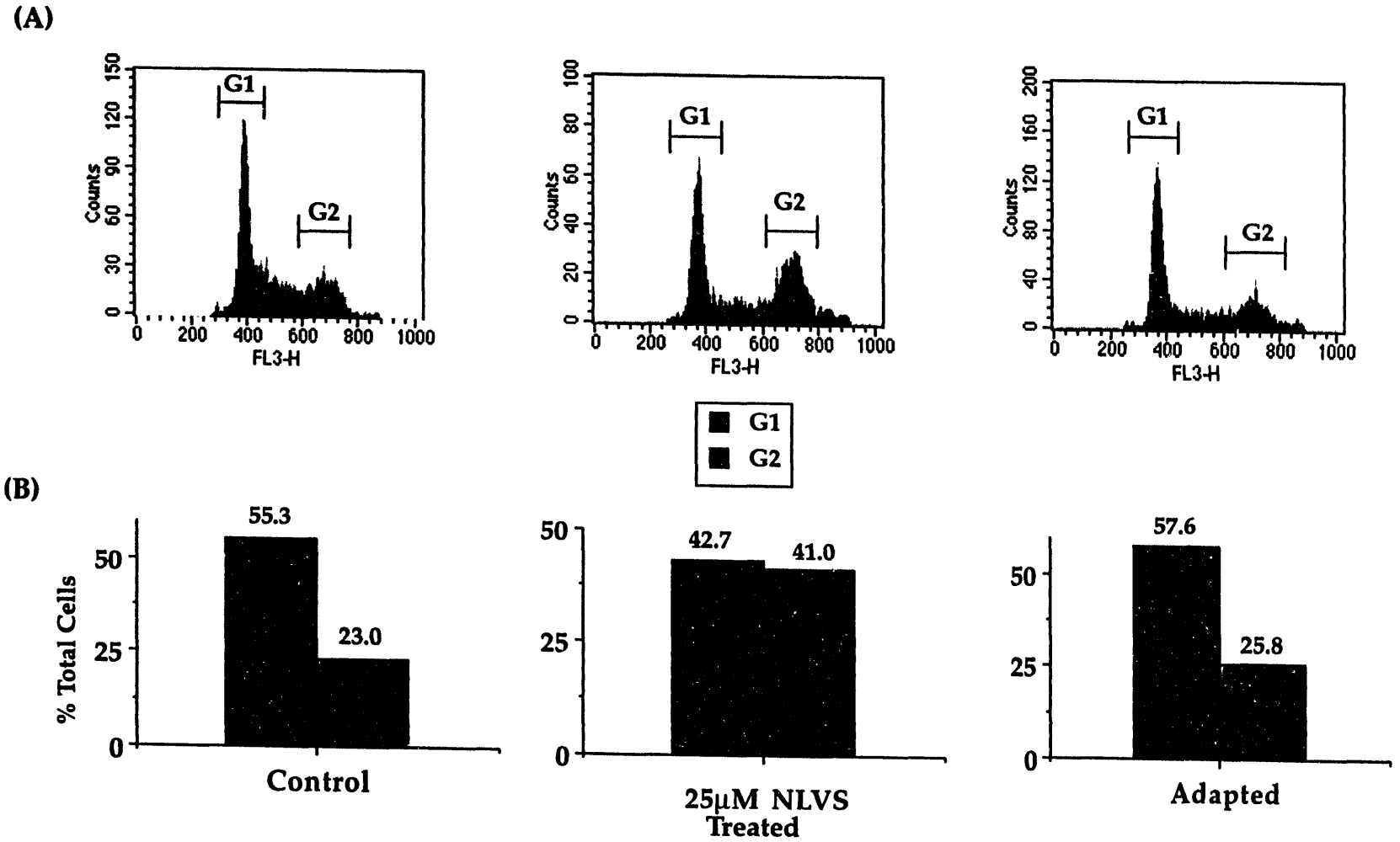
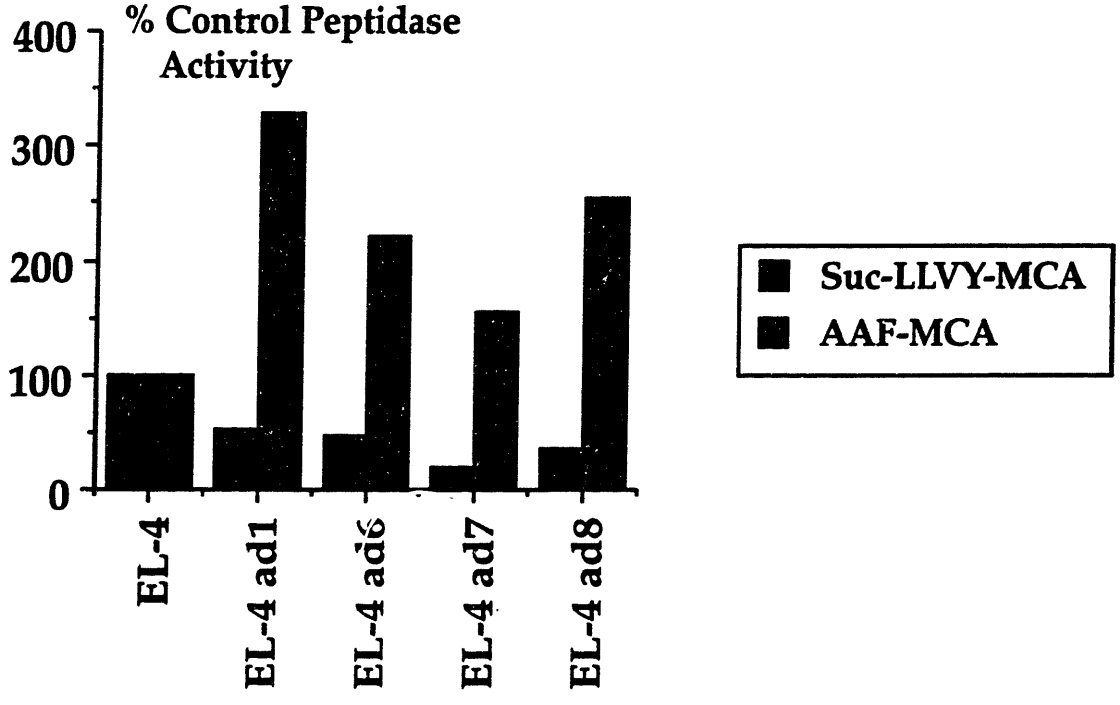

