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Proteolysis and Antigen Presentation by MHC Class II Molecules

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I. Introduction

Proteolysis is the primary mechanism used by all cells not only to dispose of unwanted proteins but also to regulate protein function and maintain cellular homeostasis. Proteases that reside in the endocytic pathway are the principal actors of terminal protein degradation. Consequently, the number of endocytic proteases must be immense, with wide tissue distributions coupled to broad and overlapping enzymatic specificity. As inferred from their intracellular location, these enzymes usually function at low pH (Chapman *et al.*, 1997).

The assignment of specific functions to the individual endocytic proteases was once thought unrealistic due to their apparent redundancy in higher organisms. However, recent technological advances, such as the generation of knockout mice and active site-directed probes, have led to a far better characterization of intracellular proteases with more unique features (Chapman *et al.*, 1997; McGrath, 1999; Turk *et al.*, 2000). Indeed, proteases are detected all along the endosomal/lysosomal route, and, accordingly, the optimal pH range for some is rather broad. In addition, the expression of certain endocytic proteases is regulated either at the level of gene transcription or enzyme maturation, and their activity is controlled by the presence of endogenous protease inhibitors. Some endocytic proteases exhibit tissue-specific expression and enzymes with distinct, rather than broad, substrate specificities have been identified. Hence, some of the "housekeeping" proteases are now recognized as having precise biological functions rather than merely carrying out bulk proteolysis (Chapman *et al.*, 1997; McGrath, 1999; Turk *et al.*, 2000).

The presentation of peptide antigens by MHC class II molecules is strictly dependent on the action of proteases (Nakagawa and Rudensky, 1999; Riese and Chapman, 2000; Villadangos and Ploegh, 2000; Watts, 2001). Class II molecules scour the endocytic pathway for antigenic peptides to bind and present at the cell surface for recognition by $CD4^+$ T cells (Watts, 1997; Wolf and Ploegh, 1995). The specialized cell types that support antigen presentation by class II molecules are commonly referred to as professional antigen–presenting cells (APCs), which include bone marrow-derived B lymphocytes,

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dendritic cells (DCs), and macrophages. In addition, class II molecules are expressed in "nonprofessional" APCs either constitutively (i.e., thymic cortical and medullary epithelial cells) or upon induction with interferon-γ (IFN-γ) (i.e., endothelial cells, intestinal epithelial cells, mast cells, and fibroblasts). Before MHC class II molecules meet their intended T-cell receptor (TCR) at the cell surface, a complex series of biosynthetic and proteolytic events ensures correct assembly, trafficking, and peptide loading of the class II molecule (Watts, 1997; Wolf and Ploegh, 1995). Accordingly, the endocytic proteases required for the function of class II molecules are found in the distinct APC types and, in some cases, are themselves IFN-γ inducible (Chapman, 1998).

MHC class II products consist of an α chain and a β chain, which dimerize shortly after synthesis in the endoplasmic reticulum (ER) to create a cleft in which antigenic peptides are captured (Watts, 1997; Wolf and Ploegh, 1995) (Fig. 1A). Newly synthesized $\alpha\beta$-dimers assemble onto a scaffold of a homotrimeric, type II membrane protein, the invariant chain (Ii). There are two isoforms of Ii in mice, p31 and p41, distinguished by alternative splicing of Ii transcripts (Koch, 1988; O'Sullivan *et al.*, 1987; Strubin *et al.*, 1986). The resulting p41 isoform contains an extra exon (exon 6b), C-terminal to Ii's trimerization domain. As we shall see below, the additional domain of p41 plays a key role in the control of protease expression and activity in APCs. In humans, these two splice variants each yield two protein products, resulting in four Ii isoforms: p31(p33), p33(p35), p41, and p43 (Cresswell, 1996). Ii prevents premature association of the class II heterodimer with peptides or unfolded segments of polypeptide chains in the ER by means of a short segment of its lumenal region, designated CLIP (Class II-associated Ii derived Peptides), which fills the peptide-binding groove of class II (Ghosh *et al.*, 1995). Interaction with Ii also facilitates folding of the $\alpha\beta$-heterodimer. The cytoplasmic tail of Ii contains targeting signals that deliver the majority of $(\alpha\beta\text{–Ii})_3$ complexes from the trans-Golgi network (TGN) directly into the endocytic pathway (Bakke and Dobberstein, 1990; Benaroch *et al.*, 1995; Lotteau *et al.*, 1990). Only 5–10% of newly synthesized class II–Ii complexes fail to be sorted and are instead delivered to the plasma membrane, followed by rapid internalization into endocytic vesicles (Castellino and Germain, 1995; Pinet and Long, 1998; Roche *et al.*, 1993; Zhong *et al.*, 1997). Thus, all elements of the endocytic pathway receive $\alpha\beta$–Ii. Likewise, the same endocytic compartments that contain class II–Ii complexes at different stages of maturation compose much of the pathway traveled by internalized antigens (Watts, 1997). Antigens that enter the endocytic pathway are processed into class II-presentable peptides by resident proteases (Chapman, 1998; Harding, 1996) (Fig. 1A).

Successful MHC class II-restricted antigen presentation not only depends on the availability of processed peptides, but also on the rate of Ii breakdown and removal. Ii must be proteolytically destroyed to free the class II peptide-

