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The Major Histocompatibility Complex (MHC) and the Proteasome-Ubiquitin Pathway in T Cell Development

by

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Submitted to the Department of Biology in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy in Biology at the Massachusetts Institute of Technology February, 2002

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ABSTRACT

The essential role of classical MHC molecules in T cell development as well as the proteasome role in antigen processing for MHC-mediated antigen presentation to T cells is well established. However, the contribution of nonclassical MHC molecules, the signals evoked by the MHC-TCR interactions, the signaling role of the proteasome-mediated proteolysis, and the regulation of the proteasome-ubiquitin proteolysis remain to be clarified. Here we address these more subtle but essential aspects of T cell development.

We obtained mice deficient for MHC molecules encoded by the H-2K and H-2D genes. KbDb -/- mice have greatly reduced numbers of mature CD8+ T cells, indicating that selection of CD8+ T cells can not be compensated for by β2m-associated molecules other than classical H-2K and D locus products. Spleen cells from KbDb -/- mice generate strong CD8+ MHC class I-specific responses after in vivo priming. Thus, a minor population of CD8+ T cells arises in the complete absence of classical MHC class I molecules. KbDb -/- animals also have self-tolerant natural killer (NK) cells that retain their cytotoxic potential.

We utilize a fetal thymic organ culture (FTOC) system with a panel of proteasome inhibitors to implicate the proteasome in thymocyte apoptosis and negative selection. We find that proteasome inhibitors do not completely block but rather delay both dexamethasone- and antigen-triggered thymocyte apoptosis. We also show that proteasome activity is increased in apoptotic thymocytes, as visualized by active site labeling of proteasomal β subunits, indicating that proteasome functions as a positive regulator in thymocyte death cascade.

We show that the deubiquitinating enzyme USP7 (HAUSP) is specifically and uniquely processed in apoptotic thymocytes. USP7 protein is highly expressed in thymus, spleen, and brain and is very similar in men and mice. Processing of USP7 does not occur in caspase 3-/- thymocytes but caspase 3 does not cleave USP7 directly. Our results suggest that thymocyte apoptosis leads to a modification of a deubiquitinating enzyme and may provide an additional link between the proteasome-ubiquitin pathway and the caspase cascade during programmed cell death.

Thesis Supervisor: Dr. Hidde L. Ploegh
CHAPTER I

General Introduction

T cell development has been extensively studied for over a decade. Despite significant advances in this field, many critical questions remain unanswered. The difficulty in dissecting the mechanism of T cell development stems from the inherent complexity of the immune system that must discriminate self from non-self (foreign) antigens to avoid self-destruction while fighting dangerous pathogens. In the case of T lymphocytes, this discrimination begins in the thymus, where lymphocytes carrying an αβ T cell receptor (TCR) become tolerant to self-epitopes represented within the thymic microenvironment and differentiate from the CD4+CD8+ double positive (DP) thymocytes into CD4+ or CD8+ single positive (SP) thymocytes (Figure 1 and (1)). The perplexing feature of T lymphocyte development is that the same receptor can induce distinct signaling pathways, resulting in either differentiation and maturation or in death. A favored hypothesis to resolve this paradox is called "the avidity model" (Figure 1). The avidity model postulates that immature DP thymocytes are positively selected to differentiate into SP T cells when their TCRs encounter low or moderate avidity thymic ligands. The default pathway is "death by neglect" that occurs when TCRs do not encounter low or moderate avidity thymic ligands and thus fail to undergo positive selection. Potentially autoreactive DP thymocytes with TCRs that bind high avidity thymic ligands die by a process called negative selection. While this avidity model does not explain all of the experimental results, it is clear that the TCR's avidity in binding to thymic ligands is an important factor in T cell selection. Positively selected T cells migrate to the peripheral lymphoid organs and participate in immune responses against foreign antigens. Signaling networks of immature and mature T cells is the fascinating means of communication between the quantity and quality of the signal delivered to TCRs by antigen presenting cells (APC).

Systems and stimuli to study T cell development

Early T cell development occurs within an embryonic thymus (2, 3). Precursor cells seed the fetal thymus by day 12 of gestation. These precursors undergo phenotypic changes, including differential expression of several cell surface proteins, which are used to break down a developmental program into a series of checkpoints (2). Perhaps the most commonly used checkpoint classification is based on the expression of CD4 and CD8 coreceptors (Figure 1). The earliest precursors are CD4+CD8+ double negative, while TCR rearrangements lead to generation of CD4+CD8+ double positive (DP) thymocytes. CD4+CD8+ DP thymocytes that avoid death by neglect and negative selection are positively selected to differentiate into CD4+ or CD8+ single positive (SP) thymocytes. The thymus provides a specialized microenvironment for thymocyte selection and maturation. The importance of interactions between immature thymocytes and thymic stromal cell components has been extensively studied (reviewed in (3)).

Fetal thymic organ cultures (FTOC) provide a controlled approach to studying regulatory mechanisms of T cell selection (2). This system allows addition of various
reagents, such as peptides, cytokines, pharmacological inhibitors, while preserving the interactions between thymocytes and thymic stromal cells. The generation of mature T cells from immature precursors has not been achieved in vitro outside the FTOC model system.

Thymocyte suspension cultures obtained from a thymus of an adult animal are often used as a model system to study some aspects of T cell development. While this system is very convenient from a practical point of view, it neglects the contribution of thymic microenvironment. In fact, it has been shown that thymocyte responses to various stimuli in suspension cultures may differ significantly from those under intact thymic microenvironment (4, 5).

A variety of triggers are used to induce thymocyte death in suspension cultures or FTOCs, with antigen-specific TCR stimulation being the most physiological. The FTOC system allows induction of thymocyte differentiation or death with antigen-specific ligands (6). Antigen-specific deletion assays in suspension cultures have also been developed (7). In vitro antigen-driven negative selection of αβ TCR transgenic thymocytes has been achieved by employing conditionally immortalized thymic cortical epithelial cell lines and dendritic cells (8).

The stimulus that is used extensively to study thymocyte apoptosis is dexamethasone, a synthetic glucocorticoid. The mechanism of dexamethasone-triggered apoptosis is not understood, but is known to require new mRNA synthesis and is dependent on ATP (9-11). Under physiological conditions in the thymus, GR- and TCR-mediated signals have been proposed to be mutually antagonistic in triggering apoptotic cascade (9-11). In this model, naturally occurring glucocorticoids cause elimination of DP thymocytes that have insufficient TCR avidity to (death by neglect). Occupancy of the TCR by low-to-moderate avidity ligands counteracts glucocorticoid-induced apoptotic signal, rescuing thymocytes from death (positive selection). The TCR occupied by high avidity ligands initiates apoptotic signaling that could not be overcome by the GR-mediated signals (negative selection). Therefore, dexamethasone employed at pharmacological concentrations is likely to "short-circuit" the mutual TCR-GR antagonism and cause cell death.

Other triggers of T cell death include ionizing radiation, exposure to the topoisomerase type II inhibitor etoposide or the PKC inhibitor staurosporine, antibodies that trigger the death receptors Fas and TNFR, crosslinking of CD3 components of the TCR by anti-CD3 or mimicking TCR engagements with the phorbol ester PMA (12). Although these stimuli may initiate different death programs, the apoptotic events that ensue are similar, such as plasma membrane phosphatidylserine exposure, nuclear DNA fragmentation, chromatin condensation, cell shrinkage and cytolyis (13).

MHC molecules present processed antigens to T cells

Thymic ligands are presented to T cells in the context of major histocompatibility complex (MHC) molecule, a phenomenon referred to as "MHC restriction". Thus, TCR specificity applies to a particular MHC-peptide combination (Figure 2 and (14)). There are two types of MHC molecules: MHC class I and class II molecules that select the CD8" and CD4" T cell repertoire, respectively (15).
MHC class I molecules are expressed in almost all nucleated cells of the body. These molecules are heterodimeric type I membrane proteins, comprised of an MHC-encoded class I heavy chain, a β2m light chain and a short (8-10 amino acids in length) peptide, all of which are essential for the formation of a stable MHC class I complex (Figure 2 and (16)). A peptide is derived from cytosolic proteins residing in APCs (17). In contrast to MHC class I complexes, MHC class II molecules are expressed primarily on the surface of specialized antigen presenting cells. Both chains of the MHC class II complexes are encoded by MHC genes and antigens are derived from proteins entering the endocytic pathway either from the cytosol or by endocytosis (18).

In mice, MHC class I proteins whose heavy chains are encoded by highly polymorphic genes H-2K, D, and L are termed "classical" or class Ia MHC. In addition to the classical H-2K and H-2D MHC class I loci, there are less polymorphic MHC class I-encoded proteins (19). Moreover, there are additional genes, clearly related more closely to MHC class I genes than to any other gene family, but not linked to the MHC gene cluster. Both MHC-linked and -unlinked class I genes of this type are called MHC class Ib genes and encode molecules that are structurally similar to the classical MHC class Ia proteins. The role of MHC class Ib genes in T cell development and immune responses remains to be clarified. Obviously, the study of MHC class Ib-mediated responses is confounded by the presence of classical MHC class Ia proteins.

Chapter II addresses T cell selection in the absence of classical MHC class I molecules.

Signaling and antigen presentation of mature T cells

Presentation of foreign epitopes by peripheral APCs to mature SP T cells occurs in a MHC-dependent fashion, similar to presentation of selecting antigens by thymic APCs to immature DP thymocytes. Furthermore, reminiscent of the avidity model for thymocyte selection, the signaling program of mature T cells depends on the avidity of MHC-peptide interactions with the TCR. For example, the so-called agonist peptides are thought to result in high avidity interactions and elicit a full immune response (20). In addition to T cell tolerance acquired during thymic selection, T cell tolerance induction continues at the peripheral environment. The mechanism of peripheral tolerance remains to be elucidated. The so-called antagonist peptides, which inhibit responses induced by agonist peptides, are thought to play a role in peripheral tolerance (21).

Many molecules participate in the decision of whether a thymocyte is to live or die or whether a mature T cell is to elicit its effector function. Costimulatory molecules on APCs are thought to modulate signals delivered by MHC class I complexes (22-24). MHC/TCR-mediated signal is usually referred to as "signal 1", while costimulatory molecule-mediated signal is referred to as "signal 2". Several crucial T cell signaling events are mediated by changes in tyrosine phosphorylation of key signaling molecules (20, 25, 26). Specifically, antigen recognition results in the activation of intracellular protein tyrosine kinases, particularly members of the Src and ZAP-70/syk kinases. The proximal events in the phosphorylation cascade are the phosphorylation of tyrosines within the cytoplasmic domains of the TCR/CD3/ζ chain complexes. A number of studies indicated that this tyrosine phosphorylation cascade might in part be triggered by the signal 1 alone.
In Chapter III we describe a "signal 1"-based tyrosine phosphorylation assay that is employed to address several question in T cell signaling, including the nature of antagonist signaling and its dependence on the avidity of MHC-TCR interactions.

The proteasome is involved in multiple tasks during T cell development and immune responses

The size and amino acid sequence of an antigen-derived peptide is crucial for its ability to bind MHC class I molecules and subsequent recognition by T cells. Proteolysis of cytosolic antigens is carried out by the proteasome, a conserved multi-subunit complex that catalyzes protein degradation in the cytoplasm and the nucleus of all eukaryotic cells. The 26S proteasome is a large protease complex comprised of an inner 20S core with two associated 19S caps (27). The eukaryotic 20S core consists of 2 copies each of 7 distinct α and 7 distinct β subunits (Figure 3 and (28)). These subunits are organized in the four hetero-oligomeric rings of the 20S particle. Three β subunits in each ring are catalytically active, with the chymotryptic-like, glutamyl peptide hydrolyzing, and trypsin-like activity attributed to β5, β1, and β2, respectively. Upon exposure to cytokines such as IFNγ, several cell types express an additional set of catalytic β subunits (β5i, β1i, and β2i) that replace β5, β1, and β2 subunits and form a new particle called the immunoproteasome (Figure 3 and (29)). The assembly of the immunoproteasome is essential for the generation of suitable peptides for MHC class I-mediated antigen presentation. While the peptide repertoire generated by the 26S particle is less well understood, the capacity the 20S proteasome to generate peptide fragments with an average length of 7-8 amino acid residues has been thoroughly investigated (30). Subunit exchanges in the immunoproteasome are thought not only to optimize class I peptide loading but also to generate epitopes in inflammatory sites which are not proteolytically generated in uninflamed tissues, avoiding autoimmunity in uninflamed tissues (31).

Processing of antigens for MHC class I-mediated peptide presentation in APCs is but one example of the proteasome role in T cell development and immune responses. Recent studies indicate that the proteasome might be a signaling node within T cells undergoing death by neglect or negative selection (32). The key proteasomal targets in this signaling cascade are largely unknown. Potential targets include IAP (inhibitors of apoptosis), NFκB, IκB, and ornithine decarboxylase (33-36). Proteasome-mediated signaling within a mature T cells has also been documented, with the NF-κB pathway being the most cited example (37).

Chapter IV addresses the role of proteasome-mediated signaling in thymocyte apoptosis.

The proteasome is a node in the proteasome-ubiquitin pathway

To be effective as a signaling step in a signal transduction cascade, any proteolytic event should be selective and, therefore, highly regulated. For example, most death pathways activate the caspase proteolytic cascade (38). For caspase-mediated proteolysis, selective caspase activation and their well-defined substrate specificity result in the selective degradation of key substrates during a cell death program. Thus, caspase activation in apoptosis does not lead to indiscriminate proteolytic degradation: structural and
regulatory proteins are cleaved in a rather specific manner. For proteasome-mediated proteolysis, the regulation of selectivity in proteolytic cleavages is not well understood.

Most proteasomal substrates are targeted for degradation by conjugation of multiple ubiquitin chains via three distinct enzymatic activities: the Ub-activating enzyme or E1, Ub-conjugating enzymes or E2s, and Ub-ligases or E3s (Figure 4 and 39). Therefore, one possibility for regulating specific proteasomal cleavages is that of selective ubiquitin conjugation. Several reports have implicated Ub-conjugating enzymes and Ub-ligases in the regulation of apoptosis (40-42). Prior to degradation of Ub-conjugated proteins, the poly-Ub chain must be removed, a reaction catalyzed by deubiquitinating enzymes (Figure 4 and 43, 44). In addition to Ub recycling, deubiquitinating enzymes can reverse regulatory ubiquitination and edit inappropriately ubiquitinated proteins. Inability to remove Ub moieties from the substrates that should be spared from degradation will render those proteins susceptible to proteasomal degradation. Hence the control of deubiquitinating enzymes is likely to be an important parameter in the control of cytosolic and nuclear proteolysis.

**Deubiquitinating enzymes (DUBs)**

Based on substrate preferences deubiquitinating enzymes can be divided into two subfamilies: Ub C-terminal hydrolases (UCHs) and Ub-specific processing proteases (USPs). UCHs hydrolyze ubiquitin C-terminal and ester of ubiquitin, while USPs release ubiquitin conjugated to polypeptides by αNH-peptide bonds or εNH-isopeptide linkages. DUB family members contain the core catalytic domain comprised of the so-called cys and his boxes. This catalytic domain is similar to a peptidase domain of Cathepsin B, a member of the papain family of thiol proteases. In the yeast genome, 17 deubiquitinating enzymes have been identified (44). Multiplicity and complexity of this family in mammals is likely to be far greater (43). DUBs have been implicated in the regulation of diverse cellular processes, such as growth and oncogenesis, development and differentiation, regulation of transcription and chromatin condensation, and neuronal memory (43). The possible role of deubiquitinating enzymes in thymocyte apoptosis has not been examined.

Chapter V addresses the role of deubiquitinating enzymes in thymocyte apoptosis.

**Caspases in thymocyte apoptosis**

Caspases form a family of cysteine proteases that cleave their substrates C-terminally of Asp residues. The role of caspases in thymocyte apoptosis remains to be clarified. TCR triggering by peptide/MHC ligands activates an active caspase in DP thymocytes (38). Inhibition of this enzymatic activity with a general caspase inhibitor ZVAD-FMK prevented antigen-induced death of DP thymocytes in FTOC from TCR transgenic mice. ZVAD-FMK also prevented apoptosis induced by an anti-CD3 monoclonal antibody and corticosteroids in FTOC of wild type C57BL/6 mice. However, the results obtained with the transgenic mice that expressed the p35 caspase inhibitor were less clear. One group showed that thymocytes from p35 mice were resistant in vitro to several apoptosis-inducing agents, with resistance correlating with the inhibition of caspase activity (45). In F5 T cell receptor transgenic mice, expression of
the p35 transgene specifically inhibited negative selection triggered by two exogenous antigens, staphylococcal enterotoxin B superantigen and an antigenic peptide. However, another group reported that p35 did not block specific peptide-induced negative selection in OT1 and HY TCR transgenic mice models (46). ZVAD-FMK could not prevent peptide-induced deletion of OT1 thymocytes, although it improved basal thymocyte survival in vitro.

The above results could be reconciled in part by postulating that different stimuli invoke different death pathways, which lead to differential caspase activation or that are even caspase-independent. In fact, one report has suggested that spontaneous death in thymocyte suspension cultures is caspase-independent (47). There are at least two major intracellular pathways leading to activation of the caspase cascade (Figure 5 and (48)). The first is initiated by cross-linking of death receptors while the second is initiated by leakage of apoptogenic proteins, such as cytochrome c and AIF from mitochondria. These pathways are not mutually exclusive, since cross-linking of death receptors can induce the release of proapoptotic molecules from mitochondria. While the dexamethasone-mediated cell death program is thought to employ a mitochondrion-dependent caspase pathway (47), antigen-driven negative selection might utilize a mitochondrion- and death receptor-independent caspase cascade.

A variety of caspase substrates in apoptotic programs elicited by a variety of stimuli in a number of cell types have been identified (38). While some of those substrates may play a role in thymocyte selection, the majority of caspase substrates crucial for execution of thymocyte apoptosis are not known.

In Chapter V we explore whether a deubiquitinating enzyme is a caspase substrate during thymocyte apoptosis.
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Figure 1. **T cell development and selection in the thymus.**

(A) T cell precursors are CD4⁺CD8⁻ double negative (DN), while TCR rearrangements lead to generation of CD4⁺CD8⁺ double positive (DP) thymocytes. DP thymocytes that avoid death by neglect and negative selection are positively selected to differentiate into CD4⁺ or CD8⁺ single positive (SP) thymocytes.

(B) The avidity model of T cell selection postulates that immature DP thymocytes are positively selected to differentiate into SP T cells when their T cell receptors (TCR) encounter low or moderate avidity thymic ligands. The default pathway is "death by neglect" that occurs when TCRs do not encounter low or moderate avidity thymic ligands and thus fail to undergo positive selection. Potentially autoreactive DP thymocytes with TCRs that bind high avidity thymic ligands die by a process called negative selection.
Figure 2. Interaction between TCR and MHC class I/peptide complexes (14). MHC class I molecules are heterodimeric type I membrane proteins, comprised of an MHC-encoded class I heavy chain, a β2m light chain and a short (8-10 amino acids in length) peptide. All of these components are essential for the formation of a stable MHC class I complex and recognition by the TCR.
Figure 3. **Structural organization of the proteasome and regulation of the proteasome activity by alterations in catalytic subunit composition** (28).

(A) Structural organization of the proteasome.

(B) Proteasome activity can be regulated by altering the composition of its three catalytic subunits. The three constitutive β subunits, 1, 2, and 5, can be replaced by LMP2 (§1i), MECL-1 (§2i), and LMP7 (§5i), respectively, in response to certain physiological stimuli.
Figure 4. The ubiquitin-proteasome pathway (39).
(A) Conjugation of ubiquitin to the target molecule.
(B) Degradation of the tagged substrate by the 26S proteasome.
(1) Activation of ubiquitin by E1. (2) Transfer of activated ubiquitin from E1 to a member of the E2 family. (3) Transfer of activated ubiquitin from E2 to a substrate-specific E3. (4) Formation of a substrate-E3 complex and biosynthesis of a substrate-anchored polyubiquitin chain. (5) Binding of the polyubiquitinated substrate to the ubiquitin receptor subunit in the 19S complex of the 26S proteasome and degradation of the substrate to short peptides by the 20S complex. (6) Recycling of ubiquitin via the action of isopeptidases.
Figure 5. **Two Principal Caspase Signaling Pathways of Apoptosis** (48).

One pathway (left) involves ligation of death receptors, resulting in the recruitment of the adaptor protein FADD through interaction between the death domains (DD) of both molecules. The death effector domain (DED) of FADD in turn recruits pro-Casp8, which is cleaved and activated at the receptor complex. Another pathway (right), which is triggered by many apoptotic stimuli, is initiated at the mitochondrion. An early, not well-understood step is the mitochondrial release of cytochrome c into the cytosol which, together with dATP, binds to the CED-4 homolog Apaf1. This event unmasks the CARD motif in Apaf1 and allows binding of procaspase-9 through CARD/CARD interaction. The mitochondrial but not the death receptor pathway is inhibited Bcl2. Antiapoptotic members of the Bcl2 family may interfere with the relocalization of cytochrome c or with the binding of cytochrome c to Apaf1. Following activation of the initiator caspase Casp8 or Casp9, the two pathways converge on the activation of effector Casp3, -6, -7, which finally cleave various death substrates. Because Casp8 cleaves Bid and generates a truncated, proapoptotic BH3-containing fragment (tBid) that induces cytochrome c release, both pathways cross-communicate. Casp8, in turn, can be also activated by Casp6 following Casp9 cleavage, thereby amplifying the apoptotic signal.
CHAPTER II

Generation and characterization of the KbDb -/- mice

This chapter describes the generation and characterization of the KbDb -/- mice. This work was published in Proc. Natl. Acad. Sci. USA, and the reprint "Major histocompatibility complex (MHC) class I KbDb -/- deficient mice possess functional CD8+ T cells and natural killer cells" is enclosed. I have also bred the KbDb -/- animals with the 2C TCR transgenic mice to obtain the 2C KbDb -/- strain. The 2C KbDb -/- animals were used in the study "Positive selection of an MHC class-I restricted TCR in the absence of classical MHC class I molecules" by Madelon Maurice. My contribution to this work was the generation of the 2C KbDb -/- founders and analysis of the cytolytic activity of CD8+ T cells isolated from the intestine of 2C RAG -/- KbDb -/- animals (Fig. 4 in the manuscript enclosed in the Appendix).
Major histocompatibility complex (MHC) class I K\textsuperscript{b}D\textsuperscript{b} –/– deficient mice possess functional CD8+ T cells and natural killer cells

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ABSTRACT We obtained mice deficient for major histocompatibility complex (MHC) molecules encoded by the H-2K and H-2D genes. H-2 K\textsuperscript{b}D\textsuperscript{b} –/– mice express no detectable classical MHC class I-region associated (Iα) heavy chains, although β\textsubscript{2}-microglobulin and the nonclassical class Ib proteins examined are expressed normally. K\textsuperscript{b}D\textsuperscript{b} –/– mice have greatly reduced numbers of mature CD8\textsuperscript{T} cells, indicating that selection of the vast majority (>90%) of CD8\textsuperscript{T} cells cannot be compensated for by β\textsubscript{2}-microglobulin-associated molecules other than classical H-2K and D locus products. In accord with the greatly reduced number of CD8\textsuperscript{T} cells, spleen cells from K\textsuperscript{b}D\textsuperscript{b} –/– mice do not generate cytotoxic responses in primary mixed-lymphocyte cultures against MHC-disparate (allogenic) cells. However, in vivo priming of K\textsuperscript{b}D\textsuperscript{b} –/– mice with allogeneic cells resulted in strong CD8\textsuperscript{T} MHC class Ia-specific alloergic responses. Thus, a minor population of functionally competent peripheral CD8\textsuperscript{T} cells capable of strong cytotoxic activity arises in the complete absence of classical MHC class Ia molecules. K\textsuperscript{b}D\textsuperscript{b} –/– animals also have natural killer cells that retain their cytotoxic potential.

Major histocompatibility complex (MHC) class I molecules, expressed on almost all nucleated cells of the body, are heterodimeric type I membrane proteins, composed of an MHC-encoded class I heavy chain, a β\textsubscript{2}-microglobulin (β\textsubscript{m}) light chain, and a short peptide 8–10 aa in length, all of which are essential for the formation of a stable MHC class I protein (1). Those MHC class I proteins whose heavy chains are encoded by highly polymorphic genes (H-2K, D, and L in mice) are termed “classical” or class I-region associated (Iα) MHC. Cell-surface MHC class Ia proteins are essential for the thymic development of CD8\textsuperscript{T} cells (2) and for providing protection against lysis by natural killer (NK) cells (3). Recent work suggests that cell-surface MHC class Ia proteins are also involved critically in maintaining the levels of memory CD8\textsuperscript{T} cells in the periphery (4, 5). For studies of these processes, mice that are totally deficient in the classical I MHC proteins would be valuable.

Mice deficient for β\textsubscript{m} or the MHC-encoded peptide transporter (TAP), a complex involved in loading peptides onto newly synthesized MHC class I proteins, do not express normal levels of cell-surface MHC class I proteins and consequently are defective in MHC class I-restricted antigen presentation and in thymic selection of CD8\textsuperscript{T} cells (6–8). These TAP –/– and β\textsubscript{m} –/– mice have been used widely as MHC class I-deficient models, but this deficiency is not complete. Thus, a sizable fraction of MHC class I heavy chains apparently folds normally and is transported to the cell surface even in the absence of β\textsubscript{m} (in particular, the H-2D\textsuperscript{b} gene product) or in the absence of TAP-dependent peptides (8–12). In addition, “empty” class I molecules are expressed on the cell surface of TAP –/– and β\textsubscript{m} –/– animals, so that total MHC I cell-surface levels are decreased at most 10-fold (12, 13). Although such empty class I molecules decay rather rapidly in ex vivo cultured cells, nothing is known about their half-life in vivo. TAP –/– and β\textsubscript{m} –/– animals also possess a limited repertoire of self-MHC class I-restricted CD8\textsuperscript{T} cells, which can be explained by their selection on the remaining low levels of MHC class I (14–17). These animals reject allogeneic skin grafts and tumors and give rise to viral and allogeneic CD8\textsuperscript{T} cell-mediated responses (14, 17–21), consistent with the continued presence of functional MHC class I proteins.