(A)
G1
G2
Counts
FL3-H
0
200
400
600
800
1000
(B)
G1
G2
% Total Cells
55.3
23.0
42.7
41.0
57.6
25.8
Control
25μM NLVS
Treated
Adapted

Figure 5.13. Multiple independent cell lines which are adapted to growth in the presence of NLVS have an increase in AAF-MCA hydrolysis activity.

Lysates were prepared from multiple independently adapted cell lines (EL-4 ad1, ad6, ad7, ad8) and hydrolysis of fluorogenic substrates was measured as described in the materials and methods section. Hydrolysis of the proteasome substrate Suc-LLVY-MCA was reduced and hydrolysis of the non-proteasomal substrate AAF-MCA was increased in all adapted cell lines tested.


% Control Peptidase Activity
400
300
200
100
0
EL-4
EL-4 ad1
EL-4 ad6
EL-4 ad7
EL-4 ad8
Suc-LLVY-MCA
AAF-MCA

11. AAF-MCA hydrolytic activity fractionates as a high molecular weight complex distinct from the proteasome

We next began purifying the proteolytic activity responsible for AAF-MCA hydrolysis. High molecular weight proteins were isolated by centrifugation of lystates from control and adapted EL-4 cells as described in the materials and methods section. These fractions were then subjected to gel filtration on the same superose 6 column described above for the fractionation of proteasomes. Fractions were assayed for AAF-MCA hydrolysis activity. Figure 5.14 shows the gel filtration profiles obtained for control and adapted 5 hour pellets assayed with AAF-MCA. The same fractions were analyzed using the proteasome substrate Suc-LLVY-MCA to indicate the elution position of proteasomes. As described above, a peak of 26S proteasome activity (fraction 17) and a peak of 20S proteasome activity (fraction 20) was observed for normal EL-4 cells. This activity was severely diminished in adapted cells. Unlike normal EL-4 cells, adapted cells showed a peak of AAF-MCA activity in fractions 14 and 15. This activity was lacking in normal EL-4 lysates and is distinct from the proteasome peaks observed with the LLVY substrate. Thus the AAF-hydrolyzing activity represents, at least in part, a protein or protein complex with larger apparent molecular weight than the proteasome.