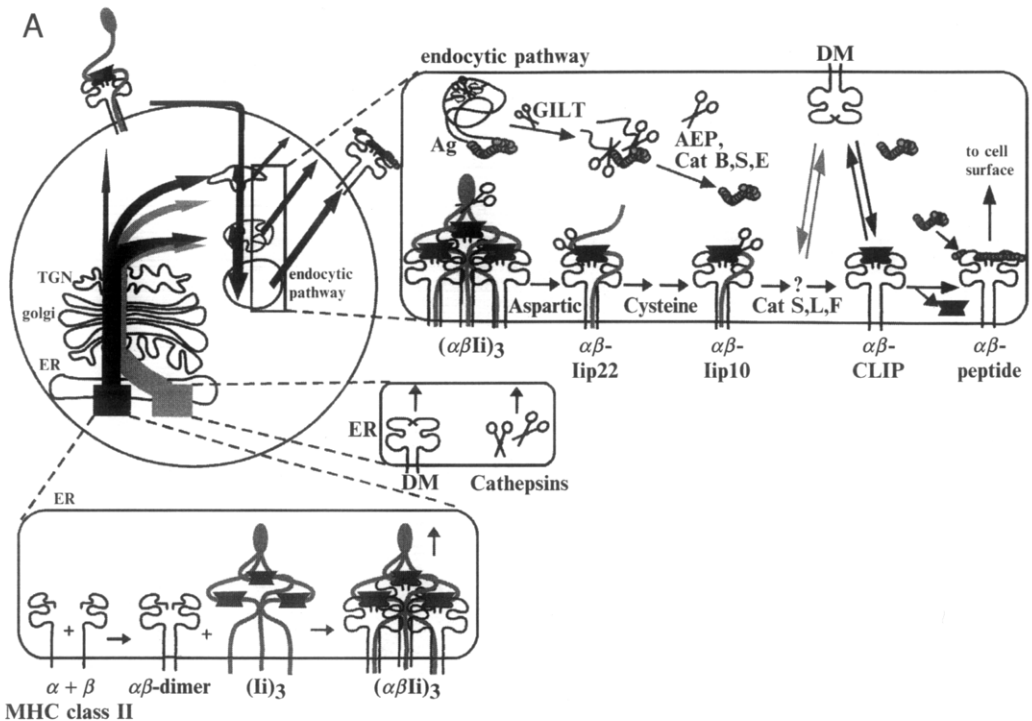
binding groove for loading of antigenic peptides. Upon arrival of αβ–Ii complexes in endocytic compartments, Ii is degraded in a stage-specific manner by a combination of aspartyl and cysteine proteases (Amigorena *et al.*, 1995; Maric *et al.*, 1994; Villadangos *et al.*, 1997) (Figs. 1A and 1B). As class II molecules themselves are poor substrates for these proteases, the peptide-binding cleft affords protection against proteolysis of its contents. To complete peptide binding, most class II alleles require interaction with the nonclassical class II dimer, H-2M/HLA-DM (referred to hereafter as DM). DM induces release of the Ii remnant CLIP and facilitates loading with antigenic peptides (Alfonso and Karlsson, 2000; Busch *et al.*, 2000; Kropshofer *et al.*, 1999). Those peptides that can no longer be dislodged by DM generate the pool of peptide-loaded class II molecules at the cell surface (Fig. 1A). Protease attack of Ii yields class II–Ii intermediates of distinct conformations required for proper trafficking, interaction with the accessory molecule DM, and acquisition of peptides (Fig. 1B). Ii proteolysis is therefore not "haphazard" but instead follows a well-defined path. The constancy of the Ii intermediates produced by proteolysis has facilitated the identification of specific proteases capable of performing the necessary cleavages to yield an αβ-dimer free to bind peptides. Cathepsins S (Nakagawa *et al.*, 1999; Riese *et al.*, 1996; Shi *et al.*, 1999), L (Nakagawa *et al.*, 1998), and F (Shi *et al.*, 2000) were all shown capable of performing a critical cleavage late in the staged destruction of Ii (see Section III). On the other hand, assigning specific functions to individual proteases in the generation of class II-restricted epitopes has been more challenging (see Section VI). Rather than relying on the function of a single protease, the generation of peptides from a given antigen may require the combination of several proteases with distinct and overlapping specificities (Villadangos and Ploegh, 2000). Nonetheless, it is likely that the processing of both antigens and Ii are executed by similar, if not the same, endocytic proteases.

Thus, the antigen presentation pathway of class II molecules has evolved to utilize the cell's proteolytic "housekeeping" machinery for its own success. Here we review those proteases required for Ii breakdown and removal from the class II αβ-dimer and those likely to be important in processing antigens into class II-presentable peptides.

II. Protease Activity in Antigen-Presenting Cells

A. Endocytic Proteases

The proteases contained in the endocytic pathway are classified into four major groups based on the active-site amino acid used by the enzyme to hydrolyze amide bonds of proteins: cysteine, aspartyl, serine, and metalloproteases. More than 16 defined intracellular acidic proteases—referred to as the cathepsins—span these four groups. By virtue of their "profession," APCs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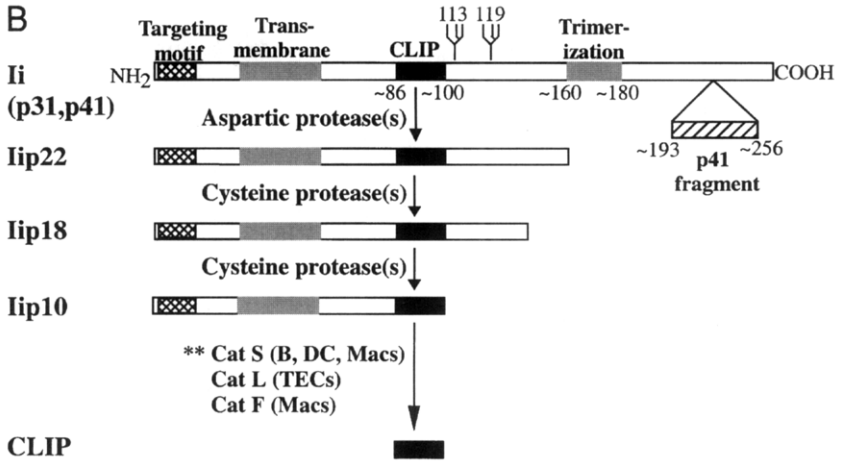


FIG. 1(A). The generation of peptide-loaded MHC class II molecules in the endocytic route. MHC class II $\alpha\beta$-dimers are synthesized in the endoplasmic reticulum (ER) where they quickly associate with homotrimers of Ii to form nonameric complexes $(\alpha\beta\text{–Ii})_3$. The CLIP region of Ii lies within the peptide-binding groove of the $\alpha\beta$-dimer. The nonameric complexes exit the ER and traverse the Golgi stacks. Once in the TGN, the majority of $(\alpha\beta\text{–Ii})_3$ complexes are delivered directly to compartments in the endocytic pathway by sorting signals in the cytoplasmic tail of Ii. A few of these complexes escape to the cell where they are then quickly internalized. In the endocytic pathway, Ii is removed in a stepwise fashion by a combination of aspartic and cysteine proteases until the CLIP region of Ii is all that remains in the class II peptide-binding groove ($\alpha\beta$–CLIP). The accessory molecule, DM, interacts with $\alpha\beta$–CLIP (black arrows)—or some intermediate between $\alpha\beta$–Iip10 and $\alpha\beta$–CLIP (gray arrows)—inducing the release of these Ii remnants. The continued interaction of DM with the now empty class II molecule preserves its structure and facilitates loading of antigenic peptides. The peptides loaded onto class II are generated from endocytosed antigens. These antigens must first be partially unfolded, possibly by the action of the thiol reductase GILT, to render the protein accessible to proteases. To date, asparaginyl endopeptidase (AEP), Cat B, Cat S, and Cat E are the only proteases shown to be important *in vivo* for the generation of some class II presentable peptides. The resulting $\alpha\beta$–peptide complexes are then deposited on the cell surface for recognition by CD4+ T cells. (B) Schematic view of Ii and its breakdown intermediates. Both the p31 and p41 isoforms of Ii are borken down in an indistinguishable manner. The nonameric class II–Ii complex $(\alpha\beta\text{–Ii})_3$ is disrupted by COOH-terminal degradation of Ii by aspartyl proteases to yield $\alpha\beta$-dimers attached to an Ii degradation intermediate of 22 residues (Iip22). All subsequent COOH-terminal processing is performed by cysteine proteases, yielding the $\alpha\beta$-associated intermediates Iip18 and Iip10. The specific proteases absolutely required for these processing steps are not known. An NH_2-terminal cleavage converts Iip10 into CLIP. This cleavage is performed by Cat S in B cells, DCs, and macrophages and by Cat L in cTECs. Cat F can digest Iip10 into CLIP *in vitro* and may function as a "backup" enzyme in macrophages that lack Cat S.