In addition to the classical H-2K and H-2D MHC class I loci, there are fewer polymorphic MHC class I-encoded proteins (encoded by genes in the O, T and M regions; refs. 22 and 23). Moreover, there are additional genes, clearly related more closely to MHC class I genes than to any other gene family, but not linked to the MHC (CD1, ZAP, FcRa, and HFE; refs. 22–27). Both MHC-linked and MHC-unlinked class I genes of this type are called MHC class Iib genes, and the molecules they encode (MHC class Iib proteins) are structurally similar to the classical MHC class Ia proteins. Many of the class Iib molecules require β\textsubscript{m} and possibly TAP-dependent peptides for their folding and transport to the cell surface (28). In some cases, presentation of peptides by class Iib proteins to αβ or γδ T cells has been shown (28).

Nonclassical MHC molecules are functionally diverse. For example, the FcRa receptor transports IgG from ingested milk across the intestinal epithelium of neonatal mice and rescues circulating IgG from degradation (24). In addition, CD1 plays a crucial role in the function of NK1.1 T cells and in their positive selection (26, 29). H-2 K\textsuperscript{a} presents N-formylated peptides derived from certain bacterial and mitochondria-encoded proteins (23, 30), whereas MHC class Ia molecules do not seem to bind such peptides (25, 30, 31). Expression of H-2 Qa-2 has been correlated with the expression of an early embryo gene, Ped, that maps in the H-2 Qa-2 region and controls the rate of preimplantation embryonic development (32). Though the functional role of Qa-2 in the immune response is not known, it binds a wide variety of peptides, and its expression depends on TAP (32). Because of the requirement of β\textsubscript{m} and TAP in the expression of nonclassical MHC molecules (22, 33), many class Iib molecules fail to be expressed on cells in animals that lack β\textsubscript{m} or TAP. Combined, these findings suggest that neither β\textsubscript{m} –/– nor TAP –/– mice can be considered ideal models for mice whose lack of MHC class Ia molecules is complete and limited to only the classical MHC class Ia molecules.

Besides their well established role as restriction elements for cell recognition, MHC class I molecules are also involved in

Abbreviations: β\textsubscript{m}, β\textsubscript{2}-microglobulin; CTL, cytotoxic T lymphocyte; FACs, fluorescence-activated cell sorter; FICIT, fluorescein isothiocyanate; Iα, I-region associated; MHC, major histocompatibility complex; MLC, mixed-lymphocyte culture; NK, natural killer; PEC, phycoerythrin; RAPHC, rabbit anti-MHC I free heavy chain; TAP, peptide transporter; TCR, T cell receptor.

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providing protection from lysis by NK cells (3, 34–36). Little is known about the development of NK cells, but a number of reports support the existence of an educational process for NK-cell tolerance, and it has been proposed that NK cells calibrate to endogenous levels of self-MHC class I during development. For example, βm−/− mice possess functional but self-tolerant NK cells, whereas βm+ mice. Con A-stimulated blasts are killed by NK cells from βm−expressing parental mice (37, 38). The mechanism of this self-tolerance is not well understood but includes the regulation of NK-cell inhibitory receptors. In addition, recent reports show that NK-cell reactivation may also depend on MHC class Iβ molecules (39, 40), which are not expressed normally in either βm−/− or TAP−/− mice. This further motivates the use of mice that are selectively deficient for MHC class Iα in such studies.

To obtain MHC class Iα-deficient animals, we took advantage of mice of the 129 strain with defective K or D genes, which account for all of the classical MHC class Iα heavy chains in mice of the H-2b MHC haplotype (4, 41). The genetic distance between these genes is only 0.2 centimorgan (42). We obtained H-2β Kβ−/− mice by crossing H-2Kb−/− with H-2Db−/− animals, crossing progeny for the rare intra-H-2 recombinant that carries both targeted genes on the same chromosome. Here, we report characteristics of these mice.

**MATERIALS AND METHODS**

**Mice and Cell Lines.** K0−/− and D0−/− single-knockout mice are reported elsewhere (4, 41). They are generated from E14TG2a embryonic stem cells derived from the 129/Ola mouse strain and injected into BAL/c mice. The H-2Kb and H-2Db, plus products of the H-2b haplotype are serologically indistinguishable from H-2Kb and H-2Db, but the H-2b haplotype differs from H-2a in the H-2T region that contains genes encoding class Iβ molecules (22). C57BL/6, BALB/c, and βm−/− strains were purchased from The Jackson Laboratory. BALB/c is an H-2Kb-expressing mouse mastocytoma derived from the DBA/2 strain. EL-4 is a T-cell lymphoma derived from (CBA × C57BL/6). T2-Kb is an H-2Kb transfectant of T2 that has an antigen processing mutation caused by a genomic deletion in the MHC class II region (43). C4.4−/− is a βm-defective mutant of EL-4 and carries a stop codon in the sixth codon of the βm gene (ref. 44; R.G. and K. Magnuson, unpublished work). RMA-S is an NK-sensitive TAP-2 defective lymphoma of RMA A strain. EL-4 is a T-cell lymphoma derived from C57BL/6. YAC-1 is an NK-sensitive lymphoma derived from the A/Sn strain, commonly used to detect NK-cell cytotoxic activity.

**Antibodies.** Anti-H-2Kc coupled to fluorescein isothiocyanate (FITC), anti-H-2Db coupled to FITC, anti-H-2Kb coupled to phycocyanin (PE), anti-H-2D4, anti-CD4 coupled to PE, anti-CD8 coupled to FITC, anti-T cell receptor (TCR) coupled to PE, goat anti-mouse IgG coupled to horsedradish peroxidase, goat anti-rabbit IgG coupled to horseradish peroxidase, anti-Qa-2, and biotinylated anti-Qa-2 antibodies were purchased from Phar-Mingen. Rabbit anti-MHC I free heavy chain (RAFHc) and antioxin 8 of K0 antibodies have been described (45).

**Metabolic Labeling and Immunoprecipitations.** Spleen cells were incubated for 30 min in methionine and cysteine-free DMEM, labeled with 1 mCi of methionine/106 cells for 45 min, and chased for 2 h in RPMI medium 1640 containing 10% fetal calf serum. Cells were lysed in Nonidet P-40 lysis buffer (0.5% Nonidet P-40, 50 mM Tris, pH 7.5/5 mM MgCl2/1 mM phenylmethylsulfonyl fluoride/2 μg/ml leupeptin/1 μg/ml aprotinin). Lysates were precleared twice with normal rabbit or mouse serum and formalin-fixed *Staphylococcus aureus*, followed by the indicated MHC class Iα or β specific reagents. Immunoprecipitates, adjusted for the total amount of incorporation as measured by trichloroacetic acid precipitation, were run on SDS-PAGE. Gels were developed with dimethyl sulfoxide/2,5-diphenyloxazole, exposed to Kodak X-Omat AR film.

**Peptide N-Glycosidase Treatment and Immunoblotting.** Tissues from different organs were homogenized in 0.5-2 ml of Nonidet P-40 lysis mix and incubated at 4°C for 1 h. Lysates were spun for 30 min at 14,000 rpm in an Eppendorf centrifuge and diluted in lysis mix to 2 mg/ml total protein. Lysates were then heat-denatured in 0.6 M 2-mercaptoethanol and 2.4% SDS at 85°C and treated for 4 h at 30°C with peptide N-glycosidase in PBS containing 25 mM EDTA/1% Nonidet P-40 and 0.1 M 2-mercaptoethanol. Lysates were then separated by SDS/PAGE and transferred to nitrocellulose. The filters were incubated with the indicated first antibodies (RAFHC 1:1000 and anti-oxin 8 of K0 antibodies 1:2000), followed by horseradish-peroxidase-coupled goat anti-mouse or anti-rabbit IgG. The immune complexes were visualized by chemiluminescence.

**Flow-Cytometry Evaluation of MHC I, CD4, CD8, and TCR Surface Expression.** Screening for K0−/− mice was done by collecting ~100 μl of tail blood into 50 mM EDTA in PBS. Red blood cells were removed by osmotic lysis. Splenocyte and thymocyte samples from K0−/− and control mice were prepared by making single-cell suspensions in RPMI medium 1640. Cells were then stained with FITC or PE-labeled antibodies in PBS containing 25% fetal calf serum and 1% normal mouse serum by suspension of unprimed mice. MHC I and TCR analyzed in a fluorescence-activated cell sorter (FACScan or FACSStar, Becton Dickinson) with CELLQUEST software. We collected ~2,500 cells per blood sample and 10,000–50,000 cells per spleen or thymus sample.

**Cytotoxic T Lymphocyte (CTL) Generation and Complement-Mediated Depletion of T Cells.** Kβ−/− βm−/− BALB/c or C57BL/6 mice were injected twice (primed) with either C57BL/6 or BALB/c splenocytes (~107 cells per 300 μl of PBS, injected i.p.) with ±10-day interval between injections. CTL were obtained by 5- to 7-day mixed-lymphocyte cultures (MLCs) with C57BL/6 or BALB/c irradiated spleen cells used as stimulators and spleen cells from either naive or primed mice as responders. For MLC, 25 × 104 responder spleen cells were mixed with 2.5 × 103 stimulator spleen cells and were used as effector cells after 5-7 days. Complement-mediated depletion of CD8+ or CD4+ T cells was done by incubating ~105 responder cells with 10 μg of the appropriate antibody for 30 min at 4°C, followed by wash and incubation with 10 μg of complement for 1 h at 37°C in 6% CO2.

**Generation of Con A Lympheblasts and NK Cells.** Spleen cells (5 × 105), from which erythrocytes had been depleted, were placed in 10 ml of complete medium with Con A (Sigma). After 2 days the cells were harvested, and viable cells were isolated by centrifugation with Lymphoprep (Nycocomb, Oslo). For cytotoxicity assays with tumor target cells, NK effector cells were generated from erythrocyte-depleted splenocytes from mice injected 1 day earlier with tilorone (Sigma). For a cytotoxicity assay with Con A lymphoblasts, NK effector cells were obtained by culturing 25 × 104 spleen cells in 10 ml of complete medium and 2000 units/ml recombinant interleukin 2 at 37°C in 6% CO2. After 5 days both nonadherent and adherent cells were removed to obtain lymphokine-activated killer cells.

**Cytotoxic 1Cr Release Assay.** The effector cells (see above) were tested at the indicated effector:target ratios, in RPMI medium 1640 supplemented with 10% fetal calf serum, against 5 × 105 51Cr-labeled target cells per well (96-well plate). Each effector:target ratio was assayed in triplicate, and assays were incubated for 3.5–4.5 h.

**RESULTS**

**Generation of Kβ−/− D0−/− Mice.** To obtain Kβ−/− D0−/− mice we used our mice were made by single K0−/− and D0−/− knockout animals on the H-2 b haplotype in the 129 mouse strain (4, 41). These animals were crossed and their offspring were screened for an intra-H-2 recombinant that carried both targeted loci on the same chromosome with the following breeding scheme.
Kb−/− animals were crossed to Dp−/− animals to obtain heterozygotes with the genotype Kp+Dp−/Kp−Dp+. To detect rare germ-line recombination events (the expected recombination frequency is 1/16) (ref. 42), we crossed these heterozygotes to the BALB/c (H-2b haplotype) strain to obtain animals with the genotype Kp−Dp−/H-2b. The screen was done by FACS analysis on blood lymphocytes. Of 155 animals tested, only one was a male that had no surface expression of either Kp or Dp proteins, as expected for the desired intra-H-2 recombination. The genetic background of these mice is therefore a mixture of the 129 and BALB/c strains.

KpDp−/− homozygotes were generated by breeding the Kp−Dp−/H-2b male with a Kp−/− female. We screened for lack of expression of H-2b alleles and crossed Kp−Dp−/Kp−Dp+ offspring to produce the double-knockout animals with a frequency of 25% (15 of 58). A line of KpDp−/− double-knockout mice was maintained by brother-sister matings. FACS analysis performed on blood lymphocytes from double-knockout mice showed no cell-surface expression of Kp, Dp, or Dp heavy chains (Fig. 1C). Thus, double-knockout animals do not have reduced viability compared with their MHC class Ia-positive littermates, as has also been observed for TAP−/− and β2m−/− animals (6, 7).

FACS analysis performed on splenocytes and thymocytes from these mice showed no cell-surface expression of Kp, Dp, or Dp heavy chains, whereas I-Aα, and Qa-2 were expressed normally (Fig. 1B and data not shown).

**Absence of Classical MHC Class I Proteins in KpDp−/− Mice.** To establish the absence of the classical MHC class I heavy chains in these mice, we analyzed whole-cell lysates from different tissues by immunoblotting using RAFCIC, also directed against the cytoplasmic tail of Kp (α-p8). As expected from their cell-surface phenotype, KpDp−/− mice do not express the approximately 43-kDa glycoprotein in any of the tissues analyzed (Fig. 2A). However, with the antisera directed against free MHC class I heavy chain, we did detect 40-kDa MHC class I heavy chain in both control and KpDp−/− samples. These glycoproteins likely correspond to the nonclassical class I heavy chains which are cross-reactive with the broadly reactive RAFCIC.

Immunoblots with the RAFCIC antisera showed an intestinespecific glycoprotein of approximately 43 kDa in C57BL/6 but not in KpDp−/− mice. Because this species was present only in wild-type animals and was not detected with the α-p8 antisera, it might correspond to an H-2 Kb splice variant (46). The use of alternative-splice acceptor sites during the removal of intron 7 in pre-mRNA splicing produces two forms of H-2Kb protein: the predominant form, derived from a transcript that was spliced at the upstream splice acceptor site for exon 8, and a Kb molecule derived from a transcript that was spliced at the downstream acceptor site for short exon 8 (46).

Immunoprecipitation experiments from 35S-labeled KpDp−/− splenocytes, Con A blasts (Fig. 2B), or T cell lines obtained from these animals confirmed the data obtained by immunoblots. Whereas MHC class I-specific antisera did not recover any immunoreactive species from cell lysates prepared from KpDp−/− mice, antibodies against MHC class I or Kb proteins or Qa-2 molecules recovered proteins of expected molecular masses (data not shown).
Thus, K^Dd−/− animals lack expression of the classical MHC class Ia heavy chain, whereas MHC class II and non-classical MHC class Ib expression seems normal.

Severe Deficiency in CD8+ T Cell Maturation in K^Dd−/− Mice. We analyzed the numbers of CD8+ TCRαβ+ T cells in splenocytes and thymocytes from double K^Dd−/−/− animals. FACS analysis showed 5–10-fold reduction in the number of peripheral CD8+ T cells, with some compensatory increase in the CD4+ T cell compartment (Fig. 3). This reduction was comparable to that seen in βm−/− animals (Fig. 3; refs. 6–8). The most obvious explanation for the small peripheral CD8+ pool is a defect in thymic development caused by the lack of classical MHC class I molecules. Indeed, the thymic CD4:CD8+ population was reduced 5-fold (to 1% of total), whereas the numbers of CD4+CD8− cells were comparable to those in control animals (Fig. 3).

To test the function of K^Dd−/− lymphocytes, we performed a series of primary MLCs with splenocytes from the K^Dd−/−/−, BALB/c (allogeneic, H-2d), and C57BL/6 (K^Dd+/+ control) animals. Cytotoxic responses were then examined on targets with the relevant haplotypes. Splenocytes from K^Dd−/−/− mice showed neither cytotoxic nor stimulatory activity (Fig. 4), consistent with the low numbers of peripheral CD8+ T cells observed by flow cytometry. We conclude that K^Dd−/− animals are severely deficient in CD8+ T cell maturation; this deficiency is caused by a block in thymic development.

Cytotoxic Activity of CD8+ T Cells from K^Dd−/−/− Mice After in Vivo Priming. Although functional CD8+ T cells are seemingly absent from K^Dd−/−/− mice, it might be possible to achieve activity from a small residual CD8+ repertoire by in vivo immunization.

The K^Dd−/−/− mice were injected (primed) twice with splenocytes from either BALB/c or C57BL/6 mice (fully allogeneic or inbred control, respectively). The splenocytes from these mice were then used as responder cells for in vitro MLC. After 5 days of MLC, we found strong cytolytic activity mediated by K^Dd−/−/− CD8+ T cells against both H-2^d (Fig. 5 and D) and H-2^k (Fig. 5 B and E) mouse target cells. This response was even stronger than that from βm−/−/− animals (Fig. 5). Similar levels of lytic activity were observed when human T2 targets transfected with mouse MHC class I molecules (H-2Kb) were used, whereas untransfected T2 cells were not killed (Fig. 5C and data not shown). These results suggest that K^Dd−/−/− CD8+ T cells directly recognize classical MHC class I antigens. Further, because of the absence of MHC class Ia-mediated selection in K^Dd−/−/− mice, such MHC class Ia-specificity may be germ-line-encoded (47).

![Image 3](#) Reduction in CD8+ TCRαβ+ T cell numbers in K^Dd−/−/− mice. FACS analysis was performed on C57BL/6, βm−/−/−, or K^Dd−/−/− splenocytes (Top and Middle) or thymocytes (Bottom) with α-CD8-FITC, α-CD4-PE, and α-TCRαβ-PE antibodies, as indicated. Numbers correspond to percentage of cells in the indicated regions.

![Image 4](#) Absence of primary cytotoxic response in MLC by spleen cells from unprimed K^Dd−/−/− mice. Effector cells were obtained by the standard 7-day MLC with spleen cells from C57BL/6, BALB/c, or K^Dd−/−/− used as stimulators and responders (as indicated). CTL-mediated cytotoxicity was measured in a standard 4-h 51Cr release assay with H-2^k P815 (Top), H-2^d EL4 (Middle), or βm−/−/− C4.4.25+ (Bottom) targets. E:T, effector:target ratio. Similar results were obtained with CTL lines specific for H-2^d and H-2^k derived from K^Dd−/−/− mice (data not shown).

A minor yet fully functional peripheral T cell population can thus develop and be activated in vivo in the complete absence of classical MHC class Ia molecules.

NK Cells in K^Dd−/−/− Mice. MHC class I proteins are important for protection against NK-cell lysis (3, 34–36). Therefore, we tested whether K^Dd−/−/− animals possess functional NK cells and whether lack of heavy chain expression would render K^Dd−/−/− cells susceptible to lysis by wild-type NK cells. NK-cell activity from K^Dd−/−/− mice stimulated with the interferon inducer tilorone was tested on NK-sensitive TAP-deficient RMA-S target cells. NK cells from K^Dd−/−/− mice were able to lyse RMA-S cells (Fig. 6A), indicating that NK cells can develop in the complete absence of classical MHC class I molecules and retain at least some of their cytotoxic function.

A similar result was obtained with YAC-1 target cells (data not shown). K^Dd−/−/− Con A blasts and βm−/−/− Con A blasts show similar levels of sensitivity, indicating that neither MHC class I free heavy chains nor βm-associated MHC class Ib molecules strongly influence sensitivity of Con A blasts to NK-cell lysis (Fig. 6B; ref. 49).

**DISCUSSION**

We have generated mice deficient for the classical MHC class Ia molecules (K^b and D^b) by intercrossing mice defective in
either K\(^b\) (H-2K\(^b\) /~) or D\(^b\) (H-2D\(^b\) /~) and screening for rare intra-H-2 recombinants. These doubly deficient K\(^b\)D\(^b\) /~ mice have a profound reduction in the numbers of peripheral CD8\(^+\) T cells, but can nonetheless generate strong MHC-specific CD8\(^+\) T cell responses after priming in vivo.

Several conclusions may be drawn from these observations. Classical MHC class I molecules are required for selection of at least 90\% of the CD8\(^+\) T cells. The contribution by \(\beta_m\)-associated, TAP-loaded class I molecules of other provenances (e.g., class Iib molecules) can thus account for no more than 10\% of CD8\(^+\) T cells. Although K\(^b\)D\(^b\) /~ mice have few peripheral CD8\(^+\) T cells, these cells are fully functional, and they must have arisen in a developmental pathway that is independent of classical MHC class Ia molecules. The apparent class I specificity of these CD8\(^+\) T cells may be germ-line-encoded, as shown by Zerrahn et al. (47), and does not depend on the presence of host class Ia molecules. The low numbers of CD8\(^+\) T cells can be ascribed to a block in thymic T cell development from the CD4+CD8\(^+\) to the CD4-CD8\(^+\) stage.

K\(^b\)D\(^b\) /~ mice would be expected to lack almost all peripheral CD8\(^+\) T cells, based on the comparison with \(\beta_m\)-/~ and TAP-/- animals (6-8). Nevertheless, we find the low numbers of CD8\(^+\) T cells striking, because class Iib molecules are structurally very similar to the classical MHC class Ia products (22, 23) and are expressed in hematopoietic cells, which have some capacity for positive selection (49). There are no arguments that would prohibit the involvement of class Iib molecules in positive selection of CD8\(^+\) T cells. However, class Iib molecules require both \(\beta_m\) and peptide for their expression and are not expressed properly at the cell surface of \(\beta_m\)-/- or TAP-/- animals. For the study of T cell MHC class Ia- and Iib-mediated T cell selection, K\(^b\)D\(^b\) /~ mice are clearly the preferred model, because their deficiency is complete and limited to only MHC class Ia molecules. Our results show that the contribution of nonclassical class Iib molecules to the development of the CD8\(^+\) T cell repertoire is minor.

The remaining pool of functional CD8\(^+\) T cells in \(\beta_m\)-/~ or TAP-/- mice has been explained by the low levels of

![Diagram](https://example.com/diagram.png)

**Fig. 5.** Cytotoxic activity of CD8\(^+\) T cells from K\(^b\)D\(^b\) /~ mice after in vivo priming. C57BL/6, BALB/c, \(\beta_m\)-/-, or K\(^b\)D\(^b\) /~ mice were injected twice in vivo with either BALB/c (A and D) or C57BL/6 (B, C, and E) splenocytes. Effector cells were obtained by a standard 6-day MLR with the indicated splenocytes used as stimulators. K\(^b\)D\(^b\) /~ effector cells (anti-BALB/c in D and anti-C57BL/6 in E) were depleted of CD8\(^+\) or CD4\(^+\) T cells with \(\alpha\)-CD8 or \(\alpha\)-CD4 antibodies and complement (D and E). CTL-mediated cytolysis was measured in a standard 4-h \(^{51}\)Cr release assay using H-2\(^d\) P815 (A and D), H-2\(^d\) EL4 (B and E), or T2-K\(^b\) (C) target cells. The controls for \(\iota\) were C57BL/6 anti-BALB/c effectors, and the controls for B and C were BALB/c anti-C57BL/6 effectors (○).

**Fig. 6.** K\(^b\)D\(^b\) /~ mice as donors of effector or target cells in NK-mediated cytolysis in vivo. (A) C57BL/6, \(\beta_m\)-/-, and K\(^b\)D\(^b\) /~ mice were injected with the interferon inducer tilorone, and splenocytes from these mice were used as effector cells 1 day after injection. NK-mediated cytolysis was measured with 4-h \(^{51}\)Cr release assay on RMA-S target cells. (B) C57BL/6 lymphokine-activated killer cells were obtained by 5-day culture of erythrocyte-depleted splenocytes. NK-mediated cytolysis was measured with 4-h \(^{51}\)Cr release assay on C57BL/6, \(\beta_m\)-/-, and K\(^b\)D\(^b\) /~ Con A blast target cells.
cell-surface MHC class I still expressed in these mice. This explanation is supported by a number of studies showing a biased specificity in these T cells, which show increased avidity for self-MHC class I molecules (14, 19). In K^D^0 --/-- mice, no MHC class Ia is present to influence the development of T cells, but we still observe the presence of a minor CD8^+ T cell pool. However, βγm or TAP --/-- mice are deficient in expression of MHC class Iβ molecules, whereas levels of class Iβ molecules seem to be normal in K^D^0 --/-- mice. Thus, the remaining population of CD8^+ T cells in K^D^0 --/-- mice may be explained by their selection on MHC class Iβ molecules, but this hypothesis will be addressed experimentally. The importance of MHC class I products in positive selection of CD8^+ T cells is well established from βγm or TAP --/-- models (2, 6–8). Our results argue for the existence of a developmental pathway of CD8^+ T cells that is entirely independent of the H-2K and D loci products. It remains to be established whether this pathway requires expression of nonclassical class Iβ products. It is a consequence of stochastic down-regulation of CD8 coreceptor after the CD4^+CD8^+ T cell. At least one class I gene product, called CD1, has been implicated in the development of mouse T cells and plays a crucial role in positive selection and function of NK1.1 CD4^+ T cells (26, 29).

The NK cells from K^D^0 --/-- animals are of considerable interest, because these develop in the absence of MHC class Iα molecules. K^D^0 --/-- spleen NK cells are able to lyse the standard NK-cell tumor target cells, such as YAC-1 and RMA-S (Fig. 6A), efficiently and have thus retained at least some cytolytic function. NK cells in K^D^0 --/-- and βγm --/-- animals probably have similar mechanisms of self-tolerance, because NK cells from βγm --/-- animals do not kill K^D^0 --/-- targets and vice versa (49). It is possible that nonclassical class Iβ molecules, acting through inhibitory NK receptors, are involved in self-tolerance of K^D^0 --/-- as well as in βγm --/-- NK cells. Of interest in this respect is the Qa-1 class Iβ molecule, which presents the signal peptide derived from H-2D to CTL in most H-2 haplotypes, except H-2^d (50, 51). Furthermore, some functions of the Qa-1 molecule are dependent on its association with β2m (52). In human cells, the NK-protective function of HLA-G can now be understood as the presentation of HLA-G's signal sequence by HLA-E. This may present a situation analogous to that proposed for Qa-1, because HLA-E and Qa-1 have similarities in their antigen binding clefts and present similar types of peptides (52, 53). Qa-1 cannot present the H-2D-derived signal sequence in K^D^0 --/-- mice, because the H-2D^β gene product is absent (the genomic deletion in the H-2D gene includes exons 1–3, coding for the signal peptide, α1 and α2), but perhaps other signal sequences may substitute. Thus, it is unlikely that signal peptide presentation by Qa-1 plays a role in self-tolerance in βγm or K^D^0 --/-- mice. What is the mechanism of this self-tolerance? Recent reports have suggested down-regulation of positive signaling pathways as an explanation for tolerance (40, 54, 55).

Killing of class I-deficient tumor targets may be explained by unregulated expression of molecules (distinct from class I) by these targets. In any case, K^D^0 --/-- animals provide a tool to study activation, inhibition, and development of NK cells. K^D^0 --/-- mice, in which the responses are mediated by classical MHC class I are absent, provide a unique system for studying the functions and immunological activities of class Iβ molecules. This type of study cannot be accomplished cleanly at present in βγm or TAP deficient mice, in which surface-expressed free MHC class I heavy chains can serve as restriction elements for immune responses (15, 16, 19, 21). Although the expression of properly folded MHC class I molecules on βγm or TAP --/-- cells may be low, T cells are able to recognize target cells with very low MHC ligand densities (56). Furthermore, βγm- and TAP-deficient animals mount protective immune responses against certain intracellular pathogens as well (8). Some of these responses arise via the CD8^+ MHC class Ia-restricted mechanism (21), whereas others employ compensatory responses by CD4^+ CTL or γδ T cells (57). K^D^0 --/-- animals will be useful in understanding the mechanisms underlying such compensatory immune responses.