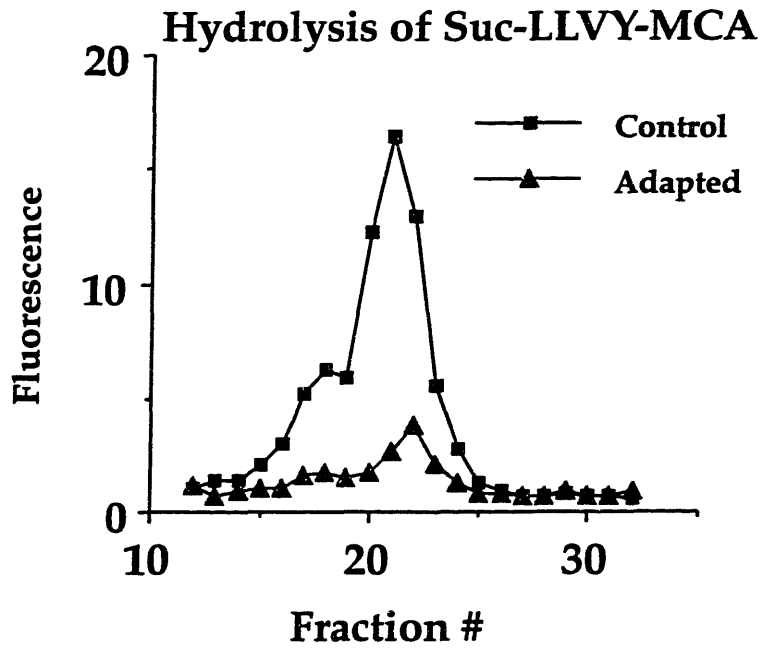
12. AAF-MCA hydrolysis activity is required for adapted cell survival

To address whether the AAF hydrolyzing activity was responsible for survival of adapted cells, it was necessary to block this proteolytic activity. Peptide chloromethyl ketones have been reported as covalent inhibitors of several classes of proteolytic enzymes (Kettner and Shaw, 1981; Rauber et al., 1988; Savory et al., 1993). We synthesized the AAF tri-peptide containing a chloromethyl ketone at the C-terminus (AAF-cmk) and tested its ability to inhibit the AAF hydrolysis observed in adapted cells. Figure 5.15 shows hydrolysis of AAF-MCA by lysates generated from adapted cells pre-treated or not with 5 μM AAF-cmk for 16 hours. AAF-cmk was also added directly to lysates generated from both normal EL-4 and adapted EL-4 cells to determine the dose-dependence of inhibition. The AAF-hydrolyzing activity observed in normal EL-4 cells was

Figure 5.14. The increased AAF-MCA hydrolysis activity found in adapted cells is a high molecular weight protein or protein complex distinct from the proteasome.

(A) Lysates obtained from control and adapted EL-4 cells were fractionated by differential centrifugation as described. Pellets from the 100,000 g centrifugation were passed over a Superose 6 column and Suc-LLVY-MCA hydrolysis activity was measured for each fraction. Note the presence of a peak of activity for the 26S (fraction 18) and 20S (fraction 21) proteasomes in control cells which is substantially reduced for adapted cells.
(B) Hydrolysis of AAF-MCA by fractions described above. Note the absence of AAF-MCA hydrolysis by proteasomal fractions (fractions 18-23) in both control and adapted EL-4 cell lysates. Hydrolysis of AAF-MCA is highest in adapted cells in fractions of higher molecular weight than that of the proteasome (Fractions 12-17). Note the absence of the AAF-MCA activity in control cells.