harbor many of these enzymes. Cathepsins B, C, D, E, F, H, K (Punturieri *et al.*, 2000), L, O, S, V, and Z (X) have all been identified in various professional APC types, and this list is certainly not complete (Riese and Chapman, 2000; Turk *et al.*, 2000; Villadangos and Ploegh, 2000). Of the enzymes identified in professional APCs, cathepsins D and E are aspartyl proteases, while the remaining cathepsins (Cat) are cysteine proteases and belong to the papain family of enzymes (McGrath, 1999). Based on chromosomal location and sequence homology, these cysteine proteases are subdivided into Cat B-like and Cat L-like subgroups (Cygler and Mort, 1997; McGrath, 1999). This classification, however, does not appear to be an accurate means of predicting which cathepsins could play a role in Ii breakdown and/or antigen processing. For instance, cathepsins S, L, and F were all shown to be capable of performing similar Ii cleavages (see below). Although both Cat S and Cat L belong to the same L-like subgroup, Cat F is distinct (Wex *et al.*, 2000). In addition to the cathepsins, a member of the legumain family of cysteine proteases, asparaginyl endopeptidase (AEP), is also present in APCs (i.e., B cells) (Manoury *et al.*, 1998) and may well be one of the more specific enzymes in terms of preferred cleavage sites. The most abundant cysteine and aspartyl proteases expressed in APCs are cathepsins B and D, respectively (Chapman *et al.*, 1997; McGrath, 1999).

The structures of cathepsins B (Musil *et al.*, 1991), L (Fujishima *et al.*, 1997; Guncar *et al.*, 1999), K (McGrath *et al.*, 1997; Zhao *et al.*, 1997), H (Guncar *et al.*, 1998), and S (McGrath *et al.*, 1998) have been determined, as well as the structures of the procathepsins B (Podobnik *et al.*, 1997), L (Coulombe *et al.*, 1996), and K (McGrath, 1999). The mature cathepsins range between 20 and 30 kDa (the proenzymes are 60–100 amino acids larger) and are composed of two equivalent sized domains, stabilized by the presence of disulfide bonds. The two domains are separated by the catalytic center that contains the active site nucleophile used to cleave bound substrates. The specificity of an enzyme is determined by the architecture of its active site. Cathepsins K, L, S, and F are endopeptidases: they hydrolyze internal amide bonds of proteins (Chapman *et al.*, 1997; McGrath, 1999; Turk *et al.*, 1997). Catalysis by endoproteases relies on a triad composed of a cysteine, a histidine, and an asparagine residue. The histidine and asparagine residues polarize the cysteine residue to generate the nucleophile that attacks the carbonyl carbon of the targeted amide bond of the substrate (Chapman *et al.*, 1997; McGrath, 1999; Turk *et al.*, 1997). Enzymatic activity is associated with an open active site approximately 15 Å in length to which substrates bind (McGrath, 1999; Turk *et al.*, 1997; Turk *et al.*, 1998). In contrast, the amino-exopeptidase activities of Cat H and Cat C, and the carboxyl-exopeptidase activities of Cat B and Cat Z, are generated by obstructing part of the active site with side chains that stabilize either the N terminus or the C terminus of the bound substrate (McGrath, 1999; Turk *et al.*, 2000). The result is hydrolysis of one or two amino acids from either peptide termini. At lower pH,

the occluding loop of Cat B is flexible, allowing this enzyme to also function as an endopeptidase (Illy *et al.*, 1997; Nagler *et al.*, 1997). A limited endoproteolytic activity has also been described for Cat H (Illy *et al.*, 1997; McGrath, 1999). However, the major endoproteases present in the endocytic pathway are the aspartic protease Cat D, and the cysteine proteases Cat S and Cat L. With the exception of AEP, the lysosomal proteases show a rather relaxed cleavage specificity, which makes the prediction of cleavage sites in antigens difficult.

B. Biosynthesis of the Active Enzyme

Lysosomal proteases are synthesized in the ER as inactive zymogens or proenzymes (McGrath, 1999; Turk *et al.*, 2000). The propiece can vary in size and is located at the N terminus of the mature enzyme. The role of the propiece in the maturation of the precursor enzyme into an active protease resembles that of the chaperone Ii in MHC class II maturation. In the ER, the inactive protease requires its propiece for efficient folding, just as the assembly and folding of the class II $\alpha\beta$-dimer are facilitated by Ii (Tao *et al.*, 1994; Vernet *et al.*, 1995). Furthermore just as the CLIP region of Ii lies in the peptide-binding cleft of the class II $\alpha\beta$-dimer, the propeptide, if of appropriate length, occupies the active site of the enzyme. Occupation of the active site prevents premature activation of the enzyme as it travels through the Golgi on its way to the endocytic pathway (Coulombe *et al.*, 1996; LaLonde *et al.*, 1999; Turk *et al.*, 1996b).

The enzymatic functions of the protease and the peptide-binding function of the class II $\alpha\beta$-dimer require that the active site of each be liberated of its occupants (the propiece and CLIP). Both of these events occur in the endocytic pathway. The cathepsins exit the ER and travel through the Golgi, where they acquire post-translational modifications including glycosylation and phosphorylation, each influenced by the presence of the propiece (Gieselmann *et al.*, 1983; Lingeman *et al.*, 1998). These modifications are required for proper sorting of proteases in the TGN. Phosphomannosyl residues of the protease bind to two types of mannose 6-phosphate receptors that shuttle between the Golgi and endocytic compartments (Kasper *et al.*, 1996; Koster *et al.*, 1993; Pohlmann *et al.*, 1995). Once deposited in an endocytic compartment, the propiece is removed spontaneously by an autocatalytic mechanism, or by the action of another protease, to yield the mature, active form of the enzyme (one chain or two chain depending on the enzyme; LaLonde *et al.*, 1999; Riese and Chapman, 2000). Thus, both the cathepsins and MHC class II molecules are rendered functional in the endocytic pathway through proteolytic events.