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Appendix to Chapter II
Positive selection of an MHC class-I restricted TCR in the absence of classical MHC class I molecules

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The H-2Ld alloreactive 2C T cell receptor (TCR) is commonly considered as being positively selected on the H-2Ld molecule. Surprisingly, 2C TCR+ CD8+ single-positive T cells emerge in massive numbers in fetal thymic organ culture originating from 2C transgenic, H-2KdKb−/− (2C x KdKb−/−) but not in fetal thymic organ culture from β2m-microglobulin−/− 2C transgenic animals. Mature CD8+ T cells are observed in newborn but not in adult 2C KdKb−/− mice. These CD8+ T cells express the αβδ integrin, which allows them to populate the intestine, a pattern of migration visualized by intrathymic injection of FITC and subsequent accrual of FITC-labeled lymphocytes in the gut. We conclude that the 2C TCR is reactive not only with H-2Ld and H-2Kd, but also with nonclassical MHC class I products to enable positive selection of 2Cαβ T cells in the fetal and newborn thymus and to support their maintenance in the intestine.

The peripheral T cell repertoire is comprised of T cells that have undergone selection for productive T cell receptor (TCR) interaction with self-MHC/peptide complexes within the thymus. The affinity of the TCR-MHC interactions determines the outcome of thymic selection: low-affinity TCR recognition of self-MHC/peptide complexes allows survival and maturation (positive selection) whereas high-affinity recognition results in elimination (negative selection) of thymocytes (1). For their prolonged survival in the periphery, mature, naive CD4+ and CD8+ T cells that leave the thymus and populate the secondary lymphoid organs require ongoing TCR interaction with their respective TCR ligands, MHC class II and class I molecules (2–5). These survival signals involve peptide-specific, low-affinity interactions of TCR with self-MHC/peptide complexes, presumed similar to those required for positive selection of thymocytes in the thymus (6, 7).

Extrathymic sites also can act as microenvironments for T cell maturation and selection, as exemplified by gut-associated lymphoid tissue (8, 9). The intestinal intraepithelial lymphocyte (IEL) subsets have phenotypical and behavioral characteristics distinct from peripheral T cells and are enriched for TCRγδ T cells (9).

Peripheral T cells exhibit high frequencies of alloreactivity toward MHC antigens to which they have not been previously exposed (10), reflecting the inherent ability of the TCR to cross-react with multiple self- and foreign MHC/peptide ligands. In the murine 2C TCR system, both self- (H-2Kd-deV8) and allogeneic (L4/2-p2ca and H-2Kbm-deV8) MHC/peptide complexes can trigger the 2C TCR (11, 12). By crossing 2C TCR transgenic mice to different H-2 backgrounds (13, 14) the positively selecting ligand was identified as Kdδ, whereas negative selection occurs in the presence of the high-affinity antigens L4/2 and Kbm. The 2C TCR remains the only TCR on murine CD8+ T cells for which the three-dimensional structure has been solved, in a complex with Kdδ (15). Selection of 2C-bearing T cells occurs neither in β2m-microglobulin−/− animals nor in fetal thymic organ culture (FTOC) from 2C transgenic on the β2m−/− background (16, 17). Furthermore, 2Cαβ thymocytes can be induced to mature in H-2Ld β2m−/− fetal thymic lobes by stabilization of free Kdδ heavy chains on thymus epithelium through addition of β2m and Kb-binding peptides (17). A surprisingly broad repertoire of structurally unrelated Kδ ligands supported the development of functionally mature 2Cαβ T cells. Therefore, these data were interpreted as consistent with a "noninterference" model of positive selection, in which the contribution of the peptide in the interaction between the MHC/peptide complex and the TCR may be limited (18, 19).

By implication of an additional, nonclassical (class Ib) MHC class I ligand in positive selection of 2C TCR-bearing T cells, we provide here an alternative explanation for degeneracy of the 2C TCR in the process of positive selection.

Methods

Mice. Mice transgenic for the 2C TCR (20) that were in the sixth generation of backcross to C57BL/6 (The Jackson Laboratory) mice were crossed to β2m−/− mice (The Jackson Laboratory) and KdKb−/− mice (21) to generate 2Cβ2m−/− and 2C KdKb−/− mice, respectively. 2C KdKb−/− mice then were crossed to 2C RAG−/− mice to generate 2C KdKb−/− RAG−/− mice. Genotyping was performed by PCR analysis for the 2C TCR transgene, and flow cytometry was used to detect the absence of Kdδ and Dp, β2m, and RAG.

FTOC. Thymic lobes were excised from fetuses at gestational day 16 (the day of the vaginal plug is considered as day 1). The lobes were placed on polycarbonate membranes (Costar) in DME supplemented with 10% FCS, penicillin, streptomycin, 2 mM L-glutamine, and 50 μM 2-mercaptoethanol. At the indicated time points, the thymic lobes were harvested and thymocytes were extruded mechanically by pressing the tissue between two glass slides. Cultures of 2Cβ2m−/− thymic lobes were supplemented with 0.5% (vol/vol) β2m−/− mouse serum instead of FCS.

Purification of IELs. Intestinal mucosal lymphocytes were prepared as described (22), with minor modifications. Briefly, the small intestine was removed and Peyers' patches were carefully excised. The intestine then was opened longitudinally, its contents were removed, and the preparation was washed in PBS/3% FCS before cutting it into ~0.5-cm-long pieces. IELs were isolated by chemical and mechanical disruption of the epithelial layer in extraction buffer (PBS, 3% FCS, 1 mM DTT, 1 mM EDTA) for 30 min at 37°C. Cells were collected and isolated on a discontinuous 40/70% Percoll (Amersham Pharmacia) gradient at 900 × g for 20 min. Cells at the 40/70% layer were collected and washed in FACS staining buffer.

Abs and Flow Cytometry. Thymocytes, splenocytes, and IELs were stained for FACS analysis in ice-cold FACS staining buffer (PBS, 0.5% BSA, and 0.02% sodium azide) with combinations of the following antibodies: phycoerythrin (PE)-conjugated anti-
CD4, FITC- or cyochrome-labeled anti-CD8a, PE-labeled anti-CD8β, FITC- or cyochrome-labeled anti-TCRβ, PE-labeled Vβ8.1/8.2, and FITC-labeled anti-LPAM-1 (α4β1 integrin) (all from Pharmingen). 2C TCR+ cells were stained with a biotinylated clonotypic antibody (IB2 mAb, generous gift of H. Eisen, Massachusetts Institute of Technology, Cambridge), followed by cyochrome-labeled streptavidin (PharMingen). IB2 F(aḃ)2 fragments were produced by using an Immunopure F(aḃ)2 preparation kit (Pierce) according to the manufacturer’s directions.

Measurement of Cytolytic Activity. IELs from 2C RAG-/-/KdD-/- and 2C RAG-/-/KdDb/-/ mice were cultured in triplicate with 5 × 10^5 [3]Cr-lydium chromium (NEN)-labeled T2-L2 target cells in 96-well round-bottom microtiter plates in the presence and absence of stimulating peptide QLSPFPFDL (synthesized on peptide synthesizer model 422, Advanced Chemtech). After 6 h of incubation at 37°C and 5% CO2, 100 μL of supernatant was collected and [3]Cr was counted in a gamma counter. The percent specific lysis was calculated as the sample release (in cpm) minus the spontaneous release (in cpm) divided by the maximal release (in cpm) minus the spontaneous release (in cpm).

Intrathymic FITC Injections. Intrathymic FITC injections were performed as described (23). Thymic lobes of 6-day-old newborn 2C KdD-/-/+ mice were injected with 10 μL of 1 mg/ml FITC in PBS. Twenty-four hours later, thymus and intestine were harvested. Thymocyte suspensions were analyzed for FITC labeling by flow cytometry. Intestinal tissue was fixed in 4% paraformaldehyde and snap-frozen in OCT embedding medium. Cryosections (5 μm thick) were air-dried, fixed in 70% ethanol, mounted in Aquapoly/Mount solution (Polysciences), and analyzed for FITC-positive cells by fluorescence microscopy. Thymocytes and gut tissue from intrathymically injected mice were used as negative controls.

Results

2C T Cells Are Generated in FTOD in the Absence of Classical MHC Class I Molecules. FTOD experiments with thymic lobes from mice transgenic for 2C TCR and deficient for both H-2Kd and H-2Dd (2C KdD-/-) revealed massive generation of CD8+ single-positive (SP) thymocytes (Fig. 1A), in numbers comparable to their KdD-/-/+ counterparts. This result was unexpected, because H-2Kd is the commonly accepted ligand for positive selection of the 2C TCR. The CD8+ SP thymocytes obtained from 2C KdD-/-/ mice expressed the transgenic TCR, as indicated by staining with the clonotypic antibody IB2 (24). Successful positive selection of thymocytes results in an increase in the levels of TCR at the cell surface. Accordingly, CD8+ SP T cells selected in 2C KdD-/-/ mice showed increased levels of TCR expression in comparison to the immature CD8+ CD4+ double-positive (DP) thymocyte population (Fig. 1A). The number of mature SP CD8+ thymocytes in 2C KdD-/-/ mice gradually increased, whereas the DP population decreased in number during the 7-day culture period, with the usual kinetics for T cell development (data not shown).

FTOD performed with thymic lobes from βm-/-/ 2C TCR transgenic animals yielded a strongly reduced number of mature CD8+ SP 2C thymocytes as compared with their KdD-/-/+ counterparts (Fig. 1A). Cultures of βm-/- thymic lobes were performed in the presence of 0.5% βm-/- mouse serum, as supplementation with FCS consistently yielded a considerable number of CD8 SP 2C+ thymocytes (30% of total lymphocytes; data not shown). Presumably, fetal βm present in FCS stabilizes mouse class I heavy chains at the cell surface of βm-/- thymic epithelial cells (25, 26) and thus would allow positive selection of 2C thymocytes. The numbers of CD8+ SP 2C thymocytes of 2C KdD-/-/ mice were not affected by supplementation with 0.5% βm-/- mouse serum as compared with cultures with FCS (data not shown). The absence of mature 2C T cells in βm-/- FTOD has been reported (17), but the effects of FCS supplementation on the numbers of mature 2C T cells were not explored.

The generation of CD8+ SP T cells in 2C KdD-/-/ mice was inhibited by F(aḃ)2 fragments of the 2C clonotypic antibody, IB2 (data not shown). Positive selection thus requires signaling via the 2C TCR. To rigorously exclude the contribution of endogenous TCRs distinct from the 2C TCR that could have resulted from inefficient allelic exclusion, we crossed 2C KdD-/-/+ mice onto a background deficient for the recombinase activating gene product RAG-1. FTOD experiments performed with these 2C RAG-/-/ mice showed maturation of 2C CD8+ SP thymocytes in numbers comparable to those observed in thymic lobes from 2C KdD-/-/+ mice expressing the full repertoire of MHC class I molecules (Fig. 1B).

Thus, 2C TCR-bearing T cells can be positively selected in the absence of the classical class I MHC molecules Kd and Dd. As the 2C TCR-bearing T cells are unable to mature in the absence of βm (Fig. 1A) (17), the light chain for both classical and nonclassical MHC class I molecules, we conclude that the selecting ligand for the 2C TCR is a nonclassical class I molecule that needs βm for its expression at the cell surface.
In Vivo Generation of 2C T Cells in the Absence of MHC Class I Molecules. We addressed the question of whether the 2C TCR uses this ligand for positive selection in vivo. In contrast to our findings in FTOC, the numbers of mature 2C\textsuperscript{+} CD8\textsuperscript{+} SP thymocytes produced in adult (2-3 months old) 2C\textsuperscript{K'D}\textsuperscript{+/-} and 2C\textsuperscript{K'D}\textsuperscript{+} mice were strongly reduced as compared with those produced in 2C\textsuperscript{K'D}\textsuperscript{+/-} and 2C\textsuperscript{RAG-1/-} K'D\textsuperscript{+/-} mice (Fig. 2). In fact, the CD4/CD8 staining patterns of adult 2C\textsuperscript{K'D}\textsuperscript{+/-} thymus were similar to those of 2C\textsuperscript{K'D}\textsuperscript{+/-} mice (Fig. 2) and were reminiscent of the phenotype for 2C TCR transgenic mice on nonselecting H-2 backgrounds (13, 14). Remarkably, the total number of thymocytes in 2C\textsuperscript{K'D}\textsuperscript{+/-} K'D\textsuperscript{+/-} mice was increased by almost 1 order of magnitude as compared with their 2C\textsuperscript{RAG-1/-} K'D\textsuperscript{+/-} counterparts (mean 220 x 10\textsuperscript{6} vs. 30 x 10\textsuperscript{6}, respectively). The significance of this difference is unknown but may reflect an intact expansion phase and defective negative selection phase in the development of 2C thymocytes in K'D\textsuperscript{+/-} animals. The numbers of CD8\textsuperscript{+} T cells among splenocytes of 2C\textsuperscript{K'D}\textsuperscript{+/-} and 2C\textsuperscript{RAG-1/-} K'D\textsuperscript{+/-} adult mice were also reduced in comparison to 2C\textsuperscript{+} and 2C\textsuperscript{RAG-1/-} mice expressing the wild-type class I repertoire. Thus, unlike the situation early development as recapitulated in FTOC, the adult K'D\textsuperscript{+/-} thymus does not support the maturation of 2C\textsuperscript{+} T cells.

The conditions for positive selection may be adequate only early in ontogeny. To address this issue, we analyzed the numbers of 2C\textsuperscript{CD8}+ T cells in thymocytes isolated from newborn 2C\textsuperscript{K'D}\textsuperscript{+/-} mice. 2C\textsuperscript{CD8}+ SP T cells were generated in thymi of newborn 2C\textsuperscript{K'D}\textsuperscript{+/-} and 2C\textsuperscript{RAG-1/-} K'D\textsuperscript{+/-} mice in numbers equal to 2C\textsuperscript{+} mice expressing the full repertoire of MHC class I molecules (Fig. 3). 2C\textsuperscript{CD8}+ SP thymocytes in 2C\textsuperscript{K'D}\textsuperscript{+/-} newborn mice had up-regulated their 2C TCR as compared with the DP population, indicating their mature status (Fig. 3). Few CD8 SP T cells were detected among thymocytes of newborn 2C\textsuperscript{K'D}\textsuperscript{+/-} mice as expected (16). We conclude that, unlike the thymus of adult K'D\textsuperscript{+/-} mice, the thymus of newborn K'D\textsuperscript{+/-} mice fully supports the maturation of 2C\textsuperscript{CD8}+ T cells.

Functionally Mature 2C T Cells in Class I-Deficient Mice Reside in the Intestine. If 2C TCR-bearing 2C\textsuperscript{+} T cells are thymically selected early in life but are present in very low numbers in adult thymi and spleens of K'D\textsuperscript{+/-} mice, what then is the fate of these early, naive 2C TCR+ thymic emigrantes? Maintenance of naive T cells requires continuous contact of the TCR with self-MHC molecules (2-5). Therefore, the thymically produced 2C\textsuperscript{CD8}+ T cells could disappear from the periphery of K'D\textsuperscript{+/-} mice, as survival signals delivered through interaction of the TCR with classical MHC class I molecules are lacking. Alternatively, they could migrate to a specific site(s) where they are maintained by ongoing interaction of their TCR with nonclassical MHC class I molecules. As high levels of nonclassical MHC class I molecules are present in the intestine (27-29), we analyzed the numbers of IELs in 2C\textsuperscript{K'D}\textsuperscript{+/-} mice. Large numbers of 2C\textsuperscript{CD8}+ T cells were found in the intestines of 4-week- and 3-month-old mice.
2C^+ K^D^+/- and 2C^+ RAG^-/- K^D^+/- mice, in numbers similar to those found in their K^D^+/- counterparts (Fig. 4A). In the absence of βm, a 10-fold reduction in the number of 2C^+ CD8 T cells was detected among IELs. These observations implicate a βm-dependent, nonclassical MHC class I molecule in the retention and maintenance of the 2C^+ CD8^+ IEL T cell population in K^D^+/- mice. IEL populations can exert direct cytolytic activity without prior antigen stimulation (30). Freshly isolated IELs from adult 2C^+ RAG^-/- K^D^+/- mice showed cytolytic activity against 51Cr-labeled L.2.43 target cells but only when the cognate peptide QLSPFPPFD (Fig. 4B) was included. The cytolytic activity of IELs from K^D^+/- and K^D^+/- backgrounds was comparable. Cytolytic activity assays with IELs from 2C^+ RAG^-/- mice on both K^D^+/- and K^D^+/- backgrounds gave similar results (data not shown). Thus, 2C^+ CD8^+ T cells that develop in the absence of classical class I molecules and populate the intestine exert ex vivo cytolytic effector function.

In animals that lack classical MHC class I molecules, a substantial CD8^+ TCRαβ IEL population remains. Detailed analysis of CD8 co-receptor expression in the IEL population of K^D^+/- mice revealed a strong reduction in CD8αβ-bearing TCRαβ T cells but preserved numbers of CD8αα-bearing TCRαβ T cells (refs. 31–33 and unpublished results). As both CD8αβ and CD8αα TCRαβ subsets disappear from IELs of βm^+/- mice, the CD8αα TCRαβ T cells likely develop through interaction with a βm-dependent, nonclassical MHC class I molecule. Similarly, the 2C^+ T cells that develop in the absence of K^+ and D^+ MHC molecules could use the CD8αα co-receptor for interaction with their selective nonclassical ligand. Although a slightly increased proportion of CD8αα-expressing IELs was observed in 2C^+ K^D^+/- mice as compared with their 2C^+ K^D^+/- littermates, a substantial proportion (42%) of 2C^+ IELs that were generated in the absence of classical class I molecules expressed the CD8αβ heterodimer at the cell surface (Fig. 5). In addition, the majority (81%) of the 2C^+ CD8 SP thymocytes that developed in K^D^+/- FTOC expressed the CD8αβ heterodimer (Fig. 5B).

**Thymically Derived Mature 2C^+ CD8 T Cells in MHC Class I-Deficient Newborn Mice Migrate to the Intestine.** The majority of the TCRαβ^-/- IEL population interacts with class I MHC/peptide complexes and, with thymic development, the matured TCRαβ^-/- IEL subsets are strongly reduced in βm^+/- transgenic T cells in their thymus, as observed in the emergence of CD8α^-/- TCRαβ^-/- transgenic IEL transgenic IEL depends on the presence of high-affinity antigens that induce negative selection in the thymus (37–39). In addition, bone marrow reconstitution of both RAG^-/- and thymectomized RAG^-/- mice showed that CD8α^-/- IELs are generated in the absence of a thymus, whereas the generation of CD4^-/-, CD8αβ^-/-, and CD4^-/- CD8αβ^-/- IEL subsets is thymus-dependent (40). In view of these findings, the 2C^+ CD8α^-/- T cells that populate the intestine in 2C^+ K^D^+/- mice likely originated from the thymus.

Possibly, these thymus-derived CD8αβ^-/- IEL T cells are instructed to migrate to the intestinal area by their interaction with the thymic epithelium. Recruitment of lymphocytes from the peripheral blood to gut mucosal lymphoid tissues is controlled largely by the interaction of the αβ2 integrin, expressed on the circulating lymphocytes, with its ligand, MAdCAM-1 (mucosal addressin cell adhesion molecule-1), expressed on the specialized endothelium in Peyers patches and lamina propria (41–43). To investigate the instructive role of the K^D^+/-.

**Fig. 5.** A large fraction of 2C^+ IELs isolated from K^D^+/- mice and the majority of CD8^+ SP mature thymocytes from K^D^+/- FTOC express CD8αβ. (A) IELs derived from 2C^+ RAG^-/- K^D^+/- and 2C^+ RAG^-/- K^D^+/- mice were gated for TCRαβ^-/- expression and subsequently examined for expression of CD8α vs. CD8β. (B) Thymocytes isolated from 2C^+ RAG^-/- K^D^+/- and 2C^+ RAG^-/- K^D^+/- FTOC were gated for CD4^-/- CD8αβ^-/- expression (SP population) and CD4^-/- CD8αβ^-/- expression (CD8 SP population) and subsequently analyzed for CD8α coexpression. Similar results were obtained in three independent experiments.
thymus in directing the 2C⁺CD8⁺ T cells to the intestine, we analyzed the expression of αβ integrin on thymocytes isolated from TTOC experiments with 2C⁺RAG⁻/⁻ K⁺D⁺⁻/⁻ mice and from newborn 2C⁺RAG⁻/⁻ K⁺D⁺⁻/⁻ mice (shown for newborn in Fig. 6A). In contrast to the DP subset, which was essentially negative for αβ integrin expression, the majority of 2C⁺ CD8⁺ SP thymocytes expressed high levels of the αβ integrin at the cell surface (Fig. 6A). Among CD8 SP thymocytes obtained from K⁺D⁺⁻/⁻ mice, the fraction and intensity of staining of αβ-expressing cells was much lower, indicating that the interaction of 2C⁺ CD8 T cells with K⁺ molecules in the thymus does not lead to uniform expression of the gut homing receptor (Fig. 6A). Thus, 2C⁺CD8 T cells that mature in a K⁺D⁺⁻/⁻ deficient thymic environment are induced to express a surface phenotype suitable for homing to the intestine.

To provide direct evidence that 2C⁺CD8 SP T cells in K⁺D⁺⁻/⁻ mice migrate from the thymus to the intestine, we labeled thymocytes of 6-day-old newborn mice in situ by intrathymic injection of FITC (23, 44). Mice that were injected extrathymically with similar amounts of FITC were used as a negative control. Twenty-four hours after injection, thymi and intestines were isolated and analyzed for the presence of FITC-positive cells (Fig. 6B and C). Thymocytes obtained from intrathymically injected mice expressed high levels of FITC as determined by flow cytometry (Fig. 6B). Splenocytes of these mice showed FITC labeling of a fraction of the total T cell population and essentially no labeling of other cell types (data not shown). In addition, FITC-positive lymphocytes were readily detectable in gut tissue sections 24 h after injection (Fig. 6C). Altogether, these data indicate that the K⁺D⁺⁻/⁻ thymus in newborns directs 2C⁺CD8⁺ SP T cells to migrate to the intestinal area in a manner that involves αβ.

**Discussion**

We here describe the positive selection of 2C⁺ TCR⁺ CD8⁺ T cells in fetal thymic lobes deficient in the classical MHC class I (class Ia) molecules H-2K⁺ and H-2D⁺ (2C⁺K⁺D⁺⁻/⁻). We conclude that a nonclassical MHC class I (class Ib) product, the identity of which remains to be identified, can interact with the 2C TCR and support positive selection in the thymus. We have shown that a TCR can interact productively with nonclassical MHC class I molecules as well as the products of at least two distinct classical MHC class I loci, H-2K⁺ and H-2L⁺. Our results further show that 2C⁺CD8αβ⁺ T cells, positively selected in the 2C⁺K⁺D⁺⁻/⁻ fetal and newborn thymus, are induced to express high levels of the mucosal homing receptor αβ on their cell surface. After export into the periphery, these 2C⁺CD8αβ⁺ T cells migrate to the intestine in large numbers.

The finding of involvement of a nonclassical class I MHC molecule in the selection of 2C TCR⁺ thymocytes is unexpected and affects the interpretation of previously published data. The requirement of MHC/peptide complexes in CD8⁺ T cell selection is commonly studied by the use of βm⁻/⁻ or transporter associated with antigen processing (TAP)⁻/⁻ thymic lobes in the FTOC system (34, 45–48). As the addition of soluble βm to the thymic lobes would stabilize expression of nonclassical as well as classical class I molecules, the use of a nonclassical class I ligand for positive selection would have been difficult to show as such, especially in view of the rather limited polymorphism of non-

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Maurice et al.

PNAS | June 19, 2001 | vol. 98 | no. 13 | 7441
classical class I products, and the TAP independence of some of their ligands.

The kinetics of selection of 2C T cells in KO DKO mice is reminiscent of the early embryonic wave of T cells that has been identified by thymectomy of newborn mice and by the analysis of fetal thymus colonization after thymus grafting (49). As compared with the adult situation, the thymic selection criteria seem to differ for this early embryonic population such that potentially autoreactive cells are included (50, 51). The earliest waves of TCR β and TCR αβ T cells that leave the thymus immediately colonize peripheral organs such as the intestine. To first "construct" the immune system at the body surfaces and cavities would confer a selective advantage to the newborn mammal, as these sites will be the first sites of antigen encounter. The thymus plays a major role in the early seeding of the intestine with both TCR β and TCR αβ T cells (52–54). The use of TCR transgenic mice has yielded unexpected dividends before, such as recently described spontaneous model for arthritis (55). Here, we uncover a surprising attribute of the 2C TCR: the crossing of this

TCR αβ transgenic receptor to a class I-deficient background allowed us to specifically visualize the early selection events of the transgenic thymocytes.

The nonclassical MHC class I ligand that supports positive selection of 2C TCR αβ cells must be expressed at adequate levels early in ontogeny; alternatively, the thymic environment itself may favor selection on this ligand early in life. During the process of positive selection on this ligand, 2C TCR αβ SP thymocytes acquire high levels of the mucosal lymphocyte integrin αβ. Although rapidly disappearing from the periphery after birth, mature 2C TCR αβ T cells are present in large numbers in the intestine of young mice. We propose that the instruction for a T cell to migrate to distant epithelia may be delivered early in thymic development and that nonclassical MHC class I products may play a key role in this process.

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CHAPTER III

Signaling of CD8+ T cells induced by recombinant MHC class I complexes

While the previous chapter focuses on immature thymocytes, here we address the signaling pathways of mature CD8+ T cells. Specifically, I have adopted an antigen presenting cell (APC)-free system to study MHC class I-TCR signaling of mature CD8+ T cells. This system relies on recombinant H-2Kb-peptide complexes refolded in the presence of a peptide of interest. These MHC class I complexes trigger CD8+ T cells, partially mimicking presentation of antigens by antigen presenting cells. A similar approach based on recombinant MHC class I complexes was employed previously to study relatively late events in T cell activation (1). I have extended this approach to study proximal signaling events with the focus on the tyrosine phosphorylation cascade.