(A)


Hydrolysis of Suc-LLVY-MCA
20
10
0
Fluorescence
Control
Adapted
10
20
30
Fraction #

(B)

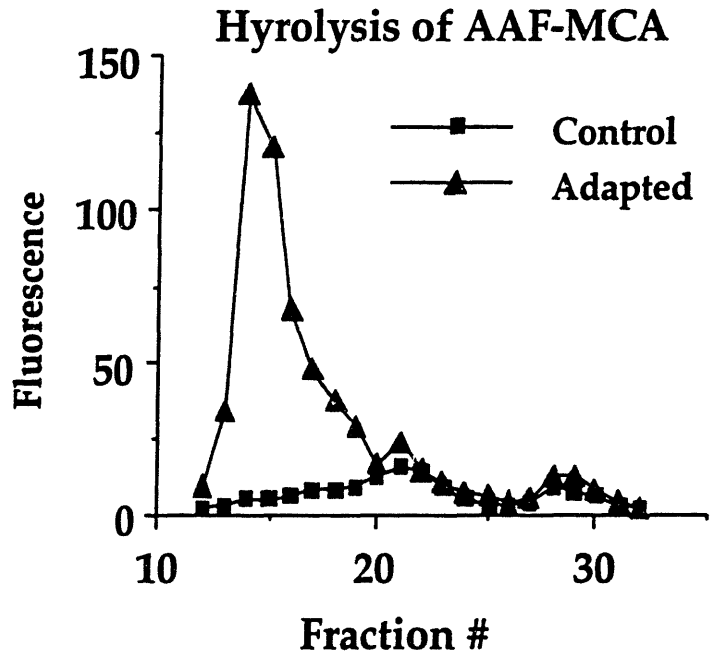
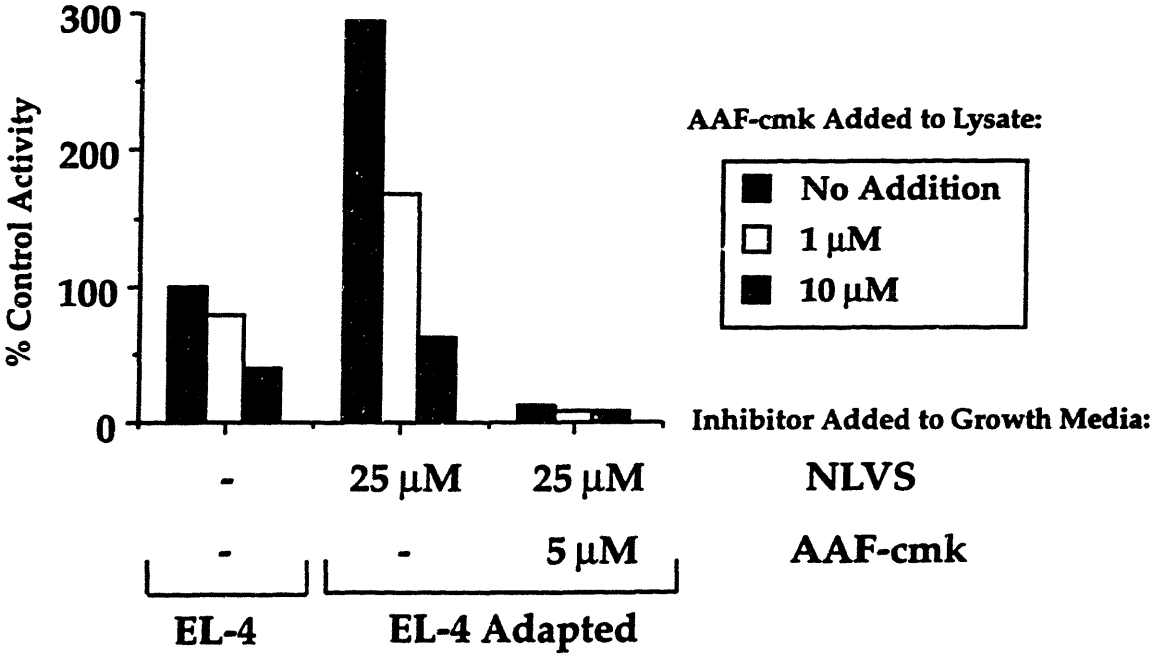

Hyrolysis of AAF-MCA
150
100
50
0
Fluorescence
Control
Adapted
10
20
30
Fraction #

Figure 5.15. The AAF-MCA hydrolysis activity is inhibited by treatment with the tri-peptide chloromethyl ketone AAF-cmk.