C. Regulation of Protease Activity

1. Intracellular pH

The inhibition of class II peptide loading with agents that raise the pH of acidic intracellular organelles was the first evidence that successful class

II-restricted antigen presentation relies on the function of endocytic proteases (Neefjes *et al.*, 1990; Wolf and Ploegh, 1995). As mentioned, the propiece of a lysosomal protease must be removed to activate the enzyme. Whether or not cleavage of the propeptide occurs by autocatalysis or by the action of another protease, an acidic pH is necessary. It is thought that the protonation of groups adjacent to the propeptide at low pH may increase the susceptibility of the propeptide to cleavage. Alternatively, the tertiary structure of the proenzyme may change with decreasing pH, rendering the propeptide vulnerable to cleavage (Riese and Chapman, 2000).

The pH in each vacuolar organelle of eukaryotes, which include endosomes, lysosomes, and the TGN, is maintained by ATP-dependent proton pumps known as vacuolar H^+-ATPases (Nelson, 1992). Neutralization of these compartments with acidotropic drugs, such as chloroquine and ammonium chloride (Thorens and Vassalli, 1986), and carboxylic ionophores such as monensin, nigericin, and X537A (Tartakoff, 1983), prevents lysosomal protease function and, in turn, class II-restricted antigen presentation. Selective inhibitors of the vacuolar H^+-ATPase include macrolide antibiotics such as bafilomycin A_1 and concanamycin B (Bowman *et al.*, 1988; Woo *et al.*, 1992; Yoshimori *et al.*, 1991). Inactivation of the vacuolar H^+-ATPase of mammalian cells with these agents affects not only pH but also the formation of endosomal carrier vesicles as well as sorting events in the TGN (Benaroch *et al.*, 1995; Mellman *et al.*, 1986). Hence, an acidic pH and/or protease function in the endocytic pathway is important for the intracellular transport of proteins, including their sorting and recycling in endosomes.

Although an acidic pH is required to remove the propiece and thereby activate the enzyme, cathepsin S is unique in that it maintains its enzymatic function at neutral pH (Bromme *et al.*, 1989; Shi *et al.*, 1992). This feature of Cat S befits its critical role in Ii processing (discussed below), a prerequisite for loading of class II molecules with antigenic peptides. Class II–Ii complexes and Cat S are present in a variety of endocytic structures, from early endosomes of relatively high pH to the acidic lysosomes (Driessen *et al.*, 1999). Earlier experiments conducted largely in B cells placed considerable emphasis on late endosomal compartments for proteolysis of Ii and maturation of class II molecules, but it is now clear that early endosomes are an essential part of the class II antigen presentation pathway (Brachet *et al.*, 1999; Pond and Watts, 1999). Hence, the pH flexibility of Cat S, in principle, enables class II peptide loading in a variety of endocytic compartments.

2. *Cytokines and Transcriptional Control*

The expression of MHC class II molecules and key accessory components of the class II antigen presentation pathway are induced by IFN-γ. Accordingly, IFN-γ regulates the expression of many of the cathepsins with critical roles in

antigen presentation, such as cathepsins S, L, and F (Chapman, 1998; Shi *et al.*, 2000). The expression of Cat B (Li and Bever, 1998), Cat H (Lafuse *et al.*, 1995), and Cat W (Chapman, 1998) is also induced by IFN-γ. The cytokine interleukin-6 (IL-6) was shown to raise the pH of early endosomes, which could influence the pattern of Ii processing in this compartment (Fuchs *et al.*, 1989), although this was not tested. Moreover, IL-6 treatment of DCs appeared to influence the types of epitopes derived from hen egg lysosome (HEL) that were presented by class II molecules (Drakesmith *et al.*, 1998). Although the mechanism by which IL-6 influences antigen presentation is not known, its alteration of endosomal pH could affect the types of peptides generated from intact antigens and/or could influence the peptide-editing functions of the accessory molecule DM.

The development of immature DCs, incapable of antigen presentation, into APCs that can support antigen presentation by class II molecules, requires the action of proinflammatory cytokines. In turn, the same cytokines that induce DC maturation also regulate the activity of proteases that participate in Ii and/or antigen processing. The proinflammatory cytokines tumor necrosis factor α (TNF-α) and IL-1β rapidly increase the activity of Cat S and Cat B in human DCs and promote class II peptide loading and presentation on the cell surface (Fiebiger *et al.*, 2001). In contrast, the antiinflammatory cytokine IL-10 attenuates the levels of both Cat B and Cat S in DCs (Fiebiger *et al.*, 2001). Like IL-6, IL-10 seems to inhibit protease activities in DCs by elevating the pH of the endocytic pathway (Fiebiger *et al.*, 2001). An earlier study demonstrated that peptide loading of the murine class II molecule I-A^k in immature DCs could be induced upon exposure to TNF-α (Inaba *et al.*, 2000). Hence, the balance of pro- and antiinflammatory cytokines in DCs, and consequently the cellular proteolytic activities, strictly correlates with antigen presentation.

Certain cathepsins exhibit tissue-specific expression that favors their participation in antigen presentation. Active Cat S is present predominantly in bone marrow-derived APCs (B cells, DCs, and macrophages). Accordingly, Cat S is the key enzyme required during late stages of Ii proteolysis in these APC types (Nakagawa *et al.*, 1999; Shi *et al.*, 1999). In contrast, active Cat L is not found in naive B cells or DCs, but rather in macrophages and cortical thymic epithelial cells (Shi *et al.*, 2000). Cat L plays a central role in Ii degradation in the thymus necessary for class II-mediated selection of maturing T cells at this location (Nakagawa *et al.*, 1998). Additionally, the cysteine protease Cat F, capable of performing the same cleavages of Ii as Cat S *in vitro*, is preferentially active in IFN-γ-induced alveolar macrophages (Shi *et al.*, 2000). The expression levels of cathepsins in macrophages can be regulated at the level of transcription and/or of enzyme maturation. Activators of macrophages can either increase or decrease intracellular levels of cathepsins, as well as induce cathepsin secretion into the extracellular space, thereby regulating the intracellular and extracellular proteolytic potential of these APCs (Liuzzo *et al.*, 1999; Wang *et al.*,

2000). Regulation of proteolysis in macrophages or DCs not only is relevant for antigen presentation but also affects the microenvironment of these APCs. Indeed, the release of cathepsins by macrophages can serve to remodel the extracellular matrix and guide cell motility.