Several crucial T cell signaling events are mediated by changes in tyrosine phosphorylation of key signaling molecules. Specifically, antigen recognition results in the activation of intracellular protein tyrosine kinases, such as members of the Src and ZAP-70/syk kinases. The proximal events in the phosphorylation cascade are the phosphorylation of tyrosines within the cytoplasmic domains of the TCR/CD3/ζ chain complexes. APC-initiated signaling results in various patterns of tyrosine phosphorylation of CD3 chains in T cells (2). Furthermore, a tyrosine kinase ZAP-70 is also phosphorylated during the T cell signaling cascade (3). Therefore, phosphorylation of the intracellular ZAP-70 and the CD3 ζ chain upon induction with recombinant class I molecules was utilized in an assay for proximal T cell signaling.

I have applied this experimental approach to study two questions in T cell signaling. The first study addresses peptide antagonism in CTL signaling. This data was published in Immunity and the reprint entitled “Peptide Antagonism and T cell Receptor Interactions with Peptide-MHC complexes” is enclosed. My contribution to this study was the biochemical characterization of antagonist-mediated T cell signaling (Fig. 5). The second study addresses the peptide-dependence in signaling of alloreactive cytotoxic T cells.

References

Peptide Antagonism and T Cell Receptor Interactions with Peptide-MHC Complexes

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Summary

We describe antagonist peptides that specifically inhibit cytolysis of T cell clones and lines that express the antigen-specific receptor of CD8+ T lymphocyte clone 2C, which recognizes peptides in association with syngeneic (Kb) and allogeneic (Lb) MHC proteins. Addition of an antagonist peptide that can bind to Kb on 2C cells decreased the tyrosine phosphorylation of CD3ζ chains elicited by prior exposure of the cells to an agonist peptide-Kb complex. Contrary to previous agonist-antagonist comparisons, the 2C T cell receptor had higher affinity for an antagonist peptide-Kb complex than for a weak agonist peptide-Kb complex. This difference is considered in light of evidence that antigen-specific receptor affinity values can be substantially higher when determined with the receptor on live cells than with the receptor in cell-free systems.

Introduction

The nature of a T cell’s response to complexes formed by peptides with major histocompatibility (MHC) proteins (pMHC complexes) can be greatly affected by variations in the peptide’s amino acid sequence (Kersh and Allen, 1986). Some peptides (agonists) elicit the full range of known responses; others (partial agonists), often differing from agonists by only one or a few amino acid residues, elicit partial responses (e.g., effector but not proliferative responses); and the principal effect of still others (antagonist peptides) is to inhibit the activity of agonists. Although a role for antagonist peptides in normal immune responses is not entirely clear, these peptides are of considerable interest because they provide the tools for exploring previously unappreciated complexities in the responses of T cells to ligation of their antigen-specific receptor (T cell receptor or TCR). We have accordingly sought to identify antagonist peptides for the CD8 cytotoxic T cell known as 2C, a clone whose TCR recognizes defined allogeneic and syngeneic pMHC complexes.

Clone 2C arose in an H-2b mouse (BALB/c strain) immunized with H-2b cells (from DBA/2 and BALB/c mice). It responds to the class I MHC protein Lb (an allorreactant) in association with naturally processed peptides (e.g., LSPFFDL, termed p2Ca; Udana et al., 1992) that derive from a ubiquitous intracellular protein (α-ketoglutarate dehydrogenase; Udana et al., 1993). Using the allorreactive cytolytic responses to Lb target cells loaded with diverse variants of p2Ca, we were unable to identify antagonist peptides. Subsequently, Udana et al. (1996) discovered that 2C CTL can respond vigorously to a synthetic peptide, SYRYQYL (here termed SYRQL), in association with Kb, which is syngeneic for 2C cells. Using highly sensitive cytolytic assays based on pulsed Kb+ target cells, we describe here several naturally occurring peptides that act as antagonists for the 2C TCR.

One of the antagonists (RGYYQYL, termed ESYVS) is of interest because of two findings. First, in contrast to the few previous comparisons between agonist and antagonist pMHC complexes, we found that the ESYVS-Kb complex is bound with about 30 times higher affinity by the 2C TCR than the weak agonist p2Ca-Kb complex. Second, the addition of the ESYVS antagonist peptide to 2C cells after they had been briefly in contact with an immobilized agonist-MHC complex (SYRQL-Kb) brought about a rapid and profound decrease in tyrosine phosphorylation of the cells’ CD3ζ chains. In considering the significance of the affinity difference between the antagonist and agonist complexes, we note that there can be substantial differences between TCR affinity values determined with the receptor on intact cells and in cell-free systems. The sequences of all the peptides discussed below and the terms used to designate them are listed in Table 1 and Figure 2 (see below).

Results

The Cytolytic Assay for Antagonist Peptides

As shown in Figure 1A, half-maximal lysis of an optimal Kb+ target cell (T2-Kb) was achieved with the SYRQL peptide at the extraordinarily low concentration of around 5 × 10^{-16} M (SD60 value). Since SYRQL and a peptide RGYYQQL (SVS) from vesicular stomatitis virus (VSV) were about equally effective in blocking the Kb-dependent cytolytic activity of an unrelated CTL clone (4G3, specific for Kb+ plus the ovalbumin peptide SIINFEKL (pOVI); Figure 1C; Table 1), it appears that the affinity of Kb for SYRQL is close to that previously measured affinity for VSV (1 × 10^{-10} M-1; Matsumura et al., 1992). In accord with the low SD60 value (Sykulev et al., 1996), we found that soluble SYRQL-Kb complexes bound to the TCR on live 2C cells with the relatively high affinity of 1 × 10^{-14} M-1 (Figure 1D; Tables 1 and 2).

To identify antagonist peptides for the 2C TCR, we took advantage of the SYRQL peptide’s potency as an agonist to pulse T2-Kb+ target cells (5 Cr labeled) with this peptide at 5 × 10^{-14} M, leaving a large number of empty Kb molecules that could be loaded with other peptides.

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target cells sensitized with pOV8 by pOV8-K⁺-specific CTL (clone 4G3) in the absence (dotted) or presence (solid curves) of various concentrations of SYRGL (filled circles) or 2C (filled squares). SYRGL and 2C alone did not elicit a cytolytic reaction with these CTL. For peptide sequences, see legend for (A).

(D) Affinity of the TCR on live 2C cells for the SYRGL-K⁺ complex. Amount of radiolabeled Fab' fragments of the clonotypic anti-2C TCR antibody (1B2) specifically bound to the TCR on live 2C cells (clone L3.100) in the absence (B) or presence (C) of various concentrations of the SYRGL-K⁺ complex. (E) Ligation of TCR molecules on 2C CTL by a clonotypic TCR-specific antibody (1B2; Kranz et al., 1984) decreases specific lysis of SYRGL-pulsed T2-K⁺ target cells. The extent of target cell lysis in the absence of the 1B2 antibody is indicated by a dotted line. About 80% of 2C TCR had to be blocked by the antibodies to decrease specific lysis of the target cells by half. Percent occupancy (%) of the TCR was calculated as $u = K_C/(1 + K_C^C)$, where $C$ is free concentration of 1B2 Fab' fragment (assumed to be the same as the total concentration) and $K$ is the equilibrium binding constant ($2 \times 10^{-11} M^{-1}$) for the reaction between 2C TCR and 1B2 Fab'. The concentration of free SYRGL was $4 \times 10^{-4} M$.

These pulsed target cells were then incubated with diverse test peptides and various CD8+ CTL that expressed the 2C TCR, including the original 2C clone and several 2C TCR⁺ T cell clones from 2C TCR transgenic mice. When added alone to T2-K⁺ cells, the tested peptides behaved either as weak agonists (high SD₃₀ values) or elicited no cytolytic activity (Figure 1A).

**Antagonist Peptides**

As evident from Figure 2, lysis of the pulsed target cells was enhanced by two peptides (agonists), unaffected by two others (null peptides), and inhibited by six others (antagonists). The antagonist effects were not always observed: it appeared that they were more consistently seen with recently derived clones than with those that had been in culture for many months, and they were not observed with the original 2C clone. Representative results are shown in Figures 3A, 3C, and 3D for three newly derived clones (L3.100, K2.30, and G3.1). We also found that polyclonal 2C TCR⁺ cell lines (derived as described below) were indistinguishable from clones L, K, and G in responding strongly to the SYRGL agonist and in their susceptibility to inhibition by the antagonist peptides (Figure 3B).

**Affinity of the 2C TCR for an Antagonist Peptide-MHC Complex**

The few comparisons that have been made between TCR binding of agonist and antagonist pMHC complexes suggest that TCR have lower affinity and form less stable complexes with antagonist than with agonist ligands (Alam et al., 1996; Lyons et al., 1996). We previously found the 2C TCR affinity for the weak agonist p2Ca-K⁺ complex to be about $3 \times 10^{-2} M^{-1}$, at the lower limit of what can be measured on live cells (Slykhuul et al., 1994a). Accordingly, we expected the 2C TCR affinity for the antagonist EVSV-K⁺ complex to be still lower, i.e., to be essentially immeasurable on 2C cells. Surprisingly, however, the affinity proved to be about $9 \times 10^{-4} M^{-1}$, or about 30-fold higher (Figure 4; Table 1).

**Comparison of Different Target Cells**

We attribute the detection of antagonist activity by a high proportion of the peptides tested to the sensitivity of the assay. It is likely that the use of T2-K⁺ as target cells was responsible for this sensitivity, as we did not observe antagonism when the peptides were tested in the same way with two other K⁺ target cells, EL4 and RCA-S. The advantage of T2-K⁺ cells was not that they were of human origin (the others are mouse cells) but rather stems from two considerations: (1) for inhibition to be observed, antagonist peptide concentrations had to greatly exceed the agonist peptide concentration used to pulse target cells (see Figure 3), and (2) much higher agonist peptide concentrations were required to pulse EL4 and RCA-S than T2-K⁺ target cells. As a result, the antagonist concentrations needed to observe inhibition could be readily achieved with T2-K⁺ but not with the other target cells. The concentrations of agonist
<table>
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<td>EVSV</td>
<td>RGYYQGL</td>
<td>K⁺</td>
<td>antagonist</td>
<td>1 x 10⁴</td>
<td>1 x 10⁹</td>
</tr>
<tr>
<td>p2Ca</td>
<td>SSPFFFDL</td>
<td>K⁺</td>
<td>agonist</td>
<td>4 x 10⁻¹⁰</td>
<td>3 x 10⁻⁹</td>
</tr>
<tr>
<td>VSV</td>
<td>RGYYQGL</td>
<td>K⁺</td>
<td>antagonist</td>
<td>1 x 10⁻⁹</td>
<td>&lt;10⁻⁹</td>
</tr>
<tr>
<td>p2VB</td>
<td>SINFEKL</td>
<td>K⁺</td>
<td>null</td>
<td>1 x 10⁻⁹</td>
<td>&lt;10⁻⁹</td>
</tr>
<tr>
<td>p2Cbb</td>
<td>VAITRIEQLSPFFFDL</td>
<td>L⁺</td>
<td>agonist</td>
<td>1 x 10⁷</td>
<td>4 x 10⁷</td>
</tr>
<tr>
<td>RL12</td>
<td>RIEQLSPPPFFDL</td>
<td>L⁺</td>
<td>agonist</td>
<td>1 x 10⁷</td>
<td>4 x 10⁷</td>
</tr>
<tr>
<td>QL9</td>
<td>QLSPPPFFDL</td>
<td>L⁺</td>
<td>agonist</td>
<td>2 x 10⁻⁹</td>
<td>2 x 10⁻⁸</td>
</tr>
<tr>
<td>p2Ca</td>
<td>LSPPPFFDL</td>
<td>L⁺</td>
<td>agonist</td>
<td>4 x 10⁻⁹</td>
<td>2 x 10⁻⁸</td>
</tr>
<tr>
<td>SL7</td>
<td>SPPPFFDL</td>
<td>L⁺</td>
<td>agonist</td>
<td>6 x 10⁻⁸</td>
<td>1 x 10⁻⁷</td>
</tr>
<tr>
<td>PL4</td>
<td>PFFDL</td>
<td>L⁺</td>
<td>agonist</td>
<td>6 x 10⁻⁸</td>
<td>1 x 10⁻⁷</td>
</tr>
<tr>
<td>p2Ca-Y4</td>
<td>LSPPPFFDL</td>
<td>L⁺</td>
<td>agonist</td>
<td>2 x 10⁻⁹</td>
<td>4 x 10⁻⁸</td>
</tr>
<tr>
<td>QL9-Y5</td>
<td>QLSPPPFFDL</td>
<td>L⁺</td>
<td>agonist</td>
<td>2 x 10⁻⁹</td>
<td>7 x 10⁻⁸</td>
</tr>
<tr>
<td>↓QL9-Y5</td>
<td>QLSPPPFFDL</td>
<td>L⁺</td>
<td>agonist</td>
<td>2 x 10⁻⁹</td>
<td>6 x 10⁻⁸</td>
</tr>
</tbody>
</table>

Note: A previous reference to unpublished results showing that the DL dipeptide could sensitize target cells for lysis by 2C CTL (cited as an addendum to Eisen et al., 1997) was mistaken, probably because the dipeptide preparations used were contaminated with traces of a strong peptide agonist.

(SYRGL) chosen to pulse target cells were based on the agonist concentration required for half-maximal lysis of these cells (SD⁰ value) with EL4 and RMA-S target cells, the SYRGL SD⁰ values were about 100 times higher than for T2-K⁺ target cells (data not shown). All of these differences reflect the greater number of empty K⁺ molecules available for peptide loading on T2-K⁺ than on EL4 and RMA-S cells. Although the total number of K⁺ molecules on EL4 and T2-K⁺ cells are approximately the same (about 5 x 10⁹ per cell; unpublished data), the peptide transporter TAP is defective in T2-K⁺ cells but not in EL4. While RMA-S is also transport defective, there are fewer K⁺ molecules on these cells than on T2-K⁺, which expresses a transfected K⁺ gene. Figure 1B provides another example that shows the advantage of T2-K⁺ over another K⁺ target cell (compare Figure 1A).

Allelic versus Syngeneic Reactions Before Udaka et al. (1998) described the SYRGL peptide, our efforts to identify peptides that inhibit the reaction of 2C cells with syngeneic (L⁺) target cells (T2-L⁺), using several L⁺-binding peptides, were fruitless (Tsomides et al., unpublished data). As shown in Table 1, these allelic targets were used to examine a series of peptides having sequences that are found in murine α-ketoglutarate dehydrogenase (shown at bottom of Table 1; see Udaka et al., 1993). Although these L⁺-binding peptides varied from 4 to 16 amino acids in length, they were all agonists for 2C cells, albeit with different degrees of efficacy; e.g., peptide concentrations for half-maximal lysis ranged from about 10⁻⁵ to 10⁻¹² M (Sykulev et al., 1994a, 1994b; unpublished data).

In Table 1, the L⁺-binding peptides are aligned from the C-terminal leucine. This alignment was used because systematic alanine substitutions of the p2Ca octapeptide and the QL9 nonapeptide had shown that the C-terminal sequence FDL is critical for binding these peptides to L⁺ (Al-Ramadi et al., 1995; Robinson and Lee, 1996). Stronger justification for viewing the peptides in register from the C terminus has emerged with the recent description of the crystal structure of L⁺ (Balandiran et al., 1997; Speir et al., 1998). Although 2C TCR affinity spans a 200-fold range from the weakest (PL4-L⁺) to the strongest binding L⁺-peptide complex (QL9-L⁺), it is striking how little the affinity values vary otherwise between complexes that differ considerably in peptide length (e.g., compare p2Cbb, RL12, p2Ca, SL7, etc., in Table 1).

How Do Antagonist Peptides Exert Their Effect? In the cytolytic assay for antagonism, the target cells were pulsed with the strong agonist peptide at about 10⁻¹⁰ M before the test antagonist peptide was added. In view of this extremely low concentration, the number of MHC-binding sites on target cells occupied by the agonist is likely to be <0.01% of total MHC sites. Hence it is most unlikely that antagonists act by competing with agonists for target cell MHC. Previous studies have led to the same conclusion (Bertoletti et al., 1994; Ewald et al., 1994; Kienerman et al., 1994).
Table 2. Differences in Equilibrium Constants for 2C TCR Interactions with Soluble Peptide-MHC Complexes Measured with the TCR on Live 2C Cells or in a Cell-Free System

<table>
<thead>
<tr>
<th>Peptide-MHC Complexes</th>
<th>TCR in a Cell-Free System</th>
<th>TCR on Live 2C Cells</th>
<th>R²</th>
<th>SDp, nM</th>
</tr>
</thead>
<tbody>
<tr>
<td>p2Ca-L²</td>
<td>3 \times 10^6</td>
<td>2 \times 10^6</td>
<td>7</td>
<td>0.5</td>
</tr>
<tr>
<td>QL-9-L²</td>
<td>3 \times 10^3</td>
<td>2 \times 10^7</td>
<td>68</td>
<td>0.005</td>
</tr>
<tr>
<td>p2Ca-K¹</td>
<td>not measured</td>
<td>3 \times 10^3</td>
<td>—</td>
<td>1.000</td>
</tr>
<tr>
<td>dEV8-K¹</td>
<td>1.2 \times 10^8</td>
<td>not measured</td>
<td>—</td>
<td>≥1.000</td>
</tr>
<tr>
<td>SYRGL-K¹</td>
<td>3 \times 10^8</td>
<td>1 \times 10^7</td>
<td>330</td>
<td>0.00004</td>
</tr>
</tbody>
</table>

*All values of equilibrium (affinity) constants shown in this table have been measured using recombinant MHC class I proteins K¹ and L¹ expressed in a Drosophila melanogaster-based expression system (Jackson et al., 1992). Soluble 2C TCR was produced in the same system (Garcia et al., 1995a, 1995b).

For peptide sequences see Table 1 and Figure 2.

K¹ values calculated from association and dissociation rate constants measured from the binding of soluble pMHC to soluble immobilized α,β-TCR by surface plasmon resonance (Garcia et al., 1995a).

K¹ values measured from direct binding of K¹-pMHC complexes or competition binding with the Fab' fragments of the 1B2 cloneotyptic antibody to the TCR on live 2C cells (see Table 1).

R² is a ratio of equilibrium binding constants for TCR-pMHC reaction measured with live cells and in a cell-free system.

SDp is a concentration of cognate peptide required to sensitize target cells (T2-K¹ or T2-L¹, respectively) for half-maximal lysis by 2C CTL; the value of SDp inversely correlates with the efficacy of cytolysis.

The reaction of soluble p2Ca-L² complex with soluble immobilized 2C TCR was also analyzed by surface plasmon resonance by Corr et al. (1994). Since the authors used the recombinant proteins produced in a different expression system, their data are not given here.

In contrast to the cell-free system, cell differences shown in this table, essentially no difference was found in the affinity values measured with the TCR on live hybridoma cells or in a cell-free system for the reaction between the TCR of the 2B4 CD4⁺ T cell clone and its pMHC ligand, a moth cytochrome peptide (MCP) in association with the I-² MHC class II molecule (Matsui et al., 1991, 1994). Whether the results with the latter system and the consistent disparities shown in the table are due to differences between CD4 and CD8, MHC I and MHC II molecules, T cells and hybridomas, or other features remains to be determined.

To determine if antagonist pMHC complexes are inhibitory because they compete with agonist complexes for binding to the TCR, we first determined the fraction of 2C cells' TCR molecules that would have to be blocked by the clonotypic anti-TCR antibody 1B2 (Fab' fragments) in order to inhibit by 50% the specific lysis of SYRGL-pulsed T2-K¹ cells by 2C CTL (in the standard 4 h cytolytic assay) (Schoch et al., 1996). As shown in Figure 1E, 50% inhibition of lysis required occupancy

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Figure 2. Effect of Diverse K¹-Binding Peptides on Specific Lysis of SYRGL-Pulsed T2-K¹ Target Cells by 2C TCR⁺ Clonal Clone L3.100

Shown is the percent inhibition (filled bars) or enhancement (striped bars) of specific target cell lysis in the presence of 1 µM of the indicated peptides. The naturally processed peptides tested were LSPFPFDL (p2Ca) (Ueda et al., 1992), LSPFPFDL (p2Ca-Y14) (Wu et al., 1995), SINIFKL (pOV8, Carbone et al., 1992); ANYIFPICV (ANY [Malanowski et al., 1999]), EVQKVYQVL (EV8, Tallquist and Pease, 1995), vesicular stomatitis virus GYGQYQGQ (VSV [van Boven and Nathenson, 1990]), and Sendai virus GYGQYQGQ (GV50 [Schumacher et al., 1991]). Other peptides were LSPFPFDL (p2Ca-Y16), SINIFKL (pOV8), ROVYQGQ (R-N6V), and ROVYQGQ (EV8).

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Figure 3. Inhibitory Effect of Various Peptides on Specific Lysis of SYRGL-Pulsed T2-K¹ Target Cells by Various CTL Clones and a Polyvalent CD8⁺ Cell Line That All Express Transgenic 2C TCR

In Figure 3 (A), L3.100, G3.1, and K2.30 and the cell line (B) were derived from the spleens of 2C TCR transgenic mice (H-2b). Peptides in (A), (C), and (D) were pOV8 (open circles), VSV (filled squares), and p2Ca-Y14 (filled triangles). Inhibition of lysis of SYRGL-pulsed T2-K¹ target cells by a polyclonal 2C CTL cell line is shown in the presence of 1 µM of the peptides indicated (B).
of about 80% of TCR molecules on 2C cells. Can antagonist pMHC complexes on T2-K" target cells occupy such a large fraction of the TCR on 2C cells under our assay conditions? The 2C TCR affinity for the EVSV-K" antagonist complex was measured to be about 9 × 10^{10} L/M (Figure 4; Table 1) or 1.5 × 10^{18} L/complex. To approach 80% occupancy of the TCR molecules in a reaction mixture [1.5 × 10^{12}; i.e., (10^9 TCR per cell) x (1.5 × 10^5 T cells per well)] with this reaction's affinity would require the density of the antagonist pMHC complexes to be about one trillion (1 × 10^{12}) per cell. However, the total number of K" molecules is only about half million (5 × 10^5) per target cell. Even if each pMHC complex engaged as many as 200 TCR molecules (Valitutti et al., 1995), competition with agonist complexes for the TCR is a most unlikely explanation for the inhibitory effect of the antagonist complexes.

Antagonist peptides on antigen-presenting cells induce altered patterns of tyrosine phosphorylation of CD3 ζ chains in T cells (Sloan-Lancaster et al., 1994, Madrenas et al., 1995). We therefore examined tyrosine phosphorylation of CD3 ζ chains in 2C cells that had been exposed first to an agonist and then to an antagonist. To separate the effects of agonist and antagonist complexes, we first confronted 2C cells for a few minutes with SYRGL-K" complexes adsorbed on plastic (96-well plates). The cells were then removed from the immobilized agonist complexes and incubated with either an antagonist (EVSV) or null (pOVA) peptide. As shown previously, the binding of extracellular peptides to MHC molecules on T cells can result in effective formation and presentation of the corresponding pMHC complexes by the T cells to each other (Walden and Eisen, 1980; Su et al., 1993). Figure 5 shows that exposure to the SYRGL-K" complex resulted in tyrosine phosphorylation of CD3 ζ chains (compare lanes 1 and 6) and subsequent exposure to the EVSV antagonist, but not to the null ligand, resulted in almost complete elimination of the agonist-induced ζ chain tyrosine phosphorylation without affecting the total amount of immunoprecipitated ζ chain (compare lanes 1, 2, and 3). The effect shown differs from that described by Reis e Sousa et al. (1996), who reported that there was less tyrosine phosphorylation of CD3 ζ chains in CTL exposed to antagonist peptide than in those exposed to agonist peptide, but more than in those exposed to null (or no) peptide.

Discussion

Two principal findings emerged from this study of peptide antagonism. First, the affinity of the 2C TCR for an antagonist pMHC (EVSV-K") was found to be higher than for a weak agonist (p2Ca-K"), although much lower than for a strong agonist (SYRGL-K"). Second, exposure of activated 2C cells to the EVSV antagonist resulted in a pronounced decrease in agonist-induced tyrosine phosphorylation of CD3 ζ chains. These findings are evaluated below with respect to various models proposed to account for the inhibitory effects of antagonist peptides. The models are largely focused on affinity and kinetics of TCR-pMHC reactions. Since affinity values measured for a given TCR under different conditions can, however, differ substantially (see Table 2, below) we evaluate these differences before considering our results in relation to the models.

In the present study and in a previous one (Sykulev et al., 1994a), the 2C TCR affinity for the agonists SYRGL-K" and p2Ca-K" and the antagonist EVSV-K"
were measured with the TCR in its natural environment on live 2C cells and with soluble pMHC complexes formed from synthetic peptides and recombinant K\(^{b}\) and L\(^{a}\) molecules produced in Drosophila cells (Figures 1D and 4; Sykulev et al., 1994a). As shown in Table 2, TCR affinity values measured under these conditions are considerably higher than those determined in cell-free systems using soluble pMHC complexes formed in the same way. One possible reason for the differences shown in Table 2 is that TCR-pMHC complexes can be stabilized by CD8 molecules (Luescher et al., 1995; Garcia et al., 1996a), which are usually abundant on intact CD8\(^{+}\) cells and generally not present in cell-free systems. Another possible reason is suggested by the convoluted nature of the cell surface membrane and the abundance of TCR molecules on the T cell surface: a pMHC complex that dissociates from a TCR molecule on the cell might thus have more opportunities than in a cell-free system to rebind to the same or neighboring TCR molecules (Valitutti et al., 1995).

Are the 2C TCR affinity values determined with intact cells ('cellular affinity') or in cell-free systems ('cell-free affinity') more useful for our present purposes? Measured on live T cells, the 2C TCR cellular affinity for SYRGL-K\(^{b}\) is about 3000 times higher than for p2Ca-K\(^{b}\) (Table 2; 1 \times 10^{-14} M \text{M}^{-1} versus 3 \times 10^{-17} M \text{M}^{-1}). This difference parallels the great difference in efficacy of the corresponding peptides in cytolytic assays with K\(^{b}\)-target cells (see Figures 1A and 1B). Similarly, the much greater effectiveness of the QL9 than the p2Ca peptide in cytolytic assays with L\(^{a}\)-target cells is in accord with the 2C TCR's having a 10 times higher cellular affinity for QL9-L\(^{a}\) than p2Ca-L\(^{a}\), but it is not in accord with this receptor's having the same cell-free affinity for these peptide-L\(^{a}\) complexes (Table 2; Sykulev et al., 1994a, 1994b). Moreover, the 2C TCR's cell-free affinity for SYRGL-K\(^{b}\) and EVSV-K\(^{b}\) differed only around 2.5-fold (Garcia et al., 1996a; see Table 2); this small difference, which is essentially within experimental error, is also not in accord with the great difference in efficacy between the corresponding peptides in cytolytic assays (Figure 1B). Differences in the binding of extracellular peptides to K\(^{b}\) and L\(^{a}\) on target cells can certainly contribute to the disparate efficacies of these peptides in cytolytic assays, but they cannot account for all of it; e.g., the million-fold difference in peptide concentration required for half-maximal lysis shown in Figure 1B. Thus, cellular affinity values for agonist pMHC complexes generally correlate more closely than cell-free affinity values with the behavior of peptides in cytolytic assays (see also Sykulev et al., 1994a, 1994b). Therefore, in asking if affinity values fit any of the models proposed to explain agonist-antagonist differences, we think it reasonable to focus on cellular affinity values rather than on cell-free values for both agonists and agonists.