Control EL-4 (EL-4) and adapted EL-4 cells grown in 25μM NLVS were treated with AAF-cmk (5 μM) or DMSO as a control (-) for 16 hours at 37°C (label at bottom of graph). Cells were then washed, lysed and AAF-cmk (0, 1 or 10 μM; see legend) added to the lysates. Hydrolysis of AAF-MCA was then measured as described in the Materials and Methods section. Fluorescence was normalized so that AAF-MCA hydrolysis by EL-4 lysates not treated with AAF-cmk was 100%. AAF-cmk inhibits AAF-MCA hydrolysis when added to lysates and when added to intact cells.


300
200
100
0
% Control Activity
AAF-cmk Added to Lysate:
No Addition
1 μM
10 μM
Inhibitor Added to Growth Media:
-
25 μM
25 μM
NLVS
-
-
5 μM
AAF-cmk
EL-4
EL-4 Adapted

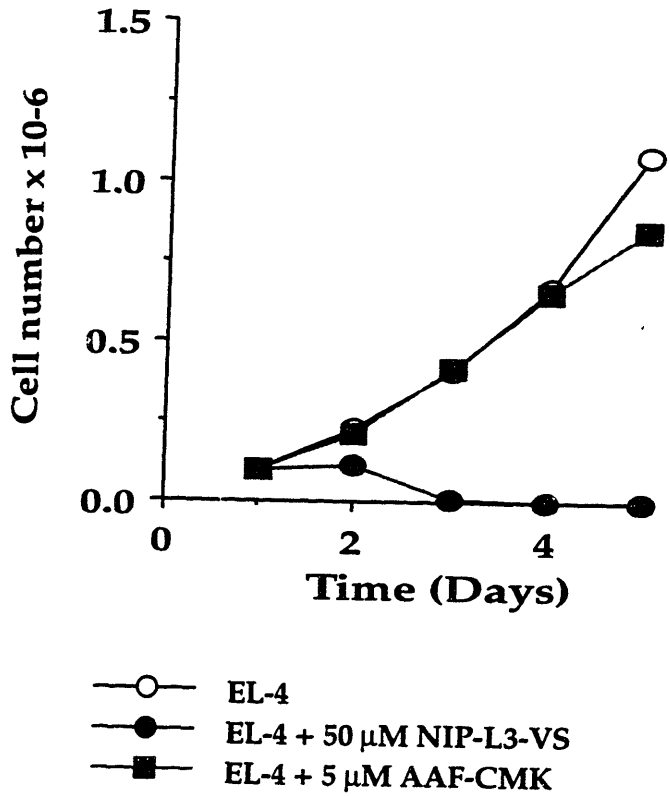
inhibited in a dose-dependent manner by the addition of AAF-cmk to the lysate. The high AAF hydrolyzing activity found in adapted cells could also be inhibited by addition of 1 and 10 μM AAF-cmk to the lysates. Pre-treatment of adapted cells with 5 μM AAF-cmk resulted in a near complete block in AAF-MCA hydrolysis. These data indicate that AAF-cmk is a potent cell-permeable inhibitor of the AAF-hydrolyzing activity observed in adapted cells.

The effects of AAF-cmk on cell growth were monitored for several days. When normal EL-4 cells were incubated with 5μM AAF-cmk, a concentration shown to block the AAF hydrolytic activity, little effect on growth was observed. The same concentration of AAF-cmk, when added to adapted cells growing in 50 μM NLVS, resulted in a nearly complete block in growth (figure 5.16). To rule out the possibility of a non-specific toxicity effect resulting from the addition of a second protease inhibitor to adapted cells, we added the tetra peptide vinyl sulfone YL_3-VS (described in chapter 3) to adapted cells growing in 50 μM NLVS. YL_3-VS is toxic to normal cell growth at concentrations of 10 μM or higher, but showed no toxic effects on adapted cell growth at concentrations as high as 50 μM (data not shown). Taken together, these results suggest that the non-proteasomal proteolytic activity measured by AAF-MCA hydrolysis and which is increased in adapted cells, is necessary for growth and survival in the absence of an active proteasome.