3. *Endogenous Inhibitors*

There are three types of endogenous cysteine protease inhibitors present in APCs that bind tightly and reversibly to the enzyme's active site: the enzyme's propiece, the cystatin superfamily of inhibitors, and a fragment of the p41 (exon 6b) isoform of Ii (discussed in Section VI; Chapman *et al.*, 1997). Both the propeptides and the cystatins bind the enzyme's active site in reverse orientation to that of natural substrates (Stubbs *et al.*, 1990). This prevents their digestion by the enzyme while occupying its active site. The propiece appears to function as an inhibitor only until the enzyme reaches an acidic compartment, upon which the propiece self-dissociates and is cleaved. There is no evidence that the released propieces reside in the endocytic pathway to regulate the activity of resident proteases.

The cystatins are perhaps the most studied endogenous cysteine protease inhibitors (Turk and Bode, 1991). They play an essential role in protecting cells and tissues from inappropriate proteolysis by enzymes that are overexpressed or escape the endocytic pathway (Chapman *et al.*, 1997; Turk *et al.*, 1997). Appropriately, the cystatins are expressed throughout the body in a tissue-specific manner and greatly outnumber cysteine proteases in the cytoplasm and extracellular spaces (Chapman *et al.*, 1997). Mutations in some cystatins or alterations in the balance of these with their cognate cysteine proteases have been implicated in several diseases (Huh *et al.*, 1999). The cystatins also inactivate cysteine proteases made by pathogens and thereby may serve as a defense mechanism against infection (Collins and Grubb, 1998; Korant *et al.*, 1998; Stoka *et al.*, 1995). On the other hand, cystatin-like protease inhibitors made by pathogens can also modulate the protease content and the processing of antigens in APCs (Manoury *et al.*, 2001). The cystatin superfamily of cysteine protease inhibitors includes the stefin subfamily (class I = cystatins A and B), the cystatin subfamily (class II = cystatins C, S, and D), and the kininogen subfamily (class III) (Brown and Dziegielewska, 1997). Two new class II cystatins have been defined, cystatin E(M) and cystatin F (leukocystatin), whose expression is restricted to hematopoietic cells (Halfon *et al.*, 1998; Sotiropoulou *et al.*, 1997). Although all of the cystatins mentioned target the cysteine proteases cathepsins B, F, K, L, S, and H, cystatin C has been shown to also inhibit the asparaginyl endopeptidase (Alvarez-Fernandez *et al.*, 1999). Intriguing is the unusually long propiece of Cat F. In addition to the normal Cat L-like propiece domain, Cat F is unique in that its propiece has an additional amino-terminal domain that resembles cystatin (Nagler *et al.*, 1999). Thus, the

cystatin-like domain of Cat F may not only serve to inhibit the zymogen form of the enzyme but also, upon its removal in the endocytic pathway, may function as an inhibitor of other cysteine proteases.

The mechanism by which cystatins inhibit cysteine proteases was revealed by determination of the three-dimensional structure of the complex of human cystatin B and papain (Stubbs *et al.*, 1990). Three highly conserved regions of these inhibitors—two rigid hairpin loops and the flexible N-terminal region of the molecule—form a wedge that binds to the active site of cysteine proteases (Turk *et al.*, 1997). For the majority of proteases exhibiting an "open" active site, the cystatins bind and inhibit the enzyme in a one-step reaction mechanism (Bjork and Ylinenjarvi, 1990; Lindahl *et al.*, 1992; Pol *et al.*, 1995; Turk *et al.*, 1994; Turk *et al.*, 1995a; Turk *et al.*, 1996a). A protease with a partially obstructed active site, such as Cat B or Cat H, is not as accessible to these endogenous inhibitors (Illy *et al.*, 1997; Quraishi *et al.*, 1999). In this case, the cystatins inhibit via a two-step reaction mechanism, consisting of an initial weak binding followed by a conformational change that displaces the occluding loop of the active site so that a tight complex forms between the inhibitor and the enzyme (Musil *et al.*, 1991; Nycander *et al.*, 1998; Pavlova *et al.*, 2000).

The recent surge of attention given to cysteine proteases by immunologists has led to the discovery that the cystatins, once thought to primarily function in the extracellular space, may also play a role in regulating the proteolytic environment of the endocytic pathway and thus, in turn, antigen presentation. Dendritic cells are perhaps the most potent APCs in the body. They are distinct in that their ability to present antigen via class II molecules is tightly coordinated with their own maturation (Thery and Amigorena, 2001). Although immature dendritic cells have the ability to take up and process antigens, the peptides generated fail to be presented in a stable fashion on the cell surface by class II molecules. Results of one study indicated that Ii degradation by Cat S is inhibited in immature DCs (from the H-2^b haplotype) by increased expression of cystatin C (Pierre and Mellman, 1998). Additional studies have shown a defect in the loading of I-A^k molecules with an HEL epitope in immature DCs, which is remedied upon induction of DC maturation with inflammatory mediators (Inaba *et al.*, 2000). It is unclear whether the peptide-loading defect seen for I-A^k in immature DCs is the result of aberrant processing of HEL caused by cystatin C inhibition of Cat S (Inaba *et al.*, 2000). It is likely that other mechanisms, besides Cat S inhibition by cystatin C, influence the trafficking of class II molecules in DCs. In Cat $S^{-/-}$, flt3-I-induced DCs (mature), $\alpha\beta$–Iip10 complexes accumulate in lysosomes, but eventually are deposited at the cell surface (Driessen *et al.*, 1999). This is in marked contrast to the fate of $\alpha\beta$–Iip10 complexes in immature DCs, in which the complexes are retained in lysosomes and then degraded, suggesting that other factors contribute to the retention of class II molecules in immature DCs (Villadangos and Ploegh,

2000). A study recently reported by Villadangos *et al.*, demonstrated that class II-peptide complexes are in fact generated in immature DCs and delivered to the cell surface. However, in contrast to mature DCs, the class II-peptide complexes deposited on the surface of immature DCs are rapidly endocytosed and then degraded. Thus, the rate of endocytosis—rather than Ii proteolysis as regulated by cystatin C—may dictate the antigen presentation capabilities of immature versus mature DCs (Villadangos *et al.*, 2001).

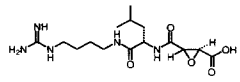
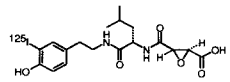
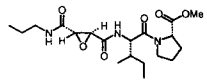
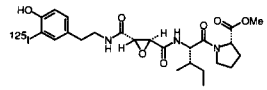
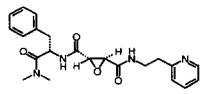
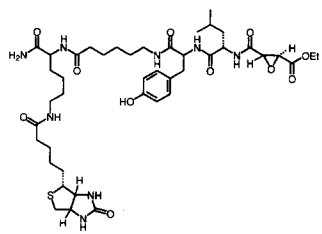
Finally, cathepsins likely regulate the activity of one another. Recently, Honey *et al.* (2001) demonstrated that in the absence of Cat S, the levels of active Cat L protein increased. Thus, Cat S could play a role in degrading mature Cat L. We have shown that competitive inhibitors can also exhibit a chaperone function *in vivo:* the p41 isoform of Ii protects a mature form of Cat L (see below). Whether this can be generalized to other protease-inhibitor complexes *in vivo* remains to be established.