According to some views, TCR have lower affinity and form less stable complexes with agonist pMHC than with agonist pMHC complexes (Lyons et al., 1996). The suggestion with respect to affinity is clearly not universally the case, as shown by the finding that the 2C TCR has about 30 times higher affinity for an agonist (EVSV-K\(^{b}\)) than a weak agonist (p2Ca-K\(^{b}\)) complex (Figure 4; Sykulev et al., 1994a). The kinetic issue is more difficult to deal with, as general procedures for determining the stability (i.e., dissociation rates) of TCR-pMHC complexes on intact T cells have not yet been developed. Nevertheless, it is unlikely that the higher affinity reaction with the antagonist EVSV-K\(^{b}\) has a faster k\(_{on}\) rate constant than the approximately 30-fold lower affinity reaction with the agonist p2Ca-K\(^{b}\).

In an interesting kinetic discrimination model (Rabinowitz et al., 1996), the binding of pMHC to TCR is assumed to result in stepwise modifications (e.g., different levels of phosphorylation of the receptor-CD3 complex); incompletely modified receptors are assumed to elicit negative signals and more extensively modified receptors are assumed to elicit positive signals (cell activation), with the balance between the two determining the extent to which a pMHC ligand acts as an antagonist or a weak or strong agonist. In this model the steady state level of the extensively modified receptor is determined not by the rate at which the ligand dissociates from the receptor per se (k\(_{off}\)) but by the relative values of k\(_{on}\) and the rate of transition (k\(_{d}\)) from an incomplete state of the receptor to a complete state. The model is thus compatible with an agonist ligand such as EVSV-K\(^{b}\) having a higher affinity and slower k\(_{off}\) than an agonist such as p2Ca-K\(^{b}\)—if, for example, the transition rate k\(_{d}\) was much slower when the receptor is liganded with an antagonist pMHC than with an agonist pMHC.

That the postulated rate of transition of ligated receptor from one state to another could vary with different pMHC ligands raises the additional possibility that the TCR might assume a different conformation when bound to an agonist or antagonist complex. This possibility is in accord with recent evidence that soluble TCR molecules dimerize on binding to agonist pMHC but not on binding to an antagonist pMHC (P. J. Travers and N. R. Gascoigne, personal communication). That a TCR can assume different conformations resulting in various T cell responses has been suggested previously (e.g., from different responses of T cells to different anti-TCR antibodies) (Rojo and Janeway, 1983; Yoon et al., 1994). The recent crystallographic studies of the 2C TCR (Garcia et al., 1996b) provide a solid basis from which to search for significant conformational differences between agonist- and antagonist-ligated TCR.

The limited evidence now available indicates that the changes in tyrosine phosphorylation of CD3 \(\zeta\) chains elicited by an antagonist pMHC complex are quantitatively reduced but qualitatively the same as those elicited by agonist complexes (Reis e Sousa et al., 1996), although actual sites of phosphorylation were not mapped. The evidence presented here based on a somewhat different experimental protocol (Figure 5) suggests, in contrast, that the antagonist complex stimulates removal of phosphotyrosine groups from tyrosine residues in CD3 \(\zeta\) chains as though from the activation of a tyrosine phosphatase (e.g., Neel, 1997). Since cellular responses to different altered peptide ligands can vary considerably (anergy, weak agonism, antagonism at one concentration and agonism at another, or antagonism only), it is possible that TCR interactions with these diverse ligands can also elicit different patterns of protein tyrosine phosphorylation.

That peptide antagonism was more readily detected
in 2C reactions with syngeneic pMHC than allogeneic pMHC raises the possibility that allogeneic and syngeneic reactions might generally differ in susceptibility to antagonism. One reason for speculating about such a difference is that in comparison with syngeneic pMHC, in allogeneic pMHC the MHC moiety generally con-
tribute more, and the peptide less, to the binding energy of TCR-pMHC interactions (Eisen et al., 1997). Thus, as in Table 1, variations in peptide length and sequence can have relatively little effect on equilibrium constants when the involved MHC is allogeneic (L1). Indeed, in some TCR reactions with allogeneic pMHC, the peptide's sequence appears not to matter at all, so long as it stabilizes the MHC moiety (Smith et al., 1997; Zhou et al., unpublished data). Even if they prove to be gener-
ally less susceptible than syngeneic reactions to peptide antagonism, it appears that some alloreactions can also be specifically inhibited by potent antagonist pMHC complexes (unpublished data).

Experimental Procedures

Cells

CTL clones and cell lines that express the rearranged α and β chain genes of the 2C TCR were derived from 2C transgenic mice (Sha et al., 1988). Isolated splenocytes were initially stimulated with irradi-
ated P815 (H-2^d) cells in K medium (RPMI supplemented with 10% heat-inactivated fetal calf serum, 10 mM HEPES, 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, 50 µg/ml amphotero-
toehanol) and restimulated 6 days later and thereafter at 1-week intervals with irradiated P815 cells (20 × 10^6 rad) in K medium con-
taining supernatants from concanavalin-A (Con A)-activated rat splenocytes. Established CTL lines were tested in cytotoxicity assays using 51Cr-labeled P815 cells and/or T2-L^K or T2-K^K cells sensitized with appropriate peptides as target cells (see below). Clones L3.100, K2.30, and G3.1 were isolated from a 2C TCR^+ cell line by limiting dilution in 96-well plates, initially with an average of 100, 30, and 1 cell per well, respectively. These clones were maintained and tested as described above.

The human mutant cell line TZ (Alexander et al., 1989), transfected with L1^T2-2^L or K^T2-K^ genes, was a generous gift from Dr. P. Cresswell (New Haven, CT). It was maintained as described else-
where (e.g., Sykulev et al., 1994a). EL4 thymoma cells (H-2^R), RAM-S cells (H-2^TAP deficient), and P815 (H-2^K) mastocytoma cells were all cultured in K medium.

Peptides

The Biopolymers Laboratory of Massachusetts Institute of Technol-
ogy synthesized the peptides used in this study (Boc method) and purified many of them by HPLC. Peptide concentrations were based on amino acid analyses or BCA assays or weight/volume.

Soluble MHC Class I Molecules

Soluble K^L and L^L class I molecules were expressed in Dro-
sophila melanogaster cells (Jackson et al., 1992) and isolated from culture supernatants as described (Sykulev et al., 1994a). Soluble peptide-K^L complexes used to study protein tyrosine phosphory-
lation in 2C TCR^+ cells were produced in E. coli according to Nathen-
son and colleagues (Zhang et al., 1992).

Cytolytic Assay

In all experiments, 51Cr-labeled H-2K^L target cells (T2-K^, EL4, or RAM-S) and various 2C CTL clones or 2C cell lines (expressing transgenic 2C TCR) were used. Usually, 5,000 target cells (in 100 µl K medium) and 25,000 CTL (in 50 µl K medium) were combined with 50 µl of PBS containing various peptides at different concentrations in round-bottom 96-well plates. The plates were centrifuged (for 1–2 min) at 500 g and then incubated at 37°C in a 5% CO₂/95% air atmosphere. After 4 h, the plates were centrifuged for 5 min and 100 µl of supernatant was harvested from each well to determine the amount of 51Cr released into extracellular medium. Percent specific lysis was calculated from the following: (51Cr release into super-
nant - spontaneous release)/total release in detergent - sponta-
nous release) × 100. Wells containing peptide were in duplicate. Similarly treated control wells (in quadruplicate) provided values for spontaneous release (CTL omitted) and total release (1% NP-40 included in medium).

To evaluate the ability of various peptides to inhibit specific lysis of K^L target cells by 2C CTL, the target cells were pulsed with the agonist peptide SYRGL by incubating them with this peptide while the cells were being labeled (100 µCi/10^6 cells/1 ml of medium). The peptide concentration was 4 × 10^-7 M for T2-K^ and 5 × 10^-6 M for the other K^L target cells. Pulsed target cells were washed three times to remove unbound peptide and unincorporated 51Cr and were mixed with various concentrations of the test peptide in 96-well plates as described above. Percent inhibition of specific lysis was calculated as 100 × (A - B)/A, where A and B are percentages of specific lysis in the absence and presence of the tested peptide.

Peptide Binding to K^L and L^L

Equilibrium binding constant of SYRGL (SYRGL) to K^L was estimated in a peptide competition assay using as a reference peptide RGIVYQVL (SVY), whose equilibrium binding (K^svy = 10^3 M^-1) was previously determined (Matsumura et al., 1992). 51Cr-labeled T2-K^L target cells were incubated with SIINFEKL (pOva) peptide (at about 10^-11 M) and various dilutions of either SYRGL or SVY; all peptides were added at the same time (unlike the conditions used to pulse target cells in the antagonist assay; see above). 400 CTLs (Walden and Eisen, 1990), which recognize pOva in association with K^L, were then added at a CTL:target cell ratio of 3:1. Percent specific lysis, determined as described above after 4 hr incubation at 37°C under 5% CO₂, was plotted as a function of concentration of competing peptide. Equilibrium binding constant of K^L for RGIVYQVL (SVY) was estimated in a peptide stabilization assay with the RAM-S cell line (100 µg/ml peptide + pOva), and the affinity of K^L for the latter peptide was measured previously (Matsumura et al., 1992).

Equilibrium binding constants of α-ketoglutarate dehydrogenase peptides to soluble L^L were measured by competition binding of these peptides and radiolabeled peptide from mouse cytomegalo-
vir (MCMV) (Reddelseh et al., 1989) to the soluble L^L. Equilibrium constant for the reaction between K^svy-L^MCMV and soluble L^L was determined in separate experiment. A detailed protocol for the assay was previously described (Matsumura et al., 1992).

Equilibrium Binding of Soluble Peptide-MHC Complexes to 2C TCR on Intact Cells

The equilibrium binding constant for the TCR-pMHC reaction was determined from the competition of soluble pMHC complexes with 131I-labeled Fab' fragments of the clonotypic anti-TCR antibody 1B2 for binding to the TCR or intact 2C cells, as previously described (Sykulev and Eisen, 1997). Soluble pMHC complexes were produced by loading emyty reconstituted K^L and L^L proteins (Jackson et al., 1992) with an appropriate peptide at a concentration (10^-10 to 10^-5 M) that was 100- to 10,000-fold higher than the specific constant for the peptide-MHC reaction (i.e., sufficient to saturate the MHC-binding sites for peptide).

Tyrosine Phosphorylation of TCR β Chains

The walls of 24-well plates were coated by incubating 1–20 µg of SYRGL-K^L complex per well at 4°C overnight. Wells were then washed with PBS, blocked with 1% BSA (in PBS) at 25°C for 30–45 min, and washed with RPMI 1640 medium. 2C cells (3–10 × 10^6 in 500 µl of RPMI 1640) were added to each well, spun at 800 rpm for 2 min, and incubated at 37°C under 5% CO₂/95% air. After 20 min, the 2C cells were transferred to Eppendorf tubes containing 50 µl of 0.1% triton X-100/test peptide (ESV or OVA) in PBS or 50 µl of PBS without peptide and incubated for 20 min at 37°C under 5% CO₂/95% air. For further controls, cells were not exposed to SYRGL-K^L but were incubated under similar conditions with test peptide (ESV or OVA) or no peptide at all. Equal volumes (500 µl) of ice-cold PBS contain-
ing 1 mM sodium orthovanadate were then added to all six
samples and the tubes were transferred to ice. Cells were pelleted at 14,000 rpm for 20 s, and pellets were lysed in 50–100 µl of lysis buffer (1% NP-40, 150 mM NaCl, 10 mM Tris-HCl [pH 8.0], 5 mM EDTA, 1 mM sodium orthovanadate, 10 µg/ml leupeptin, 10 µg/ml aprotinin, and 1 mM PMSF). Lysates were precleared with normal rabbit serum and formalin fixed Staphylococcus aureus and then immunoprecipitated with TCR δ-specific rabbit antisera (a generous gift of Dr. P. Allen, St. Louis, MO). The immunoprecipitated proteins were separated by 12.5% SDS-PAGE under reducing conditions and blotted onto nitrocellulose. The blots were probed with either the anti-phosphorylase monoclonal antibody (clone 4G10 [UBI, Lake Placid, NY]) or the TCR γ-specific antisera; in both instances they were followed by peroxidase-conjugated goat antibodies to mouse or rabbit immunoglobulins (Pharmingen) and were developed with chemiluminescence.

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TCR Recognition of Antagonist Peptide-MHC Complexes


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A tyrosine phosphorylation assay to differentiate between peptide-specific and peptide-nonspecific alloreactive cells

By Yulia Vugmeyster, Xianzheng Zhou, and Herman N. Eisen

Introduction

The mechanism of MHC allore cognition by T cells has been extensively studied (1,2). The majority of alloreactive T cells recognizes foreign MHC molecules in a peptide-dependent but not peptide-restricted manner. In this case, a peptide is thought to affect the conformational status of a MHC molecule (3). Multiple peptides can result in the conformation status required for the recognition by peptide-nonspecific alloreactive cytotoxic T cells (CTL). The existence of peptide-specific alloreactive CTLs (also called allorestricted CTLs) has also been established (4-7). These T cells are thought to recognize peptide-MHC complexes in a manner similar to syngeneic antigen-specific recognition.

Alloreactive CTLs have been extensively studied as potential effectors in anti-tumor adoptive immunotherapy (8). The ability to differentiate between peptide-specific and peptide-nonspecific CTLs is crucial for this line of research. For example, many tumor cells modify the set of peptides presented at the cell surface, as they overexpress several normal proteins. This characteristic of tumor cells be exploited for anti-tumor immunotherapy by in vitro production of tumor-specific allo-restricted CTLs and subsequent in vivo transfer of these CTL effectors into cancer-affected individuals. For this type of therapy, the establishment of peptide specificity is of great practical concern. However, it is technically challenging to distinguish between peptide-restricted and non-restricted CTLs populations using functional assays based on recognition of a peptide presented on antigen presenting cells (APC), partially due to presence of APC-endogenous peptides presented for CTL recognition. Even minor expression of these endogenous antigens might be capable of triggering CTL-mediated lysis (9).

Here, we establish an APC-independent signaling test for peptide specificity of alloreactive CTLs. This assay is based on phosphorylation of an intracellular CD3 ζchain and Zap-70 upon induction of CTLs with recombinant class I molecules refolded in the presence of different peptides (10). Several crucial T cell signaling events are mediated by changes in tyrosine phosphorylation of key signaling molecules. Antigen recognition results in the activation of intracellular protein tyrosine kinases, particularly members of the Src and ZAP-70/syk kinases. The proximal events in the phosphorylation cascade are the phosphorylation of tyrosines within the cytoplasmic domains of the TCR/CD3/ζ chain complexes followed by recruitment and phosphorylation of zeta-chain-associated ZAP-70 (11). APC-initiated signaling results in various patterns of tyrosine phosphorylation of CD3 chains in T cells (12,13). In previous studies, we have employed this approach to study zeta chain phosphorylation elicited by agonist/antagonist exposure in the case of syngeneic recognition (10). Here, we adopt this assay to differentiate between peptide-specific and peptide-nonspecific alloreactive cells.
Materials and Methods

Cell lines, peptides, and mice. T1-Kb and T1-Db, a TxB hybrid, transfected with mouse Kb or Db genes, T2-Kb, a TAP2-deficient version of T1-Kb, RMA-S/B7.1, a TAP2 deficient mouse thymoma transfected with mouse B7.1 gene, and dm2 (H-2Kd, Dd) p53 -/- mice are described elsewhere. SV9 (FAPGNYPAL), SYL8 (SIYRRYGL), and OVA8 (SIINFEKL) peptides were synthesized by the solid-phase t-butoxycarboxyl method in the Biopolymers Laboratory of the MIT and purified by HPLC.

Generation of allocoreactive T cell lines and clones. T2-Kb cells (5 x 10⁷ cells/mL) were incubated with 200 µM SV9 (FAPGNYPAL) peptides at 25 °C overnight and irradiated at 15,000 rads. The cells were washed twice and resuspended in PBS. Dm2 p53 -/- mice were immunized i.p. with peptide-loaded T2-Kb cells (10⁷ per mouse) at an interval of 10 days. Ten days after a second immunization the spleen cells (25 x 10⁶ cells) were stimulated in vitro with irradiated SV9-loaded RMA-s/B7.1 cells (5 x 10⁶ cells) for 5-6 days. The responding dm2 cells (5 x 10⁵ cells/mL) were further stimulated with peptide-loaded RMA-S/B7.1 cells (3 x 10⁵ cells/mL) in 24-well plates in complete media supplemented with 5% rat concavaculin-A supernatant and 25 mM methyl-a-D-mannopyrannoside. CD8+ CTLs were cloned into 96-well U-bottom microtiter plates at 1 cell per well using a FACS sorter and fed weekly with RMA-S/B7.1 stimulator cells loaded with SV9 peptide. The clones were expanded in 24-well plates with 5:3 responder to stimulator ratio.

CTL assay. A standard ⁵¹Cr release assay was used. Peptide-coated target cells were prepared by incubating target cells with a peptide (30 µM, unless otherwise noted) for 1 hour at 37 °C. The cells were washed and resuspended in complete media.

Tyrosine phosphorylation assay. The technique was described previously (10). Briefly, 24-well plates were coated with 20 µg of Kb-peptide complexes at 4 °C overnight. Wells were then washed with PBS, blocked with 1% BSA at 25 °C for 30-45 minutes, and washed with RPMI media. 3-10 x 10⁶ cells were added to each well, spun at 800 rpm for 2 minutes and incubated for 10 minutes at 37 °C, 6% CO₂. At the end of the incubation period, T cells were transferred to Eppendorf tubes containing equal volume of ice-cold PBS and 1 mM sodium orthovanadate. Cells were than pelleted at 14,000 rpm for 20 seconds, and pellet was lysed in 100 µL of lysis buffer (1% NP-40, 150 mM NaCl, 10 mM Tris-HCl, pH 8.0, 5 mM EDTA, 1 mM sodium orthovanadate, 10 µg/mL leupeptin, 10 µg/mL aprotinin, and 1 mM PMSF). Lysates were resolved by 10 % SDS-PAGE under reducing conditions and Western blotted onto nitrocellulose. The blots were probed with a mouse mAb against phosphotyrosine (4G10, Upstate Biotechnologies), followed by peroxidase-goat anti-mouse, and developed by chemiluminescence.
Results and Discussion

Generation of peptide-independent CTLs. Dm2 (H2-Dd, H-2Kd) p53-/- mice were immunized twice with the SV9-peptide-loaded T2-Kb cells. Ten days after second immunization the spleen cells were stimulated in vitro with irradiated SV9-loaded RMA-s/B7.1 cells for 5-6 days. The responding dm2 cells were maintained in culture with SV9-loaded RMA-S/B7.1 cells. This dm2 cell line and single-cell clones derived from this line were tested for peptide specificity in the CTL assays using T1-Kb and T1-Db APCs with or without the SV9 peptide. Dm2 line was cytotoxic against T1-Kb but not T1-Db targets (Fig.1A), establishing H-2Kb restriction for this cell line. Furthermore, SV-9-loading of the T1-Kb target had no effect on the dm2 cytolytic response. Similarly, the titration of several different Kb-binding peptides onto T2-Kb cells had no effect on the magnitude of dm2 response (Fig. 1B). Results obtained for representative dm2 clones were undistinguishable from that derived for the dm2 line. These results provide an evidence for the peptide-independence of the dm2 cell line. In addition, these results indicate that majority of CTLs generated by the above protocol are peptide-nonspecific.

Signaling of dm2 T cells is identical for different Kb-peptide combinations. To exclude the presence of an endogenous peptide on APCs that is specifically recognized by dm2 cells, we performed APC-independent signaling assays. We refolded recombinant H-2Kb/B2m complexes in the presence of several Kb-binding peptides: SV9 (immunogen), OVA8, and SYL8. These complexes were surface-immobilized and tested for their ability to elicit CD3 \( \zeta \) chain and ZAP-70 phosphorylation in dm2 T cells. All three complexes were able to activate dm2 cells, as evident from induced phosphorylation of \(~21.5\) kD and \(~70\) kD proteins, reported to be the high molecular weight form of TCR zeta chain and ZAP-70, respectively (Fig. 2). In contrast, only Kb-ova complexes are able to activate the syngeneic Kb-restricted ova-specific clone 4G3.

This result verifies that alloreactivity of dm2 cells does not depend on a specific peptide and provides a proof of concept for use of the tyrosine phosphorylation assay to study peptide dependence in CTL responses.
References
Figure 1. **CTL activity of the dm2 lines and clones against T1Kb or T2Kb targets is not peptide-restricted.** Dm2 (H-2d) p53 -/- anti-T2Kb-SV9 cell line (dm2 line) or single cell clones (dm2 clone) were used as effectors in a standard 4 hour $^{51}$Cr release CTL assay using (A) T1Kb or T1Db and (B) T2Kb targets loaded with an indicated peptide.
Figure 2. **Tyrosine phosphorylation pattern induced by recombinant H2-Kb/β2m/peptide complexes in the dm2 cells is peptide-independent.** Lysates, obtained form the dm2 (H-2d) p53-/- anti-T2Kb-SV9 cells (dm2) or from the H-2Kb-ova-restricted cell clone (4G3) induced with H2-Kb/β2m/peptide complexes refolded in the presence of an indicated peptide or with BSA, were resolved by 12.5% SDS-PAGE and western blotted with the anti-phosphotyrosine antibody (4G10).
CHAPTER IV

The role of the proteasome in thymocyte apoptosis

By Yulia Vugmeyster and Hidde L. Ploegh

Programmed cell death (apoptosis) is crucial for thymocyte development. We analyzed the role of the proteasome pathway in dexamethasone- and antigen-triggered thymocyte apoptosis in fetal thymus organ culture (FTOC) using a panel of proteasome inhibitors. Proteasome inhibitors delay thymocyte apoptosis in the FTOC system. Proteasome activity is increased in apoptotic thymocytes, as visualized by active site labeling of proteasomal β subunits, indicating that the proteasome functions as a positive regulator in the thymocyte death cascade. Results from this chapter have been submitted to the European Journal of Immunology in December, 2001 in a manuscript entitled "The ubiquitin-proteasome pathway in thymocyte apoptosis: caspase-dependent processing of the deubiquitinating enzyme USP7 (HAUSP)".
Introduction

Programmed cell death (apoptosis) plays a crucial role in thymocyte development. The outcome of thymocyte selection is determined by at least two physiological stimuli: engagement of the T cell receptor (TCR) by MHC-peptide complexes and engagement of glucocorticoid receptor by glucocorticoids, such as dexamethasone (1-3). Immature CD4⁺CD8⁺ double positive (DP) thymocytes die when their TCRs do not encounter low or moderate avidity ligands and thus fail to undergo positive selection (4). Potentially autoreactive DP thymocytes with TCRs that bind high avidity thymic ligands die by a process called negative selection (4).

The TCR-mediated signals are modulated by endogenous glucocorticoids (2, 3, 5). The stimulus that is used extensively to study thymocyte apoptosis is dexamethasone, a synthetic glucocorticoid. Under physiological conditions in the thymus, GR- and TCR-mediated signals have been proposed to be mutually antagonistic in triggering apoptotic cascade (2, 3, 5). In this model, naturally occurring glucocorticoids cause elimination of DP thymocytes that have insufficient TCR avidity to (death by neglect). Occupancy of the TCR by low-to-moderate avidity ligands counteracts glucocorticoid-induced apoptotic signal, rescuing thymocytes from death (positive selection). The TCR occupied by high avidity ligands initiates apoptotic signaling that could not be overcome by the GR-mediated signals (negative selection). Therefore, dexamethasone employed at pharmacological concentrations is likely to "short-circuit" the mutual TCR-GR antagonism and cause cell death.

A variety of other triggers can induce thymocyte death in vitro, such as ionizing radiation, exposure to the topoisomerase type II inhibitor etoposide, antibodies that trigger the death receptors Fas and TNFR, and crosslinking of CD3 components of the TCR by anti-CD3 (6).

The proteasome complex has been proposed to play a part in the regulation of the apoptotic cascade (7). The proteasome is a conserved multi-subunit complex that catalyzes protein degradation in the cytoplasm and the nucleus of all eukaryotic cells. The 26S proteasome is a large protease complex comprised of an inner 20S core with two associated 19S caps (8). Eukaryotic 20S core consists of 2 copies each of 7 distinct α and 7 distinct β subunits. These subunits are organized in the four heterooligomeric [(α1-α7)(β1-β7)(β1-β7)(α1-α7)] rings of the 20S particle. Three β subunits in each ring are catalytically active, with the chymotryptic-like, glutamyl peptide hydrolyzing, and tryptic-like activity attributed to β5, β1, and β2, respectively. Upon exposure to cytokines such as IFNγ, several cell types express an additional set of catalytic β subunits (β5i, β1i, and β2i) that replace β5, β1, and β2 subunits and form a new particle called the immunoproteasome. The assembly of the immunoproteasome is crucial for the generation of suitable peptides for MHC class I-mediated antigen presentation. However, the role of individual subunits and/or individual proteolytic activities in various cellular processes is not well understood.

Proteasome inhibitors, such as the natural product lactacystin and substrate analogue peptides modified by an aldehyde, epoxide or vinyl sulfone moiety, are valuable tools for studies of proteasomal function (9, 10). The role of the proteasome in thymocyte apoptosis has been examined in rat and murine suspension cultures of adult thymocytes (11-16). Proteasome inhibitors partially blocked cell death caused by
different stimuli. Studies of changes in proteasomal activity upon induction of apoptosis, as determined by cleavage of fluorogenic substrates, have given conflicting results. One report suggested an increase in proteasome activity upon dexamethasone or etoposide treatment (12, 15), whereas another concluded that this activity was decreased in dexamethasone-treated thymocyte suspension cultures (13). The use of fluorogenic substrates to assess proteasome activity in crude extracts is limited by the ability of other peptidases to also cleave such substrates. Purification of proteasomes from apoptotic and control cells as a means for comparison is difficult because of uncertainties inherent in the yields obtained. Finally, the role of the proteasome in antigen-driven thymic negative selection has not been addressed.