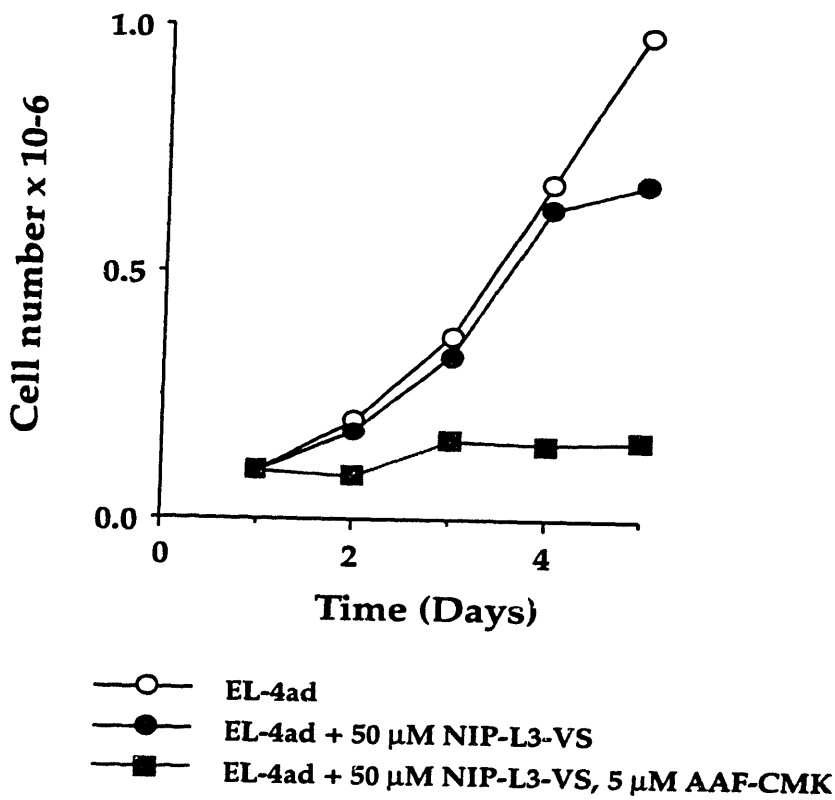
Figure 5.16. Inhibition of the AAF-MCA hydrolysis activity is toxic to adapted growth of cells adapted to NLVS.

(A) Control EL-4 cells were maintained for several days in the presence of 50 μM NIP-L3-VS or 5 μM AAF-cmk. Cells were counted daily and cell number plotted against time in culture. Note the toxicity of NLVS but not AAF-cmk to normal cell growth.
(B) Adapted EL-4 cells (maintained in 50 μM NLVS) were washed and treated with either 50 μM NLVS or 50 μM NLVS and 5 μM AAF-cmk. Growth was measured and recorded as in A. Note the complete loss of cell growth upon treatment with AAF-cmk

(A)


1.5
1.0
0.5
0.0
Cell number x 10-6
0
2
4
Time (Days)
EL-4
EL-4 + 50 μM NIP-L3-VS
EL-4 + 5 μM AAF-CMK

(B)


1.0
0.5
0.0
Cell number x 10-6
0
2
4
Time (Days)
EL-4ad
EL-4ad + 50 μM NIP-L3-VS
EL-4ad + 50 μM NIP-L3-VS, 5 μM AAF-CMK

D. Discussion

The proteasome's role in a wide range of important metabolic functions makes it indispensable for the survival of an organism, as revealed by mutational analysis in yeast. Treatment of cells with inhibitors which can block proteasome function indeed leads to cell death. However, we have found that populations of cells exist which are able to compensate for loss of proteolysis by the proteasome. The frequency with which this phenomenon occurs makes an explanation involving a genetic mutation unlikely. Biochemical analysis of proteasomes isolated from adapted cells indicates that proteasome function is severely impaired. It appears that adaptation results from the up-regulation of additional proteolytic activities whose functions must resemble those of the proteasome. This activity, when blocked using a somewhat non-specific protease inhibitor, abolishes growth of adapted cells while having little or no effect on the growth of normal cells.

The mechanism by which a cell becomes adapted to growth in the presence of inhibitor concentrations which would otherwise be toxic is not yet fully understood. We favor a scenario in which a small sub-population of cells within a normal cell culture have elevated levels of additional proteases or the ability to up-regulate such proteases. Under normal growth conditions, these cells do not have an advantage for survival over other cells, but under selective pressure of proteasome inhibitors, these cells are able to outgrow other cells that lack adequate levels of such proteases.

Once adapted, these cells are able to overcome the proteolytic blockade which results from proteasomal inhibition. Levels of ubiquitin-conjugated proteins begin to return to normal and the block in cell cycle leading to a G2 arrest is abolished. Thus, the enzyme or enzymes that are responsible for adaptation are likely to recognize and degrade proteins tagged with ubiquitin.