D. Targeting the Enzyme: Small-Molecule Active Site-Directed Probes

Experiments in the past have relied on inhibitors of broad substrate specificity to demonstrate the involvement of cysteine proteases in antigen presentation or any other physiological process. These inhibitors include the synthetic compounds leupeptin, E64, and benzyloxycarbonyl–Phe–Ala–diazomethane (Z–Phe–Ala–CHN_2). Although these are potent inhibitors of cysteine proteases, they do not serve to distinguish between the active site similarities of closely related cathepsins, such as Cat L and Cat S. Much effort has been devoted to developing analogs of these inhibitors to visualize the enzymatic activity of a particular cathepsin in distinct cell types. The enzymatic mechanism utilized by cysteine proteases is conserved and well defined. This has enabled the development of a wide range of electrophilic substrate analogs that are reactive only with the conserved active site of cysteine proteases (Bogyo *et al.*, 2000; Greenbaum *et al.*, 2000). These inhibitors consist of a peptide specificity determinant attached to an electrophile that becomes irreversibly alkylated when bound in close proximity to an attacking nucleophile (Bogyo *et al.*, 2000). Such mechanism-based inhibitors include vinyl sulfones (Palmer *et al.*, 1995), epoxysuccinic derivatives (Barrett *et al.*, 1982), and dimethyl ketone derivatives (Pliura *et al.*, 1992; Shaw, 1994; Shaw *et al.*, 1986). By engineering these inhibitors to contain affinity labels, such as radioactive iodine or biotin, they can be used as probes with which to measure the protease activity in distinct tissues (Bogyo *et al.*, 2000; Greenbaum *et al.*, 2000) (summarized in Table I). Such active site-directed probes not only measure the function of known cathepsins but also serve to identify new active proteases in a given pathway.

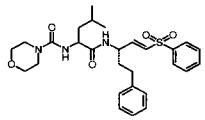
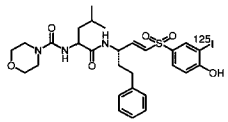
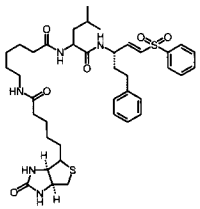
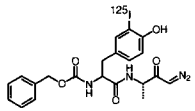
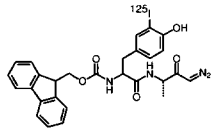
Affinity-labeled versions of the active site-directed inhibitors *N*-morpholinurea–leucine–homophenylalanine–vinyl sulfone–phenyl (LHVS) and JPM-

TABLE I
ACTIVE SITE-DIRECTED PROBES AND AFFINITY LABELS

Inhibitor	Specificity	Affinity label	Membrane permeable
A. Epoxysuccinates			
1. E-64			
	General	None	Yes
2. JPM-565			
	General	^{125}I	No
3. CA-074			
	Cat B	None	Yes/no
4. MB-074-OMe			
	Cat B	^{125}I	Yes
5. CLIK148			
	Cat L	None	Yes
6. DCG-04			
	General	^{125}I, biotin	No

(continues)

TABLE I *(continued)*

Inhibitor	Specificity	Affinity label	Membrane permeable
B. Vinyl sulfones			
7. LHVS 	Cat S (>>>>B>L)	None	Yes
8. LHVS-PhOH 	Cat S (>>>>B>L)	^{125}I	Yes
9. Biotin-LHVS 	Cat S (>>>>B>L)	Biotin	Yes
C. Diazomethyl ketones			
10. ZYA-CH_2N_2 	General	^{125}I	Yes
11. FmocYA-CH_2N_2 	General	^{125}I	Yes

Note: The authors thank Dr. H. S. Overkleeft for his assistance in preparing Table I.

565 (both iodinated and biotinylated) have been used successfully to obtain a profile of the cathepsins that are active in distinct APC types (Driessen *et al.*, 1999, No. 108; Shi *et al.*, 2000, No. 95; Lennon-Dumenil, submitted for publication). Both inhibitors covalently bind the active sites of cysteine proteases. The membrane-permeable peptide vinyl sulfone, LHVS, specifically inhibits Cat S at nanomolar concentrations and Cat B to a lesser extent (Palmer *et al.*, 1995). At higher concentrations, the specificity of LHVS extends to additional cysteine proteases, including Cat L (Felbor *et al.*, 2000; Lennon-Dumenil *et al.*, submitted for publication). JPM-565 is an analog of the natural product E-64, which itself is a peptide epoxide that inhibits most of the known lysosomal cysteine proteases by covalently modifying the active-site nucleophile (Barrett *et al.*, 1982). JPM-565 is not membrane permeable and exhibits the same general specificity for cysteine proteases as E-64 (Bogyo *et al.*, 2000; Greenbaum *et al.*, 2000). Active cysteine proteases present in cell lysates are covalently modified by the JPM-565 or LHVS affinity-labeled probes. The labeled enzymes can be separated by SDS–PAGE and visualized either by autoradiography (when labeled with ^{125}I-JPM-565 or ^{125}I-LHVS; Bogyo *et al.*, 2000; Driessen *et al.*, 1999; Shi *et al.*, 2000) or by blotting with streptavidin–horseradish peroxidase followed by chemiluminescense (when labeled with LHVS^{biotin} or JPM-565^{biotin}) (Greenbaum *et al.*, 2000; Lennon-Dumenil *et al.*, 2001; and P.W.B., unpublished data). Since the covalent modifications by LHVS and JPM-565 are mechanism based, labeling with these probes is proportional to the enzymatic activity of the protease targeted (Palmer *et al.*, 1995). More recent developments include the synthesis of fluorescent probes that afford multiplexing and visualization of proteolytic activities in living cells (Bogyo, personal communication).