Here we analyze the role of proteasomes in thymocyte apoptosis by using a fetal thymic organ culture (FTOC) system, a more physiological model of thymocyte development than adult suspension cultures. We assess the ability of proteasome inhibitors to block dexamethasone-induced apoptosis of fetal thymocytes. Using active site labeling of proteasomal β subunits, we analyze proteasomal activity in apoptotic thymocytes. Using the FTOC system, combined with the use of TCR transgenic mice, we address the role of the proteasome in antigen-driven negative selection of T cells.
Materials and Methods

Mice and Reagents. C57BL/6 mice were purchased from Jackson Laboratory. 2C mice and OT-1 mice were obtained from Dr. H. Eisen (MIT, Cambridge MA) and Kristin Hogquist (University of Minnesota), respectively, and were typed by FACS or PCR. Dexamethasone and etoposide were purchased from Sigma Chemical Corporation and used at 0.5 μM and 30 μM, respectively. Lactacystin was purchased from Dr. E. Corey (Harvard University, Cambridge MA), ZL3VS, NLVS and 125I-NLVS were synthesized as described previously and were used at 20 μM (17). Epoxomycin was obtained from Affinity, UK, and was used at 5 μM. ZVAD-fmk was obtained from Enzyme Systems, Dublin, CA and was used at 50 μM. Annexin-V-FITC, propidium iodide (PI), anti-CD4-PE, anti-CD8-cyochrome, and anti-CD8-FITC were purchased from Pharmingen and were used according to the manufacturer's instructions. Anti-20S serum was a gift of Dr. J. Monaco (University of Cincinatti, OH), anti-rabbit-HRP was obtained from Southern Biotechnology Associates. Peptides were synthesized on an Advanced ChemTech instrument using standard FMOC chemistry.

Fetal Thymic Organ Culture (FTOC). Thymic lobes of gestation-day 16 (d16) fetuses were placed on polycarbonate membranes (Costar, Corning NY) in DME medium supplemented with 10% fetal calf serum, penicillin, streptomycin, 2 mM L-glutamine and 50 μM 2-mercaptoethanol. For dexamethasone apoptosis induction, d16 thymic lobes from C57BL/6 or TCR transgenic mice were cultured for 4 days and exposed to 0.5 μM dexamethasone for time periods indicated in the text. For peptide-induced apoptosis, thymic lobes from 2C or OT-1 TCR transgenic mice were incubated for 4-5 hours with 50 μM SIYRYYG or SIINFEKL (ova) peptide, respectively. At the indicated time points, thymocytes were extruded mechanically by pressing the lobes between two glass slides. 2-4 or 8-10 identically treated lobes were pooled for FACS analysis or for HAUSP immunoblotting, respectively.

FACS analysis. For annexin V staining, 5x10^5 - 10^6 thymocytes were incubated with anti-CD4-PE and anti-CD8-cyochrome antibodies in the ice-cold FACS staining buffer (PBS, 5% FCS, 0.5 μL NMS). Cells were washed in the annexin V binding buffer (2.5 mM CaCl2, 10 mM Hepes, 140 mM NaCl, pH 7.4) and stained with annexin V-FITC for 15 min at 25 °C. For PI staining, cells were incubated with anti-CD4-PE and anti-CD8-FITC in the FACS buffer and washed with PBS. 1 μg/mL PI was added right before FACS analysis. Samples were analyzed using Becton Dickinson FACScan and software. As previously reported, apoptotic cells displayed reduced levels of CD4 and CD8 markers, lower FSC and higher SSC (18, 19). Therefore, a wide gate (including smaller apoptotic cells with reduced CD4 and CD8 expression) was used for the annexin V phenotype analysis.

125I-NIP-L3VS (125I-NLVS) labeling. Synthesis and iodination of NLVS were described previously (17). Thymocytes were lysed by glass bead (Sigma, 106 microns) lysis in the Lysis Buffer (50 mM Tris, pH 7.4, 2mM dithiothreitol, 5 mM MgCl2, 2mM ATP) and nuclei were removed by centrifugation. For competition assays, thymocytes were pretreated with 20 μM lactacystin, NLVS or ZL3VS for 3-4 hours. For 125I-NLVS
labeling, lysates, normalized for protein concentration (10-20 μg of total protein), were incubated with $^{125}$I-NLVS, diluted to a final concentration of $2.5 \times 10^4$ Bq/μL, at 37 °C for 2 hours; samples were resolved by 12% reducing SDS-PAGE. For 2D, non-equilibrium pH gradient SDS-PAGE (NEPHGE), the samples were prepared by adding urea (8M final concentration) and NEPHGE sample buffer with Pharmacia ampholite pH 3-10. 2D NEPHGE SDS-PAGE analysis was performed as previously described (20).

**Immunoblotting.** 20-50 μg of total protein was loaded onto each lane of 12% reducing SDS/PAGE and transferred onto a PVDF membrane. The filters were incubated with the indicated first antibodies (anti-20S 1:5000), followed by 1:5000 HRP-coupled goat anti-rabbit IgG. The blots were developed by chemoluminescence.
Results

Dexamethasone-induced thymocyte apoptosis in FTOC.

To examine the consequences of inhibition of proteasomal proteolysis in thymic development, we utilized a fetal thymus organ culture (FTOC) system in combination with a panel of proteasomal inhibitors. The FTOC system is a more physiological model for thymic development than thymocyte suspension cultures employed in earlier studies.

Thymic lobes of gestation-day 16 (d16) fetuses were cultured on filter membranes and proteasome inhibitors were added to FTOC on day 4 of culture, when a large population of CD4⁺CD8⁺ double positive (DP) thymocytes was evident. Thymic lobes pretreated with proteasome inhibitors and their controls were exposed to 0.5 μM dexamethasone 3-4 hours after addition of proteasome inhibitors. The percentage of apoptotic cells within the DP population was assessed by flow cytometry using annexin V staining at different time points after induction of apoptosis. Dexamethasone-treated thymic lobes contained a high percentage of apoptotic DP cells (Fig. 1). After 5.5 hours of treatment, the majority of DP cells were in early apoptosis, showing increased annexin V expression (Fig. 1A), while preserving cellular integrity, as evident from the lack of propidium iodide (PI) staining (data not shown). Furthermore, this treatment resulted neither in a significant change in the percentage of viable DP thymocytes nor in the reduction in the total number of viable cells, as determined by trypan blue exclusion (data not shown). Treatment with a solvent alone had no effect on thymic lobes over the time course of the experiment (data not shown). The addition of different proteasome inhibitors prior to dexamethasone treatment resulted in partial reversion of the annexin V⁺⁹ phenotype (Fig. 1C), i.e. in a reduction in the number of apoptotic cells. This result is in agreement with previous findings obtained in suspension cultures of adult thymocytes and for etoposide treated thymic lobes (data not shown and (11)). The compounds used represent three chemically distinct classes of inhibitors: the natural products lactacystin (a β-lactone) and epoxomicin (an epoxyketone), and the synthetic inhibitor NLVS (a vinylsulfone-based inhibitor). The structures of these compounds are shown in the appendix. All of these inhibitors result in irreversible, covalent inactivation of proteasomal β subunits (10, 17, 21). Proteasomes thus play a role in apoptosis of both fetal and adult thymocytes.

Of all the above proteasome inhibitors tested, lactacystin was the least toxic to fetal thymocytes (data not shown). In contrast, longer treatment (> 11 hours) with either NLVS or epoxomicine alone resulted in an annexin V⁺⁹ percentage similar to that observed for dexamethasone-treated cells (data not shown). Therefore, only lactacystin was used for analysis of later time points after induction of apoptosis. Thymic lobes treated with dexamethasone for 16 hours lost most of their DP cells, as evident from the dramatic decrease in both absolute cell number and percentage of DP cells (Fig. 2A, top and middle panels). Although lactacystin partially restored the relative fraction of DP thymocytes (Fig. 2A, top panel), the total number of DP thymocytes was still low as compared to untreated lobes (Fig. 2A, middle panel). Furthermore, lactacystin-pretreated, dexamethasone-induced DP cells exhibited an annexin V⁺⁹ phenotype (Fig. 2A, bottom panel). We conclude that lactacystin does not block but rather delays apoptosis of DP thymocytes in FTOC.
Treatment of thymic lobes with proteasomal inhibitors resulted in incomplete block of proteasomal activity, as judged by a competition assay performed on fetal thymocyte lysates with cold lactacystin and radiolabeled active site directed probe 125I-NLVS (Fig. 2B, upper panel) (17). Covalent modification of proteasomal β subunits with lactacystin precludes their subsequent modification by radiolabeled NLVS. Thus residual labeling with 125I-NLVS reflects the number of β subunits that were left unmodified by lactacystin and remain active. The geometry of the FTOC system might preclude complete access of proteasome inhibitors to all susceptible cell populations. Not surprisingly, lactacystin blocked a significantly greater percentage of proteasomal activity in adult thymic suspension cultures (Fig. 2B, lower panel). Accordingly, lactacystin toxicity was greatly increased in adult suspension cultures and precluded analysis of late time points. The relatively low efficiency of lactacystin in prevention of cell death in FTOC at late time points was in contrast to the effect observed with ZVAD-fmk, a general caspase inhibitor. Consistent with previous reports, ZVAD-fmk rescued the number and percentage of viable DP cells and the annexin V positive cells (Fig. 2A) (22, 23). The ability of ZVAD-fmk to rescue cells from apoptosis is not diminished by lactacystin treatment (Fig. 2A). Therefore, it is unlikely that the relatively low efficiency of lactacystin in prevention of DP cell death at late time points is due to proteasome inhibition in thymic stroma.

To address the activity of proteasomal β subunits in dexamethasone-induced cell death directly, we analyzed proteasomal activity in treated and untreated thymic lysates using the radiolabeled active site probe 125I-NLVS (17). As shown in Fig. 3, the activity of proteasomal β subunits was increased significantly in the dexamethasone-exposed thymocytes as early as after 4 hours of treatment, consistent with the increase in hydrolysis of a fluorogenic substrate LLVY-MCA reported previously (12). Taken together, our data suggest that the proteasome functions as a positive regulator of dexamethasone-induced thymocyte apoptosis.

Surprisingly, ZL3VS, a vinylsulfone-based inhibitor different from NLVS, had little effect on dexamethasone-induced apoptosis in fetal thymocytes. In 2 out of 3 experiments ZL3VS-pretreated dexamethasone-induced lobes had the same percentage of annexin V positive cells as non-pretreated dexamethasone-induced lobes (Fig. 4A). One experiment resulted in a small reduction in the percentage of annexin V positive cells when dexamethasone-induced lobes were pretreated with ZL3VS (data not shown). To analyze the difference in NLVS and ZL3VS β subunit labeling, we performed a competition assay on thymocyte lysates with cold lactacystin, NLVS, or ZL3VS and radiolabeled active site directed probe 125I-NLVS. Since β2 and β2i subunits are relatively poor substrates for ZL3VS and NLVS (9), we focused on the β1, β1i, β5 and β5i subunits. Our result suggested that ZL3VS might preferentially label the immunoproteasome subunits β1i and β5i, with the least preference for the β1 subunit (Fig. 4B and 4C). This correlation between reduced β1 preference in ZL3VS labeling and reduced cell death inhibition by ZL3VS suggests that the ratio of different peptidase activities might be important for the proteasome function in the apoptotic cascade.
Antigen-induced thymocyte apoptosis.

The possible role of the proteasome in antigen-mediated apoptosis or thymic negative selection has not been explored, likely due to limitations of the thymocyte suspension approach. Earlier studies were performed in adult thymocyte suspension cultures using the phorbol ester PMA to mimic TCR engagement (11). We employed the FTOC system with 2C TCR transgenic mice of H-2b background. Recognition of SIYRVYGL peptide bound to the H-2Kb molecule by the 2C TCR results in negative selection of 2C-expressing DP thymocytes (24). This FTOC system allowed us to address a role of the proteasome in negative selection using a physiological approach.

Consistent with published data, 2C fetal thymic lobes had a high percentage of CD8⁺ SP cells, with less than 50% of the cells being CD4⁺CD8⁺ double positive (25). Thymic lobes of gestation-day 16 fetuses cultured for 4 days on filter membranes were incubated with 50 μM SIYRVYGL peptide for 4-5 hours. This resulted in 2-3-fold decrease in the relative percentage of DP thymocytes (Fig. 5, top panel). More than half of the remaining DP thymocytes exhibited the annexin V⁺ phenotype (Fig. 5, bottom panel). Pretreatment with lactacystin, NLVS or epoxomicine (not shown) partially restored the percentage of DP thymocytes (Fig 5, top panel). However, cells that were apparently rescued in this manner still showed the annexin V⁺ phenotype (Fig. 5, bottom panel) and, therefore, were destined to die. ZVAD-fmk pretreatment restored both the percentage and annexin V phenotype of antigen-stimulated DP cells (Fig. 5), as reported previously (22, 23). Thus, similar to results observed upon dexamethasone treatment, proteasome inhibitors delay antigen-triggered cell death in FTOC. We conclude that the proteasome plays a part in antigen-triggered negative selection.
Discussion

Here we examine the role of proteasome-mediated proteolysis in thymocyte apoptosis. Earlier studies on the role of the proteasome in apoptosis were performed in suspension cultures of adult thymocytes where antigen-driven programmed cell death was not examined (11-16). We utilize the FTOC system, a more physiological system for thymocyte development, to study the role of proteasome in thymic selection. Our results implicate the proteasome in apoptosis of fetal thymocytes, not only when triggered by dexamethasone (Fig. 1 and Fig. 2), but also when induced by peptide-MHC complexes (Fig. 5), two physiological stimuli in T cell development. We find that proteasome inhibitors do not completely block but rather delay both dexamethasone- and antigen-triggered thymocyte apoptosis. One explanation is the incomplete inhibition of proteasome function attained by such compounds. The existence of a proteasome-independent branch in the thymocyte apoptotic pathway is also a possibility.

Active site directed labeling of proteasomal β subunits upon dexamethasone induction of apoptosis indicates that proteasome activity is increased on average two-fold in apoptotic cells (Fig. 3), in line with the ability of proteasome inhibitors to delay thymocyte apoptosis (Fig. 1, 2, and 5). The observed increase in proteasome activity is consistent with the earlier results obtained with cell-permeable fluorogenic substrates (12, 15). However, a fluorogenic substrate approach to the assay of proteasome activity in cell extracts is less specific than active site labeling, as other cytosolic peptidases can cleave such substrates. A decrease in proteasomal activity in dexamethasone-induced thymocytes, as examined by assaying cell extracts or purified proteasomes for cleavage of proteasome-specific substrates, has also been reported (13). This apparent discrepancy may be attributable to loss of activity in the course of proteasome purification as done in these earlier experiments.

The increase in proteasomal activity might be a crucial checkpoint in the apoptotic program, leading to degradation of signaling, survival, or transcription factors. What are the proteasomal targets that are crucial for the execution of this program? While the majority of proteasomal substrates are processed into small peptides, more selective cleavages can also occur. The generation of the active form of transcription factor NFκB from its inactive precursor and degradation of IκB, responsible for retention of NFκB in the cytosol, are the examples of a selective cleavage and a peptide processing, respectively (26, 27). Another set of targets may be anti-apoptotic molecules of the IAP (inhibitor of apoptosis) family that are selectively lost in a proteasome-dependent manner from apoptotic thymocytes. IAP degradation may relieve the inhibition of active caspases, triggering caspase-mediated apoptotic signaling (16). While proteasomal degradation of NFκB, IκB, and IAP is thought to be Ub-dependent, Ub-independent proteasomal degradation has also been implicated in the apoptotic cascade. Grassilli et al. have suggested that proteasomal degradation of ornithine decarboxylase, known to be Ub-independent, leads to an imbalance in polyamine metabolism and initiates cell death (28).

Finally, our data suggest a correlation between proteasome inhibitors’ β subunit substrate preference and efficiency of cell death inhibition (Fig. 4). The significance of this observation needs to be explored by further experiments. However, it is tempting to speculate that by the ratio of different peptidase activities (possibly reflected by the
composition of an active β subunit pool) or the ratio of immunoproteasome to regular proteasome complexes might attenuate the proteasome function in the thymic apoptotic cascade.
References


Appendix to Chapter IV

The structures of the proteasomal inhibitors

lactacystin

NLVS

epoxomicin

ZL_{3}VS
Figure 1. **Short-term effect of proteasome inhibitors on dexamethasone-induced thymocyte apoptosis in FTOC.** (A) and (B) Thymic lobes of gestation-day 16 fetuses were exposed to 0.5 M dexamethasone on day 4 of culture for the indicated times. The percentage of apoptotic cells (pooled from 2-4 identically treated lobes) within the CD4⁺CD8⁺ population was assessed by flowcytometry, using annexin V staining as described in Materials and Methods. The shaded box represents the background apoptotic population, defined as the percentage of annexin V positive cells in untreated lobes. A significant variation in the background apoptotic levels was observed. This time course is a representative experiment. (C) Thymic lobes pretreated for 3-4 hours with the indicated proteasome inhibitors (20 M NLVS, 20 M lactacystin, 5 M epoxomycin) and their controls were exposed to 0.5 M dexamethasone for 5.5 hours and analyzed for annexin V staining as in (A). The graph is representative of at least 3 independent experiments.
Figure 2. **Long-term effect of lactacystin on dexamethasone-induced thymocyte apoptosis in FTOC.** (A) Thymic lobes pretreated with either 20 M lactacystin or 50 M ZVAD-fmk for 3-4 hours and their controls were exposed to 0.5 M dexamethasone for 16 hours, and analyzed for the percentage of CD4⁺CD8⁺ cells (top panel), the absolute number of CD4⁺CD8⁺ cells (middle panel), and the percentage of annexin V positive cells (bottom panel) as in Fig.1. The figure is representative of at least 3 independent experiments. (B) Thymic lobes or thymic suspension cultures were preincubated with 20 M lactacystin for 3-4 hours, lysed and labeled with the ¹²⁵I-NLVS, as described in Materials and Methods. Radiolabeled proteasomal subunits were resolved by reducing 12% SDS-PAGE.
Figure 3. **Activity of proteasomal subunits in dexamethasone-induced thymocyte apoptosis.** Thymic suspension cultures were exposed to 0.5 M dexamethasone for the indicated time. Lysates, normalized for total protein concentration, were split in half and analyzed for proteosome activity by $^{125}$I-NLVS labeling of subunits (top panel) or for steady-state proteosome levels by immunoblotting with ant-20S antiserum (middle panel), as described in Materials and Methods. The intensity of subunit labeling (§5, §5i, and §1 for each lane) or anti-20S blot (all anti-20S-reactive subunits for each lane) was quantified by densitometry (bottom panel), with the zero-time point arbitrarily set to unity.
Figure 4. ZL3-VS inhibition of lymph node proteasome. (A) LY325118 treated for 3-4 days with 20 M NLVS or ZL3-VS.

(B) and (C) Thymic suspension cultures were precipitated with 20 M Iodoacetamid, NLVS or ZL3-VS (as indicated) for 3-4 hours, lysed and labeled with the 125I-NLVS, and described in Materials and Methods. Radioiodinated proteasomal subunits were resolved by reducing 1D 12% SDS-PAGE (B) or 2D NEPHGE (C).
Figure 5. **Effect of proteasome inhibitors on antigen-induced thymocyte apoptosis in FTOC.** Thymic lobes of gestation-day 16 fetuses from 2C TCR transgenic mice were cultured for 4 days on filter membranes and incubated with 50 M SIYRYYGL peptide for 4-5 hours. Where indicated, the lobes were pretreated with 20 M lactacystin, 20 M NLVS, or 50 M ZVAD-fmk for 3-4 hours. The percentage of CD4⁺CD8⁺ cells (top panel) and annexin V positive cells (bottom panel) was assessed by flowcytometry, as in Fig.1. The figure is representative of at least 3 independent experiments.
CHAPTER V

Deubiquitinating enzymes in thymocyte apoptosis: involvement of USP7 (HAUSP)

By Yulia Vugmeyster, Anna Borodovsky, Madelon M. Maurice, Margo H. Furman, and Hidde L. Ploegh

Programmed cell death (apoptosis) is crucial for thymocyte development. The activity of deubiquitinating enzymes in murine apoptotic thymocytes was examined by active site labeling. We show that the deubiquitinating enzyme USP7 (HAUSP) is specifically processed upon dexamethasone-, γ-irradiation-, and antigen-induced cell death. This processing event appears to be a specific proteolytic cleavage of USP7. We show that HAUSP is highly expressed in thymus, spleen, and brain and is very similar in men and mice. Processing of HAUSP does not occur in caspase 3/-/ thymocytes or upon pretreatment of wild type thymocytes with the general caspase inhibitor ZVAD-fmk. Thus, our results suggest that thymocyte apoptosis leads to modification of deubiquitinating enzymes and may provide an additional link between the ubiquitin-proteasome pathway and the caspase cascade during programmed cell death. Results from this chapter have been submitted to the European Journal of Immunology in December, 2001 in the manuscript entitled "The ubiquitin-proteasome pathway in thymocyte apoptosis: caspase-dependent processing of the deubiquitinating enzyme USP7 (HAUSP)".
Introduction

Recent studies implicate the ubiquitin (Ub)-proteasome pathway in thymocyte apoptosis (1-6). Multiple components of this pathway may be involved in the execution of cell death. However, the regulation of selectivity of proteasomal degradation is not well understood.

Most proteasomal substrates are targeted for degradation by conjugation of multiple ubiquitin chains via three distinct enzymatic activities: the Ub-activating enzyme or E1, Ub-conjugating enzymes or E2s, and Ub-ligases or E3s (7). Therefore, one possibility for regulating specific proteasomal cleavages is that of selective ubiquitin conjugation. Several reports have implicated Ub-conjugating enzymes and Ub-ligases in the regulation of apoptosis (8-10). Prior to degradation of Ub-conjugated proteins, the poly-Ub chain must be removed, a reaction catalyzed by deubiquitinating enzymes (11). In addition to Ub recycling, deubiquitinating enzymes can reverse regulatory ubiquitination and edit inappropriately ubiquitinated proteins (11). Inability to remove Ub moieties from the substrates that should be spared from degradation will render those proteins susceptible to proteasomal degradation. Hence the control of deubiquitinating enzymes is likely to be an important parameter in the control of cytosolic and nuclear proteolysis. The possible role of deubiquitinating enzymes in apoptosis has not been examined.

DUBs family members contain the core catalytic domain comprised of the cys and his boxes. This catalytic domain is similar to a peptidase domain of Cathepsin B, a member of the papain family of thiol proteases. In the yeast genome, 17 deubiquitinating enzymes have been identified (12). Multiplicity and complexity of this family in mammals is likely to be far greater (11). Based on their substrate preferences, deubiquitinating enzymes can be divided into two subfamilies: Ub C-terminal hydrolases (UCHs) and Ub-specific processing proteases (USPs). DUBs have been implicated in regulation of diverse cellular processes, such as growth and oncogenesis, development and differentiation, regulation of transcription and chromatin condensation, and neuronal memory (11).

Proteasomal targets that are crucial for the execution of apoptotic programs and are potential substrates for E1, E2s, E3s, and deubiquitinating enzymes remain to be identified. An example is an anti-apoptotic molecules of the IAP (inhibitor of apoptosis) family that are selectively lost in a proteasome-dependent manner from dexamethasone or etoposide-treated thymocytes (6). IAPs can inhibit apoptosis induced by a variety of stimuli, in part via direct inhibition of active caspases. Auto-ubiquitination and degradation of IAPs by proteasomes might be key events in the death program. These and other studies also establish a link between the caspase cascade and the ubiquitin-proteasome pathway during programmed cell death (10).

Most death pathways involve activation of the caspase proteolytic cascade (13). Caspases are cysteine proteases that cleave their substrates C-terminally of Asp residues. Caspases are classified as initiator (for example caspase 8, 9 and 12) and effector caspases (for example caspase 3, 6, and 7) based on their structure and order of activation in the proteolytic death cascade. Studies of various caspase knockout mice show that, for any given caspase, defects in apoptosis may be both cell type- and stimulus-dependent.
(14). The most severe defects are observed in the brains of caspase 3 -/- or caspase 9 -/- animals, whereas other embryonic tissues in these mice are apparently normal (15-17). In fact, caspase 3-deficient thymocytes are sensitive to diverse apoptotic stimuli. However, caspase 3 and 9 are required in multiple cell types for execution of some of the typical nuclear and morphological changes associated with completion of apoptosis. For caspase-mediated proteolysis, selective caspase activation and their well-defined substrate specificity result in selective degradation of key substrates during cell death program (13). Thus, caspase activation in apoptosis does not lead to indiscriminate proteolytic degradation: structural and regulatory proteins are cleaved in a rather specific manner.

Here we examine the activity of ubiquitin-specific proteases (USP) upon apoptosis induction in murine thymocytes. We show that USP7 (HAUSP) is specifically processed upon dexamethasone- and γ-irradiation-induced cell death and during negative selection. While other USPs might also be affected, this apparent cleavage event is unique for USP7. Furthermore, we show that USP7 is highly expressed in thymus, spleen, and brain, organs that rely on apoptosis for development. The processing of HAUSP is not observed in caspase 3 -/- deficient thymocytes or upon pretreatment of wild type thymocytes with a general caspase inhibitor ZVAD-fmk. Thus, our results suggest a role for deubiquitinating enzymes in thymocyte apoptosis and demonstrate a potential link between caspase and ubiquitin-proteasome proteolytic death cascades.
Materials and Methods

Mice and Reagents. C57BL/6 mice were purchased from Jackson Laboratory. 2C mice and OT-1 mice were obtained from Dr. H. Eisen (MIT, Cambridge MA) and Kristin Hogquist (University of Minnesota), respectively, and were typed by FACS or PCR. Caspase 3-/- and caspase 12-/- mice were the gift of Dr. R. Flavell (Yale U., New Haven CT) and Dr. J. Yuan (Harvard Medical School, Boston MA), respectively. Dexamethasone and etoposide were purchased from Sigma Chemical Corporation and used at 0.5 μM and 30 μM, respectively. NLVS was synthesized as described previously and was used at 20 μM (18). ZVAD-fmk was obtained from Enzyme Systems, Dublin, CA and was used at 50 μM. UbVS and 125I-UbVS were described previously (19). Annexin-V-FITC, propidium iodide (PI), anti-CD4-PE, anti-CD8-cyochrome, and anti-CD8-FITC were purchased from Pharmingen and were used according to the manufacturer's instructions. Anti-HAUSP r201 serum and USP7 cDNA in the pCI-neo vector were the gift of Dr. R. Everett (Medical Research Council, Glasgow UK), anti-20S serum was a gift of Dr. J. Monaco (University of Cincinnati, OH), anti-cIAP1 and anti-PARP were purchased from R&D Systems, anti-rabbit-HRP and anti-goat HRP were obtained from Southern Biotechnology Associates. PARP cDNA was obtained from Dr. J. Yuan. Peptides were synthesized on an Advanced ChemTech instrument using standard FMOC chemistry.