The presence of an increased AAF-MCA hydrolyzing activity in adapted cells is likely to be important for the adaptation phenomenon. The fact that hydrolysis of a single substrate increases in adapted cells in no way indicates that adaptation is the result of increased activity of a single proteolytic enzyme. The free amino terminus of the AAF-MCA substrate makes it likely to be a target for a number of N-terminal peptidases. Likewise, the ability of the tri-peptide

chloromethyl ketone to block adapted cell growth may not result from the inhibition of a single protease.

Future work must focus on the molecular characterization of the enzyme or enzymes responsible for adaptation. Initial studies are well underway to purify factors with AAF hydrolyzing activity. Recently, a high molecular weight protease complex, larger than the proteasome, was found in the archaebacteria *Thermoplasma* (Tamura et al., 1996). Interestingly, this protease complex, named tricorn protease, has preference for the AAF-MCA substrate containing a free amino terminus, an observation that prompted our use of a similar substrate as the corresponding inhibitor. Like the activity observed in adapted cells, it is sensitive to inhibitors which contain a chloromethyl ketone functional group. While these findings do not provide a direct link between the Tricorn protease and the activity observed in adapted cells, the high molecular weight protease activity which is activated in adapted cells may represent a mammalian homolog of the bacterial tricorn protease.

We have also found that the phenomenon of adaptation is not limited to the EL-4 cell line. We have succeeded in adaptation of the RBL-5 derived lymphoma cell line RMA. These cells may serve as tools to further examine the role of the proteasome as well as other proteases in such metabolically important functions as cell cycle regulation and antigen presentation.

Finally, the ease with which the peptide vinyl sulfones are synthesized and derivatized has made the discovery and characterization of the adaptation phenomenon possible. Peptide aldehydes, due to their increased cross-reactivity and non-specific toxicity are less likely to be useful for such studies. In addition, the reversibility of inhibition observed with peptide aldehydes may make removal by multi-drug resistance transporters a possible mechanism of adaptation. Lactacystin, on the other hand, although highly specific for the proteasome, is difficult to synthesize and thus the cost of obtaining sufficient quantities for such a study is prohibitive, at least at present.

VI. FUTURE DIRECTIONS

Although inhibitors of the proteasome have become widely used to dissect the role of the proteasome in many cellular processes, there still remains much which can be done with these types of pharmacological agents. The work presented in this thesis establishes the utility of the peptide vinyl sulfones as active site-directed probes for the study of proteasome function. The structure-function studies presented here allow for the correlation between enzymatic inhibition and inhibitor binding. Further, the *in vivo* application of the peptide vinyl sulfones has helped to uncover a novel mechanism by which a virus escapes host immunity. Finally, the prolonged treatment of cells with the peptide vinyl sulfones lead to the discovery of additional proteolytic systems that may exist in normal cells and allow for survival in the absence of the proteasomal activity. The exact physiological role of these newly discovered activities remains to be established.

Compounds such as the peptide vinyl sulfones, which contain a peptide backbone similar to a protein substrate, can be equipped with an appropriate tag for biosynthetic labeling, and can covalently modify proteasomal active site nucleophiles, offer the possibility of designing inhibitors which target specific proteasomal β subunits. The groundwork of such a study is described in chapter 3. Further C-terminally modified peptides may be synthesized with a variety of different amino acid sequences using modern combinatorial methods. Subsequent labeling of proteasome subunits with mixtures of compounds could identify inhibitors which show selective binding properties. Subunit-specific compounds could then be used to correlate subunit binding with inactivation of individual peptidase activities of the proteasome. Furthermore, such subunit-specific reagents would allow for the generation of pharmacological "knock-outs", in which the role of individual proteasomal peptidase activities in various metabolic processes such as antigen presentation could be explored.

While there can be no denying the importance of the proteasome in the generation of antigenic peptides for presentation via MHC class I molecules, many of the details of the proteasome's role in this process remain a mystery. A wide range of inhibitors with differing specificity for proteasome active sites may be extremely valuable in deciphering the details of antigen processing required for an infected cell to be recognized by a cytotoxic T-lymphocyte. Work on the

generation of such reagents is already underway and may someday be the focus of a thesis such as this one.

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