In addition to the E-64 analog JPM-565, other peptide–epoxide-based inhibitors include the Cat B-specific inhibitors CA030 and CA074. These compounds were designed to mimic a carboxy-terminal dipeptide by replacing the terminal guanidinobutylamine of E-64 with a proline residue and terminating the chain with a free carboxylic group (Murata *et al.*, 1991; Towatari *et al.*, 1991). CA030 and CA074 bind with their peptide portion to Cat B's active site in inverse orientation to that observed for other epoxide-containing inhibitors of the E-64 family (Turk *et al.*, 1995b). The specificity of CA030 and CA074 is achieved through a tight binding composed of two H bonds between the C-terminal proline of CA030 and two highly conserved histidine residues from the occluding loop of Cat B (Turk *et al.*, 1995b). To increase membrane permeability of these Cat B-specific inhibitors, an analog in which the proline free-acid group of CA-074 was converted to its methyl ester was generated and used *in vivo* to measure Cat B activity (Buttle *et al.*, 1992). Recently, a new peptide epoxide, MB-074, was developed by replacing the isopropyl moiety of CA-074 with a phenol group as a site for attachment of radioactive iodine, yielding an affinity-labeled probe with which to study Cat B activity (Bogyo *et al.*, 2000).

A series of epoxysuccinate-based Cat L- and Cat S-specific inhibitors was also developed, designated CLIK (Katunuma *et al.*, 1999). The representative inhibitor of this series, CLIK148, was shown to be the first E-64 derivative using both prime-site and non-prime-site interactions along the enzyme's active-site cleft (Tsuge *et al.*, 1999). CLIK148 inhibits Cat L exclusively. The specificity for Cat L comes from the N-terminal pyridine of CLIK148 (Tsuge *et al.*, 1999). Finally, using E-64 as a model, analogs were synthesized by varying the core peptide recognition sequence while adding affinity tags at distal sites (Greenbaum *et al.*, 2000). The resulting probes, DCG-03 and DCG-04, maintained the same general cysteine protease specificity as the parental E-64 compound. Moreover, a library of DCG-04 derivatives was constructed in which the P2 position (leucine), the main specificity determinant for many cysteine proteases, was replaced with different amino acids. This library was used to obtain the activity profiles for multiple proteases in crude cellular extracts (Greenbaum *et al.*, 2000).

A final group of well-characterized, active site-directed inhibitors are the membrane permeable, diazomethyl ketones, such as Cbz–Tyr–Ala–CN_2. As with the inhibitors above, Cbz–Tyr–Ala–CN_2 irreversibly binds to the active site of cysteine proteases in proportion to their activity. An iodinated version of this compound, Cbz–^{125}ITyr–Ala–CN_2, has been used to demonstrate the activity of cysteine proteases in living cells (Mason *et al.*, 1989a,b). Another analog of the peptidyl diazomethane probe, fluoren-9-ylmethoxycarbonyl (Fmoc)–[I_2]Tyr–Ala–CHN_2, was produced by blocking the N terminus with a Fmoc group to permit further modifications of the probe without damaging the diazomethane group (Crawford *et al.*, 1988; Xing *et al.*, 1998). An iodinated form of Fmoc–[I_2]Tyr–Ala–CHN_2 reacted specifically with Cat B and Cat L, but not with Cat S. Use of this affinity-labeled inhibitor revealed that unlike Cat B and Cat L, the active site of Cat S was restricted in that it could not accommodate the bulky di-iodotyrosine of the inhibitor (Xing *et al.*, 1998).

The advances in inhibitor and probe design summarized above provide an exciting prospect for the assessment of protease profiles in cell populations available in limited numbers, such as subsets of professional APCs, and should allow the construction of a complete catalog of the proteases relevant for antigen presentation. Further refinement of inhibitors may ultimately yield compounds sufficiently selective to block a single protease in living cells. This approach nicely complements the genetic tools that are now available.

E. Cathepsin-Deficient Mice

Perhaps the most precise tool for determing which cysteine proteases are required for antigen presentation by class II molecules is a mouse in which an individual cathepsin gene has been "knocked out." Mice deficient in Cat B

(Deussing *et al.*, 1998), Cat D (Saftig *et al.*, 1995), Cat S (Nakagawa *et al.*, 1999; Shi *et al.*, 1999), Cat L (Roth *et al.*, 2000), Cat S/L (Shi *et al.*, 2000), and Cat S/B (Driessen *et al.*, in press) have been generated. The class II antigen presentation pathway in these mutant mice, including intracellular trafficking, Ii processing, antigen processing, and peptide loading and presentation, have been studied in detail. These *in vivo* models for protease function quickly exposed the limitations of *in vitro* studies utilizing cultured cells and cysteine protease inhibitors as a means to assign function to individual cathepsins. Although cathepsins B, D, and L were all implicated by *in vitro* studies to be important in the degradation of protein antigens into class II presentable peptides, none appear indispensable *in vivo* (Deussing *et al.*, 1998). Likewise, the ability of Cat B or Cat D to digest Ii *in vitro* is irrelevant for Ii processing *in vivo*. We now know that Cat S in bone marrow-derived APCs (Nakagawa *et al.*, 1999; Shi *et al.*, 1999) and Cat L in cortical thymic epithelial cells (Nakagawa *et al.*, 1998) are required for a rate-limiting cleavage step late in the staged breakdown of Ii *in vivo*. The phenotypes of these mice with regard to antigen presentation are discussed in detail in the following sections.

III. Proteolytic Digestion of Ii

The primary function of Ii is to ensure that peptide-receptive class II molecules end up in the endocytic pathway. That class II molecules depend on Ii in order to function is somewhat paradoxical: the chaperone function of Ii in the formation of class II $\alpha\beta$-dimers is required to create the peptide-receptive cleft, yet the physical complex formed between Ii and the $\alpha\beta$-dimer prevents class II molecules from binding peptides. Thus, the function of proteases in the destruction of Ii, once $\alpha\beta$–Ii complexes reach the endocytic pathway, is indispensable. The requirement for Ii does not abruptly end once class II molecules are delivered to their peptide-binding destination. Ii contains signals that retain class II molecules in endocytic compartments until peptide-loading events ensue (Cresswell, 1996). In addition, occupation of the class II peptide-binding groove with the CLIP fragment of Ii preserves the peptide-receptive status of the class II $\alpha\beta$-dimer, thereby preventing degradation of an empty class II molecule, until antigenic peptides are loaded with (or without) the assistance of DM (Alfonso and Karlsson, 2000). Hence, proteases must coordinate their digestion of Ii in such a way as to preserve the peptide-receptive state of the class II $\alpha\beta$-dimer. Which are the key endocytic proteases that participate in the stepwise degradation of Ii?