Fetal Thymic Organ Culture (FTOC). Thymic lobes of gestation-day 16 (d16) fetuses were placed on polycarbonate membranes (Costar, Corning NY) in DME medium supplemented with 10% fetal calf serum, penicillin, streptomycin, 2 mM L-glutamine and 50 μM 2-mercaptoethanol. For dexamethasone apoptosis induction, d16 thymic lobes from C57BL/6 or TCR transgenic mice were cultured for 4 days and exposed to 0.5 μM dexamethasone for time periods indicated in the text. For peptide-induced apoptosis, thymic lobes from OT-1 TCR transgenic mice were incubated for 4-5 hours with 50 μM SIINFEKL (ova) peptide. At the indicated time points, thymocytes were extruded mechanically by pressing the lobes between two glass slides. 2-4 or 8-10 identically treated lobes were pooled for FACS analysis or for HAUSP immunoblotting, respectively.

FACS analysis. For annexin V staining, 0.5-1 x 10^6 cells were washed in the annexin V binding buffer (2.5 mM CaCl2, 10 mM Hepes, 140 mM NaCl, pH 7.4) and stained with annexin V-FITC for 15 min at 25 °C. 1 μg/mL PI was added right before FACS analysis. Samples were analyzed using Becton Dickinson FACSCAN and software. As previously reported, apoptotic cells displayed lower FSC and higher SSC (20, 21). Therefore, a wide gate including smaller apoptotic cells was used for the annexin V phenotype analysis.

UbVS labeling. Synthesis and iodination of UbVS were described previously (18, 19). Cells were lysed by glass bead (Sigma, 106 microns) lysis in the glass bead lysis buffer (50 mM Tris, pH 7.4, 2mM dithiothreitol, 5 mM MgCl2, 2mM ATP) and nuclei were removed by centrifugation. 0.5x10^6-1.5x10^6 cpm of 125I-UbVS was incubated with 10-20
μg of total protein for 40 min-1 hour at 37 °C and samples were resolved by 10% reducing SDS-PAGE.

**Immunoblotting** For analysis of HAUSP tissue expression, organs were homogenized with glass slides in ice-cold PBS, cells were separated from debris by centrifugation, depleted of red blood cells, and lysed in the Lysis Buffer containing 0.5-1% of NP-40. For analysis of apoptotic thymocytes, glass bead lysis was performed. 20-50 μg of total protein was loaded onto 10-12% reducing SDS/PAGE and transferred onto a PVDF membrane. The filters were incubated with the indicated first antibodies (anti-HAUSP r201 1:1500, anti-PARP 0.5 μg/mL, anti-cIAP1 0.4 μg/mL, anti-IFN 1:5000), followed by 1:5000 HRP-coupled goat anti-rabbit or rabbit anti-goat IgG. The blots were developed by chemoluminescence.

**In Vitro Cleavage Assays.** USP7 or USP7-derived and PARP constructs were used as templates for coupled transcription/translation using a T7 kit (Promega) and [35S-met] (Promega), with ~1 μg of DNA per reaction. In some cases, 35S-labelled USP7 was incubated with unlabelled 3 μM UbVS for 40 min-1 hour at 37 °C. 35S-labelled USP7 and PARP were incubated with the bacterial lysates, expressing caspase 3 (provided by Dr. J. Yuan) for 1 hour at 37 °C. The reaction were terminated by addition of SDS-PAGE loading buffer and heating at 95 °C for 5 min. The reaction products were analyzed on the reducing 10% SDS-PAGE.

**Activation-Induced Cell Death and In Vitro Deletion Assay.** H-2Kb-ova specific 4G3 T cells (10⁶ cells/mL) or OT-1 thymocytes (5 x 10⁶ cells/mL), were incubated for 24 hours or 15 hours, respectively, with antigen-presenting DAP cells (3 x 10⁵ cells/mL) untreated or pretreated with 12 μg/mL ova peptide for 3 hours. When indicated, an incubation media contained 48 Units/mL of recombinant IL2. Non-adherent T cells were separated from APCs and lysed in the Lysis Buffer containing 1% of NP-40. Analysis of APC cultures without T cells indicated that usually < 5% of non-adherent cells were APCs. Samples were analyzed for HAUSP expression by immunoblotting and percentage of cell death was quantified by FACS analysis of annexin V staining, as described above.

**Preparation of thymic RNA pool and RT-PCR.** A single cell suspension from a thymus of a three-month-old C57BL/6 mouse was pelleted and resuspended in 1 mL of Trizol reagent. RNA was extracted by addition of 200 μL of Chloroform (by spinning at 13K rpm for 15 minutes at 4 °C), precipitated with 500 μL of isopropanol (by spinning at 13K rpm for 10 minutes at 4 °C), washed with 75% ethanol and resuspended in 100 μL of water. First strand cDNA synthesis was performed using Gibco Superscript II kit according to the manufacture instructions for the random hexamer method. A fragment of mouse USP7 was amplified with the Pfx enzyme (with Ta of 60 °C, extension time = 3.5 minutes, 35 cycles) using a forward primer MH3.KB (containing a KpnI and BamHI sites): G GGG TAC GGA TCA GCG GGC GAG CAG CAG CTG AGC and a reverse primer C5.X (containing XhoI site): CCG CTC GAG GGC ACT CCT CTT TGG GGC TTT GTT GAA, corresponding to nucleotides 231-252 and 3244-3270, respectively in the human USP7 sequence. The amplified fragment was cloned into pcDNA 3.1 using KpnI and XhoI sites. This plasmid was used as a template in the
subsequent PCR reaction (under identical conditions) that introduced the "missing ends" to the mouse USP7 using a forward primer E5.Kpn: GG GGT ACC ATG AAC CAC (CAG); AAA GCG GGC GAG CAG CAG CTG AGC and a reverse primer E3.Xho: CCG CTC GAG TCA GTT ATG GAT TTT AAT GGC CTT TTC AAG GTA AGT GTA GCG ACT CCT CTT TGG GGC TTT GTT GAA. This PCR product containing a full-length USP7 coding sequence was cloned into pcDNA 3.1 using Kpn I and Xho I restriction sites. The sequencing was performed with the set of sense (residues 210-233, 451-472, 1169-1192, 2573-2596, 2056-2088, 3326-3348 and anti-sense (929-952, 1326-1349, 1857-1880, 3326-3348, 2566-2589, 3810-3831) primers designed from human USP7 sequence.

Site directed mutagenesis and generation of HA-tagged USP7 constructs. Site directed mutagenesis of human USP7 construct in pCI-neo vector was performed with a QuikChange Site-Directed Mutagenesis Kit (Stratagene), according to the manufacture's protocols using the Pfx enzyme and a forward SDM.sense primer: G AAT CAG GGA GCG ACT GCT TAC ATG AAC AAG AGC CTG and a complementing SDM.antisense primer (18 PCR cycles with 16 minutes/cycles and Tm=55 °C, Te=68 °C). The sequences of both strands of USP7.SDM construct were verified using a forward primer TGA AGT CTT TGT ACA GGC GGA TGC and a reverse primer GGG ACG CTT TTA GAC GAA TCA TC.

HA-tagged constructs were obtained by a Pfx-catalyzed PCR reaction using a pCI-neo-USP7 template with a forward primer BKHA (containing a BamH I restriction, a Kozak sequence, an HA tag, and a 203-233 nucleotides of human USP7 sequence): CG GGA TCC ACC ACC ATG TAC CCC TAC GAC GTG CCC GAC TAC GCC AAC CAC CAG CAG CAG CAG CAG CAG CAG AAA and reverse primers E3.Xho and 196.1 (containing a stop codon, an Xho I restriction site, and residues 173-196 of human USP7): CCG CTC GAG TCA CTG TAC AAA GAC TTC AAA GGT AAC for the HA-USP7 or HA-USP7.SDM and HA-USP7.1-196 constructs, respectively. These PCR products were cloned into pcDNA 3.1 using the BamH I and Xho I restriction sites.

Immunofluorescence. For HAUSP localization in thymocytes, freshly isolated thymocytes were plated onto polylysine (1mg/mL)-coated cover slips placed onto 24-well plates (2-4 x 10^6 cells/well) in complete RPMI media. The plates were spun at 3,500 rpm for 5 min to allow the cells to attach to the slides. Cells were washed once with PBS-Ca-Mg (PBS supplemented with 0.1 g/L CaCl_2(H_2O)) and 0.1 g/L MgCl_2(H_2O) and fixed with 4% solution of paraformaldehyde (10 minutes, room temperature). Slides were washed, permeabilized for 10 minutes in the blocking buffer (5% goat serum, 0.1% Triton X in PBS-Ca-Mg), and stained with the polyclonal anti-HAUSP or anti-Kb p8 antisera (1:200 in the blocking buffer). After 3washes, slides were stained with the secondary goat anti-rabbit-FITC antibody (1:200 in the blocking buffer), washed again 3 times, and mounted with the Aquapoly/Mount solution (Polysciences Laboratories). Microscopy analysis was performed with a Bio-Rad MRC 1024 confocal laser scanning microscope.

For localization of HA-tagged HAUSP constructs in MGU373 cells, 5 μg of DNA was transfected into MGU373 cells plated onto 100 mm dishes in complete DME media using the calcium phosphate method. 24 hours post transfection, cells were plated in each well
of glass chamber slides (Nalge Nunc International Laboratories) and incubated overnight to allow the cells to attach to the slide. Cells were washed 3 times in PBS and fixed with 3.7% paraformaldehyde (10 minutes, room temperature). Slides were washed 3 times with PBS, permeabilized for 10 minutes in the blocking buffer (3% BSA, 0.5% saponin in PBS), and stained with anti-HA antibody (1:800 in the blocking buffer) for 30 minutes at 37 °C. After 3 washes, slides were stained with the secondary anti-mouse Alexa™568 (Molecular probes, 1:500 in the blocking buffer), washed again 3 times in PBS and once with water, mounted with the Aquapoly/Mount solution (Polysciences Laboratories) and examined in a Nikon microscope with an X 40 objective lens and appropriate filters.

**Metabolic labeling and immunoprecipitations.** 293 cells were plated onto 100 mm dishes (2 x 10⁶ cells/plate) and in 24 hours were transfected with 10 µg of an HA-tagged construct using the Fugene-6 method, according to the manufacturer’s instructions. 24 hours post-transfections, cells were incubated for 60 minutes in methionine and cysteine-free DME media, labeled with 1 mCi [³⁵S] methionine/cysteine for 45 min, and lysed in either NP-40 lysis mix (0.5% NP-40, 50 mM Tris, pH 7.5, 5 mM MgCl₂, 1 mM PMSF, and 1 µg/mL aprotinin) or glass bead lysis buffer (50 mM Tris, pH 7.4, 5 mM MgCl₂, and 2mM ATP). Lysates were precleared twice with normal rabbit and mouse serum and formalin-fixed Staphylococcus aureus, followed by anti-HA immunoprecipitations. Precipitates, adjusted for total cell numbers, were run on 11% SDS-PAGE. Gels were developed using DMSO/PPO and exposed to Kodak X-OMAT AR film.

**Retrovirus-mediated USP7 gene transfer into MGU373 cells and UV-induced apoptosis of MGU373 cells.** USP7 and USP7.SDM were excised from the pcDNA 3.1 with Not I restriction digest, followed by Mn blunt and Xho I digest. These fragments were inserted into the pMIG vector (mouse stem cell virus IRES-GFP) digested with EcoR I, blunted with Mn, and digested with XhoI; the resulting vectors were called RV-GFP-USP7 and RV-GFP-USP7.SDM. GPG packaging cells were transfected with RV-GFP-USP7 and RV-GFP-USP7.SDM constructs using the Effectene method (QIAGEN) and day 4 viral supernatant was used to infect MGU373 cells. Virally-infected MGU373 cells were sorted twice on the FL1 channel by FACS sorting to enrich for cell population expressing relatively high levels of GFP and USP7 proteins; the resulting cell lines were called MGU373-GFP-USP7 and MGU373-GFP-USP7.SDM. MGU373, MGU373-GFP-USP7 and MGU373-GFP-USP7.SDM cells were induced to apoptose with the ten-minute UV pulse (UV Stratalinker 2400, Stratagene) and examined for PI or annexin V apoptotic markers by FACS analysis as described above.
Results

USP7 (HAUSP) is processed during thymocyte apoptosis.

Identification of proteasomal targets and possible regulatory mechanisms that control proteasomal processing are crucial for dissection of the thymic apoptotic program. Few experiments have so far addressed the matter. The evidence that anti-apoptotic molecules of the IAP (inhibitor of apoptosis) family are selectively lost in a proteasome-dependent manner from dexamethasone or etoposide-treated thymocytes (6) has been used to implicate IAPs in the execution of thymic programmed cell death. Most proteasomal substrates are targeted for degradation by conjugation of multiple ubiquitin chains (7). Therefore, regulation of proteasomal processing during apoptosis is likely to stem from selective ubiquitin conjugation or removal. Several reports have implicated Ub-conjugating enzymes and Ub-ligases in the regulation of apoptosis (8-10), while the role of deubiquitinating enzymes (USP) has not been explored.

To examine the involvement of ubiquitin specific proteases (USPs) in thymocyte apoptosis we have used a radiolabeled active site directed probe, $^{125}$I-UbVS, which covalently modifies the active site of several mammalian and yeast deubiquitinating enzymes (19) and Appendix. Unlike proteasome inhibitors, UbVS is not cell-permeable and cannot be employed for labeling in intact cells. Therefore, analysis was performed on cell lysates using $^{125}$I-UbVS. Lysates, prepared from thymocytes induced to undergo apoptosis with dexamethasone for the indicated time period, were labeled with $^{125}$I-UbVS. USP activities of induced and uninduced lysates were analyzed as shown in Fig 1A. The most striking and reproducible difference in labeling pattern was the disappearance of a ~135kD polypeptide with the concomitant appearance of a lower MW polypeptide, evident as early as after 3.5 hours of induction. The kinetics of this apparent cleavage were similar to the kinetics of phosphatidylserine exposure, as judged by annexin V staining (Fig 1B, Chapter 4). Several other USPs appear to be affected in the apoptotic thymocytes. For example, $^{125}$I-UbVS labeling of ~110kD polypeptide appears reduced (Fig. 1A). Two identified mammalian USPs, USP8 (UBPY) and USP7 (HAUSP), are reported to have a MW in the 130kD range (22, 23). Both the full-length protein and its cleaved product were reactive with anti-HAUSP polyclonal rabbit serum (Fig.1B), as determined by immunoblotting. This identifies the 135 kD polypeptide as HAUSP/USP7 and the lower MW form (HAUSP*) as a HAUSP/USP7 cleavage product. The upper polypeptide cross-reacting with anti-HAUSP/USP7 serum has been observed previously and is not reactive with UbVS (Fig. 1B) (22). Cleavage of the ~135kD band was also observed during $\gamma$-irradiation (Fig. 1C) and etoposide-triggered apoptosis (data not shown) with kinetics similar to that of phosphatidylserine exposure (data not shown). Likewise, HAUSP is partially cleaved during antigen-triggered thymocyte apoptosis (Fig. 1D), albeit less efficiently than during dexamethasone-induced death. However, we were unable to detect HAUSP cleavage during activation induced cell death (AICD) of a mature T cell clone (Fig. 2). Likewise, all attempts to detect HAUSP cleavage in various transformed cell lines have failed, with some cell lines expressing barely detectable HAUSP levels (Fig. 2). We conclude that HAUSP is selectively cleaved during apoptosis of murine thymocytes. The cleavage of HAUSP occurs relatively early in the apoptotic cascade. Furthermore, the processed polypeptide remains enzymatically active,
since it is modified by the active site directed probe UbVS. Immunoblotting analysis of various murine tissues showed that HAUSP expression is high in brain, spleen, and thymus (Fig. 1E), organs that rely on apoptosis for development. As expected, preincubation of thymocyte suspension cultures with NLVS substantially reduced HAUSP cleavage (Fig. 3A), consistent with the ability of proteasome inhibitors to partially block/delay thymocyte apoptosis (Chapter IV). Next we addressed cellular localization of HAUSP in murine thymocytes. The nucleus occupies most of the cell volume in this cell type (Fig. 4A); therefore a confocal microscopy analysis of HAUSP expression was performed. Confocal microscopy analysis of murine thymocytes suggested a nuclear localization of HAUSP, likely excluding the nucleoli (Fig. 4B), consistent with earlier experiments performed in the Hep2 and HFL cell lines (22).

**Role of caspases in HAUSP cleavage.**

First, we analyzed the effect of the general caspase inhibitor, ZVAD-fmk, on HAUSP processing during dexamethasone-induced thymocyte apoptosis. Consistent with the potency of ZVAD-fmk in blocking thymocyte apoptosis (Chapter 4), HAUSP was not cleaved after ZVAD-fmk pretreatment of thymic suspension cultures (Fig. 3B).

Next, we examined whether HAUSP cleavage depends on the activity of specific caspases. Remarkably, no HAUSP processing was evident in the thymocytes of caspase 3 deficient animals, as examined by both active site $^{125}$I-UbVS labeling and Western blots (Fig. 5A). Immunoblotting analysis of the dexamethasone-treated lysates showed that caspase 3 -/- thymocytes exhibit at least some biochemical hallmarks of apoptosis, such as cleavage of poly(ADP-ribose) polymerase (PARP), a protein involved in DNA repair, and degradation of cIAP1, a anti-apoptotic molecule of the IAP (inhibitor of apoptosis) family (Fig. 5A). HAUSP processing was unaffected in caspase 12-/- thymocytes (Fig. 5A). PARP processing in caspase 3 -/- thymocytes has been observed previously and demonstrates their susceptibility to apoptosis (15). Combined, these results suggest that the specific cleavage of HAUSP observed during thymocyte apoptosis is dependent on caspase 3.

Next, we examined HAUSP cleavage in the *in vitro* translation assay. *In vitro*-translated HAUSP was incubated with the bacterial lysates expressing active caspase 3. We did not observe HAUSP cleavage in this assay (Fig. 5B). In contrast, poly(ADP-ribose) polymerase (PARP) protein, a known caspase 3 substrate, was processed as expected. Note that *in vitro*-translated HAUSP retains its enzymatic activity, as shown by labeling with UbVS (Fig. 5B). This result suggests that HAUSP is not cleaved directly by caspase 3.

**Cloning of a murine HAUSP homologue and construction of HA-tagged HAUSP constructs.**

A mouse homologue of USP7 was cloned by RT-PCR, with thymic RNA pool used as a template. Human and mouse USP7 are strikingly conserved at both the DNA and protein level with about 90% similarity (Fig. 6). Unfortunately, the mouse USP7 clones contained a nonsense mutation that precluded expression of a full-length mouse USP7. However, high sequence similarity between the human and mouse proteins
prompted us to perform further analysis with available human USP7 clones. First, we obtained an inactive form of human USP7, "USP7.SDM" by replacing a postulated active site Cys residue (codon 223) with an Ala residue using site directed mutagenesis (22). Next, we introduced an N-terminal HA tag to the full-length human USP7 (HA-USP7), USP7.SDM (HA-USP7.SDM), and a deletion mutant lacking peptidase domains (HA-USP7.1-196) (Fig. 7A). The myc-tagged version of the USP7 and USP7.1-212 deletion mutant, lacking peptidase domains and containing a "non-classical" TNF-Receptor-Associated Factors (TRAF) domain, has been reported previously (24). In vitro translation reactions of all three HA-tagged HAUSP constructs contained polypeptides of expected MW (Fig. 7B). Furthermore, UbVS modified "HA-USP7" but not "HA-USP7.SDM" gene product (Fig. 7C). This result verifies that Cys 223 is the active site for the USP7 isopeptidase. In-vitro-translated products were immunoprecipitatable with an anti-HA monoclonal antibody. Transient transfections of HA-tagged constructs into 293 cells yielded proteins of the expected molecular weights (Fig. 8). Furthermore, several polypeptides appear to co-immunoprecipitate specifically with HA-USP7.1-196 gene products. The identity of these proteins is not known at present.

**Intracellular localization of HA-tagged USP7 constructs.**

Ha-tagged constructs were transiently expressed in MGU373 astrocytoma cell line. 24-48 hours post-transfection, their expression was analyzed by immunofluorescence using an anti-HA monoclonal antibody. Approximately, 50% of the HA-USP7-transfected cells displayed a perinuclear staining pattern. The rest of HA-USP7 expressing cells stained outside of nuclear region. The diversity of staining patterns is illustrated in Fig. 9A. This result appears to contradict previously published data with myc-tagged USP7 constructs transiently transfected into COS cells, where predominantly nuclear staining was observed (24). HA-USP7.SDM-transfected cells displayed a set of staining patterns similar to that observed for the cells transfected with the "wild type" HA-USP7 construct. The HA-USP7.1-196 deletion construct was found outside of the nucleus even more so than the full length constructs (Fig. 9B). These data is in agreement with the bipartite nuclear localization signal (NLS) at the residue 343 of USP7 protein, as identified by the PSORT software (http://psort.nibb.ac.jp/form2.html). The predicted NLS motif is not consistent with the data published for the myc-tagged USP7.1-212, since this deletion mutant lacks the predicted NLS but displays a predominantly nuclear staining upon transient transfections into COS cells (24).

**Retroviral-mediated gene transfer approach to study USP7 function.**

To address a possible role of HAUSP in programmed cell death, we examined the effect of USP7.SDM expression on UV-induced apoptosis of MGU373 astrocytoma cells. We utilized a retrovirus expression system to obtain stable cell lines MGU373-USP7 and MGU373-USP7.SDM, expressing human USP7 and USP7.SDM gene products, respectively (Fig. 10A and 10B). Annexin V staining was used as a measure of cell death upon exposure to UV radiation. Neither MGU373-USP7 nor MGU373-USP7.SDM cells differed from the control MGU373 cells in their susceptibility to UV-induced apoptosis (Fig. 10C). This result is perhaps not too surprising, considering that no detectable
HAUSP cleavage occurred in several transformed cell lines (Fig. 2). We have attempted to perform retrovirus-mediated gene transfer into either adult thymocytes (via intrathymic injections) or into FTOCs. These attempts have failed, with the viral titer being a likely limiting factor in these gene transfer experiments (data not shown).
Discussion

Here we examined the role of deubiquitinating enzymes in thymocyte apoptosis. Earlier studies on the role of the ubiquitin-proteasome pathway thymocyte apoptosis focused primarily on the proteasome complex itself (1-5). Most studies indicate that the proteasome is a positive regulator in thymocyte apoptosis, since proteasomal activity is increased upon induction of cell death.

The increase in proteasomal activity might be a crucial checkpoint in the apoptotic program, leading to degradation of signaling, survival, or transcription factors. Other players in the Ub-proteasome pathway, such as Ub-ligases, Ub-conjugating enzymes, and deubiquitinating enzymes might modulate the execution of the proteasome checkpoint. Selective ubiquitin conjugation and removal are likely to be important parameters in the control of cytosolic and nuclear proteolysis. Several reports have implicated Ub-conjugating enzymes and Ub-ligases in the regulation of apoptosis. Induction of Ub-conjugating enzyme activity has been observed during adenovirus-or aggregated LDL-induced apoptosis (8). Fas associates with the Ub-conjugating enzyme UBC-FAP (9). The Ub-ligase, Nedd4, undergoes caspase 3-mediated cleavage during anti-fas or etoposide induced apoptosis in Jurkat T cells (10). The suggestion that Ub removal could play a regulatory role would therefore seem entirely reasonable. In addition, several deubiquitinating enzymes physically associate with the proteasome (19, 25). Regulation of this binding event provides a potential route for modulating the substrate accessibility to proteasomal complex and could bring about changes in proteasomal activity.

Here we assess the activity of ubiquitin-specific proteases (USPs) upon induction of apoptosis in murine thymocytes by active site directed labeling using [125I]-UbVS. UbVS is a C-terminally modified Ub derivative that is a highly specific irreversible inhibitor of this class of enzymes. UbVS covalently modifies active sites of 6 of the 17 known UBP's in yeast and of a significant number of mammalian USPs as well (19). We show that USP7 (HAUSP) is specifically processed soon after the onset of dexamethasone, γ-irradiation induced cell death and during negative selection. While the processing event appears to be a proteolytic cleavage of USP7, a change in a post-translational modification, although less likely, could not be excluded. It is unlikely that HAUSP processing is merely a side effect associated with elevated proteolytic activity during apoptosis, since programmed cell death does not lead to indiscriminate proteolysis; rather specific structural and regulatory proteins are cleaved (26). HAUSP processing appears to be cell-type specific since it is observed only in primary thymocytes. Furthermore, we show that HAUSP expression is high in brain, spleen, and thymus, organs that rely on apoptosis for development perhaps more so than other tissues. HAUSP appears to be the only deubiquitinating enzyme that is specifically processed during thymocyte apoptosis. In addition, a remarkable sequence similarity between human and mouse USP7 proteins points to a conservation of an important, albeit unidentified, function. In man, the HAUSP gene has been mapped to regions rearranged in leukemia and deleted in solid tumors, conditions that may be driven by defects in the cell death program (27). Importantly, the processed HAUSP retains its enzymatic activity, since the fragment that is produced can still be modified by the active-site directed probe UbVS. It is tempting to speculate that HAUSP substrate recognition
rather than its catalytic domain is modified by proteolytic cleavage during apoptosis. While not much is known about substrates and interacting partners of specific USPs, the sequence diversity outside the conserved catalytic domain suggests distinct functions for individual family members (11, 12). Removal of (part of) a regulatory domain would presumably affect the ability of the cleaved USP to deubiquitinate a key substrate(s), promoting subsequent proteasomal degradation of this substrate. Removal of a regulatory domain might also result in altered intracellular distribution of USP7 and, consequently, in altered pool of accessible substrates.

Our results underscore the need for further studies on HAUSP intracellular distribution and on identity of HAUSP nuclear localization signal. Earlier studies (24, 28) as well as our confocal microscopy analysis on HAUSP localization in thymocytes suggest a nuclear localization for this protein. However, heterogeneity of HAUSP distribution observed in transient transfection experiments suggests that HAUSP localization might be partially cell cycle-dependent and subject to dynamic control.

What are the possible interacting partners or substrates of HAUSP? HAUSP has been identified as an ubiquitin-specific protease that binds to a herpesvirus regulatory protein Vmw110 (28). HAUSP is distributed in the nucleus in a micropunctate pattern, and is dynamically associated with the PML nuclear domains during viral infection. Our data suggest that N-terminal part of the protein is removed during apoptosis. The HAUSP N-terminus contains a "non-classical" TNF-Receptor-Associated Factors (TRAF) domain (24). Thus, HAUSP processing during apoptosis is likely to result in loss or altered functionality of HAUSP's TRAF domain. TRAF family proteins regulate several functions of the TNFR superfamily and could bind variety of proteins, such as protein kinases and Ub-ligases (29). TRAF family members are potential interacting partners for HAUSP, a possibility that now needs to be explored by experiment. Since in previous studies HAUSP was shown to localize mostly to the nucleus, it was thought to participate in the relatively late stage of TNF signal transduction cascade. Zapata et al. speculate that HAUSP could regulate or could be regulated by TRAFs that translocate to the nucleus as a step of certain signal transduction pathways (24).

Remarkably, HAUSP processing is not observed in caspase 3 deficient thymocytes. Caspases are classified as initiator (for example caspase 8, 9 and 12) and effector caspases (for example caspase 3, 6, and 7) based on their structure and order of activation in the proteolytic death cascade (13, 14). Caspase 3 is an effector that is thought to mediate certain nuclear and morphological changes associated with apoptosis (16, 30). Therefore, potential HAUSP targets may include nuclear regulatory proteins/transcription factors that are rescued from ubiquitin-proteasome degradation.

The identification of HAUSP binding partners is crucial for understanding of HAUSP role in apoptosis. Our immunoprecipitation experiments performed in 293 cells transfected with HA-tagged HAUSP constructs, point to several candidate polypeptides. In addition, a preliminary yeast two-hybrid screen with the N-terminal part of USP7 resulted in several potential binding partners (Madelon Maurice, unpublished results).

How is HAUSP processing positioned in the apoptotic pathway with respect to caspase 3 activation and signaling by other components of Ub-proteasome pathway? Consistent with the proposed regulatory function of HAUSP processing, its processing occurs quite early in the death program, with kinetics reminiscent of that seen for the loss of plasma membrane asymmetry. Note that caspase 3 activation during apoptosis occurs
even before phosphatidyl serine exposure (31). Although our in vivo data indicate that HAUSP processing is dependent on caspase 3, our in vitro data indicates that caspase 3 may not cleave HAUSP directly. The majority of structural and regulatory proteins processed during programmed cell death can be cleaved by caspase 3 (30). The exception to the “caspase 3 cleavage rule” appears to be degradation of cIAP1, an IAP family member, the degradation of which is dependent on the proteasome but is independent of caspase 3. Thus, our results suggest that degradation of cIAP1 during dexamethasone-induced thymocyte apoptosis is upstream of caspase 3 activation, which, in turn, is upstream of HAUSP processing. In contrast, Clem et al. suggest that c-IPA1 is cleaved by caspase 3 to produce a proapoptotic C-terminal fragment in virus-induced apoptosis (32). IAPs can inhibit apoptosis induced by a variety of stimuli, in part via direct inhibition of active caspases (33, 34). Thus, it is likely that IAPs degradation during apoptosis is executed by different effectors in cell-type- and stimulus-dependent manner. In apoptotic thymocytes, Ub-ligase activity of cIAP1 results in self-ubiquitination and degradation of IAPs by proteasomes (6). In addition, Ub-ligase activity of IAPs could provide an additional route for caspase inhibition via ubiquitination and targeting for subsequent proteasomal degradation (35). Of note, the results obtained for IAP do not provide a direct functional connection, but rather a correlation between IAP degradation and thymocyte apoptosis. Likewise, our studies underscore the importance of functional studies on the role of deubiquitinating enzymes in thymocyte apoptosis. While this report focuses on HAUSP, other USPs may also be involved. Availability of USP genetic knockouts and of cell-permeable specific inhibitors of USPs will be crucial for further studies on the role of these enzymes in programmed cell death. These studies may lead to novel therapeutic strategies for treatment of diseases stemming from defects in the apoptotic pathway.

In summary, multiple components of the ubiquitin-proteasome pathway may be involved in the execution of programmed cell death. Players other than the highly conserved proteasome itself are likely to provide tissue- and stimulus- specificity and facilitate integration with the caspase proteolytic cascade. We here provide a first report on possible involvement of deubiquitinating enzymes in thymocyte apoptosis by showing that HAUSP is an early substrate of a caspase-3 dependent processing. Furthermore, our results may provide a potential link between the caspase and ubiquitin-proteasome proteolytic death cascades.
References


Appendix to Chapter V

The structure of Ubiquitin Vinyl Sulfone (UbVS)

![Ubiquitin Vinyl Sulfone (UbVS) structure](image)
HAUSPs were prepared as described in Materials and Methods. HAUSPs processse product is labeled as

![Image of gel electrophoresis with bands labeled HAUisp-UVS, HAUisp-UVS+V, and anti-HAUisp.]

**Figure 1.** LS7 (HAUSPs) processing during lymphocyte apoptosis. (A) Lanes 1-10: UVS

dexamethasone

cells

- +
- +

E

- +
- +

D

- +
- +

C

- +
- +

B

- +
- +

A

Label: 15S-UVS
Figure 2. Analysis of USP7 (HAUSP) expression and processing in cell lines. Lysates obtained from the indicated cell lines were analyzed for HAUSP expression either by immunoblotting with HAUSP antiserum (WB) or by labeling with $^{125}$I-UbVS as described in Materials and Methods. When indicated, cells were induced to apoptosis with γ-irradiation for 10-20 minutes (γ), UV-irradiation 0.5-2 minutes (uv), 0.5-1 μM dexamethasone (dex), 30 μM etoposide (eto), 0.5 μM staurosporine (st), or 10 ng/mL TNF- and 10 μg/mL cycloheximide (tnf). Cell death was quantified by FACS analysis using annexin V staining as described in Materials and Methods. USP7 is indicated by an arrow.
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Figure 3. **NLVS and ZVAD-fmk inhibition of USP7 (HAUSP) processing.** Thymocytes, pretreated with either (A) 20 M NLVS or (B) 50 M ZVAD-fmk, were induced to undergo apoptosis with 0.5 M dexamethasone for 5.5 hours, lysed, and labeled with \(^{125}\text{I}-\text{UbVS}\) as described in Materials and Methods. Radiolabeled proteins were resolved by reducing 10% SDS-PAGE.
Figure 4. **Intracellular localization of USP7 (HAUSP) in murine thymocytes.** (A) Distribution of nuclear staining in murine thymocytes was analyzed using a standard immunofluorescence analysis with DAPI stain. (B) Thymocytes stained with either anti-HAUSP or anti-Kb (anti-p8) serum were analyzed by confocal laser scanning microscopy as described in Materials and Methods.
Figure 5. **The role of caspase 3 in HAUSP processing.** (A) Thymocytes from wild type (wt), caspase 3 -/-, or caspase 12 -/- mice were induced to undergo apoptosis with 0.5 M dexamethasone for 5.5 hours and lysed as described in Materials and Methods. Lysates were split and labeled with [35S]-UbVS or analyzed by immunoblotting using the anti-HAUSP, anti-cIAP1, or anti-PARP antibodies, as indicated. (B) *In vitro*-translated [35S]-labeled HAUSP or PARP was incubated with the bacterial lysates expressing an active caspase 3. When indicated, HAUSP was incubated with unlabeled UbVS. The reaction products were analyzed by reducing 10 % SDS-PAGE.
Figure 7. Construction and in vitro translation of HA-tagged USP7 (HAUSP) constructs. (A) The full length human USP7 protein containing the indicated domains, the USP7 cys 223 to ala mutant obtained by site-directed mutagenesis (USP7.SDM), and the USP7 deletion mutant lacking a cys box, a his box, and a C-terminus (USP7.1-196) were constructed and HA-tagged as described in Materials and Methods. (B) These constructs were used as templates in the in vitro translation reactions and were resolved by reducing 10% SDS-PAGE (direct load). The products of the in vitro translation reactions were subsequently immunoprecipitated (IP) with the anti-HA antibody, as described in Materials and Methods. (C) In vitro translated HA-USP7 and HA-USP7.SDM were incubated with unlabeled UbVS as in Fig. 5.
Figure 8. **Transient transfections of HA-tagged USP7 (HAUSP) constructs into 293 cells.** HA-tagged USP7 constructs were transiently transfected into 293 cells using a Fugene-6 method. 24 hours post-transfection, 293 cells were labeled with [35S]-methionine/cysteine and lysed in either NP-40 or glass bead lysis buffer. The HA-tagged constructs were immunoprecipitated with the anti-HA antibody and were resolved by reducing 11 % SDS-PAGE as described in Materials and Methods. The possible binding partners of the HA-USP7.1-196 protein are indicated by arrows.
Figure 9. Intracellular localization of HA-tagged USP7 constructs in MGU373 astrocytoma cells. HA-tagged constructs were transiently expressed in MGU373 astrocytoma cell line. 24-48 hours post-transfection, their expression was analyzed by immunofluorescence using an anti-HA monoclonal antibody as described in Materials and Methods. (A) A representative set of anti-HA staining patterns is shown for the HA-USP7 and HA-USP7.SDM transfections. (B) A representative set of anti-HA and DAPI double staining patterns is shown for the HA-USP7.1-196 transfections.
Figure 10. Retroviral-mediated gene transfer of USP7 and USP7.SDM into MGU373 astrocytoma. MGU373-USP7 and MGU373-USP7.SDM stable cell lines, expressing human USP7 and USP7.SDM gene products, respectively, were obtained using the retrovirus expression system containing the IRES-GFP cassette and either the wild type USP7 protein (RV-GFP-USP7) or the cys 223 to ala mutant form of USP7 protein (RV-GFP-USP7.SDM). (A) GFP expression in MGU373-USP7 and MGU373-USP7.SDM cell lines was analyzed by FACS. (B) USP7 expression in MGU373-USP7 and MGU373-USP7.SDM cell lines was analyzed by immunoblotting with the anti-HAUSP serum. (C) MGU373, MGU373-USP7 and MGU373-USP7.SDM were induced to apoptosis by UV-radiation with the ten-minute UV pulse and examined for annexin V apoptotic markers by FACS analysis at the indicated times after the pulse.
CHAPTER VI

General Conclusions and Future Directions

*Thymocyte development in the absence of classical MHC class I molecules*

In chapter II we characterize K\(^b\)D\(^b\) -/- mice that are deficient in all classical MHC class I molecules. K\(^b\)D\(^b\) -/- mice have a 5-10 fold reduction in the numbers of peripheral CD8\(^+\) T cells, but can nonetheless generate strong MHC-specific CD8\(^+\) T cell responses after priming *in vivo*. Several conclusions can be drawn from these observations. Classical MHC class I molecules are required for selection of > 95% of the CD8\(^+\) T cells. The contribution by \(\beta_2\)m-associated, TAP-loaded class I molecules of other provenance (e.g. class Ib molecules) can thus account for no more than 5% of CD8\(^+\) T cells. Although K\(^b\)D\(^b\) -/- mice have few peripheral CD8\(^+\) T cells, these cells are fully functional, and they must have arisen in a developmental pathway that is independent of classical MHC class I molecules. These CD8\(^+\) T cells are likely to be selected by non-classical MHC class Ib molecules. Alternatively, the apparent class I specificity of these CD8\(^+\) T cells may be germ-line encoded and does not depend on the presence of host class I molecules (1).

*T cell signaling in the presence of antagonist peptides*

In chapter III we examine the patterns in CD3 \(\zeta\) chain tyrosine phosphorylation in 2C cells that had been exposed first to agonist and then to high-affinity antagonist peptide-MHC complexes. Contrary to previous findings, exposure to these antagonist complexes resulted in an almost complete elimination of the agonist-induced CD3 \(\zeta\) chain tyrosine phosphorylation. Previous results indicated that the changes in tyrosine phosphorylation of CD3 \(\zeta\) chain elicited by antagonist complexes were quantitatively reduced but qualitatively the same as those elicited by agonist complexes (2). Our result suggests that the agonist complex stimulates removal of phosphoryl groups from tyrosine residues in CD3 \(\zeta\) chains as though from the activation of a tyrosine phosphatase.

*Thymocyte apoptosis in the presence of proteasome inhibitors*

In chapter IV we examine the role of proteasome-mediated proteolysis in thymocyte apoptosis. We utilize a fetal thymic organ culture (FTOC) system with a panel of proteasome inhibitors to implicate the proteasome in thymocyte apoptosis, not only when triggered by dexamethasone, but also when induced by peptide-MHC complexes. We find that proteasome inhibitors do not completely block but rather delay both dexamethasone- and antigen-triggered thymocyte apoptosis. One explanation is the incomplete inhibition of proteasome function attained by such compounds. The existence of a proteasome-independent branch in the thymocyte apoptotic pathway is also a possibility. We also show that proteasome activity is increased in apoptotic thymocytes, as visualized by active site labeling of proteasomal \(\beta\) subunits, indicating that proteasome functions as a positive regulator in thymocyte death cascade.
The system and reagents utilized in this study allow for a more physiological and a more specific assessment of the role of proteasomal proteolysis in thymocyte apoptosis. Active site directed labeling of proteasomal β subunits is a more specific assay to assaying proteasome activity than a fluorogenic substrate approach used in earlier studies (3, 4), as other cytosolic peptidases have been shown to cleave such substrates as well. The FTOC system is a more physiological model for thymic development than thymocyte suspension cultures employed in earlier studies, as several reports have suggested that responses of thymocytes in suspension cultures may be different to those within an intact thymic microenvironment (5, 6). Furthermore, limitations of the thymocyte suspension approach preclude analysis of the role of the proteasome in antigen-driven apoptosis.

**Deubiquitinating enzymes in thymocyte apoptosis**

The regulation of proteasome function during thymocyte apoptosis is poorly understood. Other members of the proteasome-ubiquitin pathway are likely to confer specificity and selectivity in proteasomal cleavages. In chapter V, we examine the activity of deubiquitinating enzymes in murine apoptotic thymocytes by active site labeling. We show that the deubiquitinating enzyme USP7 (HAUSP) is specifically processed upon dexamethasone-, γ-irradiation-, and antigen-induced cell death. This processing appears to be unique to USP7. We also show that USP7 protein is highly expressed in thymus, spleen, and brain and is very similar in men and mice. Processing of USP7 does not occur in caspase 3-/− thymocytes but caspase 3 does not cleave USP7 directly. Our results suggest that thymocyte apoptosis leads to a modification of a deubiquitinating enzyme and may provide an additional link between the proteasome-ubiquitin pathway and the caspase cascade during programmed cell death.

While these results do not provide a functional link between USP7 processing and thymocyte apoptosis, it is unlikely that USP7 processing is merely a side effect associated with elevated proteolytic activity during apoptosis, as programmed cell death does not lead to indiscriminate proteolysis (7). The uniqueness of USP7 processing across the set of examined deubiquitinating enzymes, cell-type specificity of this processing event, a possible link to a caspase cascade and TRAF signaling pathway, as well as the high expression of USP7 in T cell lineage and high conservation of USP7 amino acid sequence point to a potential functional significance of USP7 processing event.

**Modes of USP7 action in thymocyte apoptosis**

How is the processing of USP7 positioned in the apoptotic pathway with respect to caspase 3 activation and signaling by other components of Ub-proteasome pathway? Our experiments in caspase 3 -/− thymocytes indicate that USP7 processing is caspase 3-dependent, while degradation of cIAP1, an IAP family member, does not require an active caspase 3. IAPs can inhibit apoptosis induced by a variety of stimuli, in part via direct inhibition of active caspases. (8, 9). In apoptotic thymocytes, Ub-ligase activity of cIAP1 results in self-ubiquitination and degradation of IAPs by the proteasome (10). In addition, Ub-ligase activity of IAPs could provide an additional route for caspase inhibition via ubiquitination and targeting for subsequent proteasomal degradation (11). Assuming that the proteasome, cIAP1, caspase 3, and USP7 are all linked in the same
apoptotic pathway during dexamethasone-induced thymocyte cell death, our results suggest that 1) proteasome activation and proteasomal-mediated degradation of cIAP1 is upstream of caspase 3 activation; 2) caspase 3 activation is upstream of USP7 processing. The first conclusion is consistent with a recent report that investigates the effect of proteasomal inhibition on mitochondrial membrane potential in thymocytes and provides evidence that the proteasome acts at a pre-mitochondrion and pre-caspase stage of apoptosis (12).

We propose two mutually exclusive pathways that link the proteasome, IAPs, caspase 3, and USP7 in the thymocyte death pathway (Fig. 1). If caspase 3-mediated USP7 processing disables USP7-mediated deubiquitination of IAP proteins, USP7 serves as an amplifier in the apoptotic pathway (Fig. 1A). Alternatively, caspase 3 activation may lead to enhancement of USP7 ability to promote IAP deubiquitination, sparing IAPs from proteasome-mediated degradation and providing negative feedback control (Fig. 1B). In this case USP7 is a feedback sensor. The existence of a negative feedback loop may explain the fact that proteasome inhibitors delay rather than block thymocyte apoptosis. If the proteasomal activity is decreased below some minimal "healthy" level, USP7 activity is relatively low, IAP ubiquitination is not counter-balanced, caspase 3 inhibition by IAPs is relieved, all of which ultimately lead to cell death. Our preliminary coimmunoprecipitation experiments with the HA-tagged the "wild type" USP7 (HA-USP7) and a USP7 deletion mutant lacking the peptidase domain (HA-USP7.1-196) suggest that "wild type" USP7 protein may have a folding conformation that precludes interactions with some interacting partners. Caspase 3-dependent USP7 processing may therefore promote USP7 interaction(s) with a new binding partner(s), favoring the feedback IAP-dependent pathway for USP7 action.

As discussed in Chapters IV and V, the proteasomal targets that are crucial for the execution of the apoptotic program remain to be identified. IAP family members are just the tip of the iceberg of possible candidates. One such possibility is IκB, responsible for retention of a transcription factor NFκB in the cytosol (13). Several reports suggest that NF-κB may be important for the survival of immature thymocytes (13-15). USP7 processing may either disable or enable interactions between IκB and USP7, leading to another possible feedback (Fig. 1D) or amplification mechanisms (Fig. 1C), respectively. Interestingly, NFκB and IAP signaling could be interconnected, since the function of some IAP members is under NFκB control (16, 17).

USP7 may act on multiple (as yet unidentified) substrates during execution of an apoptotic program. Furthermore, USP7-substrate interaction(s) may be indirect and rely on adapter molecules. As discussed in Chapter V, USP7 contains a "non-classical" TNF-Receptor-Associated Factors (TRAF) domain (18). TRAF family members could interact with other TRAF-containing molecules as well as with a variety of other proteins, such as protein kinases and Ub-ligases (19). Thus, the TRAF domain of USP7 may mediate interaction with various adaptor proteins during apoptosis. An intriguing candidate for such an adaptor protein is RIP2, a Receptor Interacting Protein family member. These adapter molecules have Ser/Thr kinase activity and are involved in variety of signaling pathways, including NFκB activation and cell death, in part attributable to specific interactions with members of the tumor necrosis receptor (TNFR) family (20). RIP2 was shown to interact with cIAP1 and TRAF family members (20). Overexpression of RIP2
signaled both NFκB activation and cell death. Thus, RIP2 provides a route to regulate the interactions of USP7 partners.

Finally, processing of USP7 is likely to release the N-terminal fragment, which contains a polyglutamine stretch (21). Proapoptotic effects of polyglutamine-containing polypeptides are the subject of intense research, in part due to the implications in Huntington's disease (22, 23). A hallmark of this condition is the proteolytic production of N-terminal fragments of huntingtin protein. Huntingtin contains polyglutamine repeats that can form ubiquitinated aggregates in the nucleus and cytoplasm of the affected neurons. Caspases appear to directly cleave huntingtin and generate the toxic polyglutamine-containing fragments. Similarly, the thymocyte apoptotic program may be modulated by caspase-mediated release of the polyglutamine fragment of USP7.

In summary, USP7 may be either a positive or negative regulator in the cell death program and its effector function may rely on multiple interactions with a diverse set of proteins. Further experiments will be required to clarify the position of USP7 in the apoptotic cascade.

**Complexity and compensatory mechanisms in T cell development**

T cell development is a complex process, governed by multiple inputs from intricate signaling cascades. The MHC interactions with the TCR is thought to provide the specificity in signaling, while other signals, such as interaction between costimulatory molecules and endogenous glucocorticoids, modulate and fine tune the signaling response. It is the relative magnitude of multiple signals that determines the quality of response and, ultimately, thymocyte fate or mature T cell effector function. While part of the signaling cascade might be initiated by MHC-TCR interactions alone (Chapter III), multiple interactions are required for proper thymocyte development. The complexity of the system also ensures the existence of compensatory mechanisms at most nodes of the T cell signaling pathway. For example, a single T cell could bind and sample multiple distinct MHC-peptide complexes. The signals initiated by different MHC-peptide complexes could either accentuate or antagonize each other (Chapter III). While interaction with a particular set of these complexes might not be sufficient for rescue from death by neglect, the thymic microenvironment presents diverse sampling opportunities. The absence of classical MHC class I does not completely abolish the development of CD8+ T cells, as non-classical MHC class I molecules apparently select some CD8+ T cells (Chapter II). Likewise, glucocorticoid receptor (GR) knock-in mice that are deficient in glucocorticoid-induced gene transcription have grossly normal thymocytes even though glucocorticoid-dependent thymocyte apoptosis is impaired (24). The caspases are thought of as proteolytic effectors in T cell apoptosis. However, studies of various caspase knockout mice show that, for any given caspase, defects in apoptosis may be rather subtle and cell type- and stimulus-dependent (25). Another proteolytic apoptotic machinery is the proteasome-ubiquitin pathway (Chapters IV and V). Proteasome inhibition by proteasome inhibitors results in a delay rather than a block of thymocyte apoptosis, which might partially be explained by existence of the proteasome-independent signaling pathway (Chapter IV). Other members of the proteasome-ubiquitin pathway, such as ubiquitin ligases and deubiquitinating enzymes (DUBs) are likely to be responsible for the regulation of the proteasome function in apoptosis.
(Chapter V). While specific substrates and roles of these enzymes remain to be discovered, recent evidence points to a significant functional redundancy. For example, none of the DUB single, double, and triple deletion mutants in yeast has any detectable phenotype (26). Finally, the caspase and the proteasome proteolytic pathways are intertwined in thymocyte apoptosis, in part via Ub-ligases and DUB components of the proteasome-ubiquitin pathway (discussed in Chapter V).

Future directions to study USP7 function in thymocyte apoptosis

The T cell compensatory mechanisms and functional redundancy obviously complicate the dissection of the apoptotic signaling cascade. For example, future studies of USP7 function obviously include the generation of the USP7 deficient mice. However, based on functional redundancy of DUB family members, USP7 knockout animals are unlikely to have detectable defects in thymocyte development. The differences between wild type and USP7/-/- T cells are likely to be rather subtle, such as changes in kinetics of apoptosis or lack of some morphological changes associated with apoptosis in a stimulus-specific context. An identification of USP7 substrates in either a two-hybrid screen or in co-immunoprecipitation experiments might reveal additional information on USP function. In fact, initial co-immunoprecipitation experiments have identified several potential USP7 binding partners for further purification and mass-spectroscopy analysis (Chapter V). In addition, identification of the USP7 processing site and/or cleavage mediator might be important for understanding the role of USP7 in thymocyte apoptosis and for establishing a link between USP7 processing and the caspase cascade. Since USP7 processing has only been detected in primary thymocytes, purification of a processed form of USP7 from apoptotic thymocytes with subsequent radioactive sequencing is an obvious approach to identify the cleavage site. To this end, we have shown that both forms of USP7 (processed and unprocessed one) could be pulled down with streptavidin-coated agarose beads when the thymocyte lysates are labeled with the DUB-specific active site biotinylated probe, biotin-UbVS (Fig. 2).

Finally, the study of the compensatory mechanisms themselves is quite intriguing and complicated, due to a "chicken and egg" problem. Is the real role of non-classical MHCs to select extrathymic T cells and thymocytes that are destined to "non-classical" immune response sites, such as intestine, or merely to compensate for deficiency in the classical MHCs? The easiest model to study the role of nonclassical MHCs (class Ia) is MHC class Ia knockout mice, where dominant classical MHC responses are absent. However, the results obtained from the class Ia deficient mice would describe how MHC class Ia behaves in the absence of class Ia, which is not necessarily the case in its presence. Similarly, the characterization of USP7 knockout mice might only reveal its exclusive role but not the details of its "day-to-day routine". But we'll take whatever we can learn, won't we?
References:


include CLAP1, NF-kB, and UBD (C and D). Since caspase 3 does not cleave LSP7 directly, a cleavage mediator, "<b>c</b>" is proposed.

significant molecule, leading to either decrease or increase in the LUB-dependent proteasome degradation of this molecule. Possible LUB-LUB interaction and a feedback loop is a feedback sensor in apoptosis. Caspase 3-mediated LUB processing can either enable or disable interactions between LUB and a possible links between the proteasome, caspase 3, and LUB in the lytic apoptotic cascade. (a) and (c) LUB and an amplifier of apoptosis.
Figure 2. Purification of USP7 from murine thymocytes. Thymocytes from C57BL/6 mice (5 x 10^7 cells) induced to undergo apoptosis with 1 M dexamethasone for 6 hours (lanes 2, 4, 6, and 8) or their controls (lanes 1, 3, 5, and 7), were lysed in 100 L of NP-40 lysis buffer as described in Chapter V. Lysates were precleared three times with 40 L of streptavidin-coated agarose beads and labeled with 3 M biotin-UbVS using a protocol for UbVS labeling described in Chapter V. Biotin-conjugated deubiquitinating enzymes were precipitated (IP) with 40 L of streptavidin-coated agarose beads and both pellet (lanes 1-2) and supernatant (lanes 5-6) proteins were loaded on the 10% SDS-PAGE. To test the efficiency of this pull-down procedure, biotin-conjugated deubiquitinating enzymes were re-precipitated (re-IP) from the supernatant of the first pull-down with 40 L of streptavidin-coated agarose beads. Pellet (lanes 3-4) and supernatant (lanes 7-8) material from this re-precipitation were also loaded on the same SDS-PAGE. Resolved polypeptides were transferred onto a PVDF membrane, the filters were incubated with the HRP-coupled streptavidin (1:5000) and developed by chemoluminescence. The processed and unprocessed forms of USP7 are indicated by arrows.
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