A. Cat B and Cat D Are Not Required for Ii Proteolysis

Early experiments employing protease inhibitors of broad specificity revealed the importance of both cysteine and aspartyl proteases in Ii

breakdown (Amigorena *et al.*, 1995; Blum and Cresswell, 1988; Maric *et al.*, 1994; Neefjes and Ploegh, 1992). As Cat B and Cat D are the most abundant endocytic proteases, they were predicted to be the major enzymes involved in Ii proteolysis. Indeed, *in vitro*, both Cat B and Cat D could digest Ii away from $\alpha\beta$–Ii complexes either in cell lysates or in purified preparations (Avva and Cresswell, 1994; Mizuochi *et al.*, 1994; Reyes *et al.*, 1991; Roche and Cresswell, 1991). However, the commercially available enzyme preparations used for these initial *in vitro* studies proved impure, as they were contaminated with other, less abundant cathepsins. Experiments using pure preparations of both Cat B and Cat D failed to reproduce the original results—neither enzyme was capable of removing Ii from $\alpha\beta$–Ii complexes synthesized *in vitro* (Riese *et al.*, 1996). Still, these *in vitro* experiments were flawed because they did not emulate the *in vivo* levels and/or ratios of enzymes to substrates, nor could the role of other proteases in initiating Ii breakdown or in rendering Ii cleavage sites accessible to key enzymes be assessed. Which enzymes participate in Ii proteolysis *in vivo*?

To correctly identify the proteases involved in Ii breakdown *in vivo*, mutant mice that are deficient in distinct cathepsins were analyzed. The proteolytic digestion of Ii in APCs isolated from mice that lacked either Cat B (Deussing *et al.*, 1998) or Cat D (Villadangos *et al.*, 1997) was unaffected. In fact, the phenotype of these mice with regard to the rate of Ii digestion, and the types of proteolytic intermediates generated, was indistinguishable from that of wild-type mice. Hence, despite their copious levels in APCs, neither Cat B nor Cat D is essential for normal Ii removal *in vivo*.

B. Conversion of $\alpha\beta$–Ii to $\alpha\beta$–Iip10

The proteolytic conversion of $\alpha\beta$–Ii molecules into $\alpha\beta$–peptide complexes was largely dissected by performing pulse–chase experiments on APCs followed by immunoprecipitation of class II–Ii complexes after different times of chase. To reveal the proteolytic steps that remove Ii from class II molecules during their maturation, the APCs analyzed in these biochemical experiments were treated with specific protease inhibitors, resulting in the accumulation of Ii breakdown intermediates (Morton *et al.*, 1995; Neefjes and Ploegh, 1992; Villadangos *et al.*, 1997). These represent "normal" intermediates of Ii processing (i.e., occur in uninhibited cells): stalling Ii processing by inhibition of specific proteases merely allows their capture and detection. The Ii fragments that remain associated with class II molecules are coprecipitated with the $\alpha\beta$-dimer and can be visualized by SDS–PAGE. The identity of these Ii processing intermediates was determined by their molecular weights and their reactivity with antibodies specific for distinct regions of intact Ii. A unique feature of certain class II alleles (i.e., murine I-A^b, human DR1) when bound to more terminal Ii breakdown intermediates as well as peptides is the stability of

the complex in SDS. The intact complex can be visualized by SDS–PAGE if the immunoprecipitated complexes are not boiled prior to separation (Sadegh-Nasseri and Germain, 1991). Such experiments conducted on both mouse and human APCs have led to a model for the stepwise degradation of intact Ii into the final end product, CLIP, as depicted in Fig. 1B (Driessen *et al.*, 1999; Riese and Chapman, 2000; Villadangos *et al.*, 1999).

The first step in Ii processing involves unraveling the class II–Ii nonameric complex that is delivered to early endosomes. The framework of this complex is the Ii homotrimer, held together via trimerization domains located C-terminal to CLIP (Cresswell, 1996). Each individual Ii in the homotrimer is attached to one class II $\alpha\beta$-dimer via its CLIP region and the dimer's peptide-binding groove. Proteolytic cleavage of the carboxy-terminal region of Ii results in disruption of the homotrimer—and thus the nonameric complex—into monomeric $\alpha\beta$–Ii complexes. Noncysteine proteases are responsible for these early Ii processing events, as the cysteine protease inhibitor leupeptin does not prevent elimination of the C-terminal trimerization region (Blum and Cresswell, 1988; Maric *et al.*, 1994; Villadangos *et al.*, 1997). However, the identity of the protease(s) involved remains unknown. Although Cat $D^{-/-}$ mice exhibited no defect in Ii processing (Villadangos *et al.*, 1997), inhibitors of aspartic proteases affect Ii breakdown (Maric *et al.*, 1994; Zhang *et al.*, 2000). Cat E, or possibly AEP, may participate in these early cleavage events.

All steps subsequent to the first carboxy-terminal cleavage are leupeptin sensitive and thus rely on cysteine proteases. Several class II-associated Ii breakdown intermediates accumulate in APCs treated with leupeptin (Blum and Cresswell, 1988; Neefjes and Ploegh, 1992). The first predominant intermediate is a 22- to 24-kDa (Iip22) N-terminal fragment resulting from removal of the COOH-terminal trimerization domain by an aspartic protease. This fragment spans residues 1 through ~160 of intact Ii and is detected more readily in human APCs and in H-2^d haplotype mice (Villadangos *et al.*, 1997). The second major Ii intermediate that accumulates in the presence of leupeptin is approximately 10 kDa (Iip10). The Iip10 fragment begins at the very NH_2-terminus of Ii and extends just through to the COOH-terminus of CLIP (~100 residues; Villadangos *et al.*, 1997). A processing intermediate of 18 kDa also accumulates in bone marrow-derived mouse APCs (Villadangos *et al.*, 1997). The data support a precursor/product relationship between these Ii processing intermediates, with Iip22 preceding Iip18, which proceeds Iip10. The cysteine proteases absolutely essential for conversion of Iip22 into Iip10 *in vivo* are still not known.

C. Generation of $\alpha\beta$–CLIP Complexes

Treatment of APCs with leupeptin uncovers two key Ii processing steps that require cysteine proteases: the conversion of $\alpha\beta$–Iip22 into $\alpha\beta$–Iip10,

and further digestion of αβ–Iip10 into the degradative end product αβ–CLIP. αβ–CLIP is not generated in the presence of leupeptin. Since the cysteine protease Cat S is expressed primarily in APC, and its expression is induced by INF-γ (which upregulates class II expression), Cat S was considered as a likely participant in these late stages of Ii breakdown. Indeed, treatment of B cells and DCs with the Cat S-specific inhibitor LHVS (Bromme and McGrath, 1996) resulted in the accumulation of αβ–Iip10 complexes (Riese *et al.*, 1996; Villadangos *et al.*, 1997). Moreover, αβ–Iip10 complexes isolated from LHVS-treated APCs could be converted *in vitro* into αβ–CLIP by digestion with recombinant Cat S (Riese *et al.*, 1996). Hence, αβ–Iip10 (not αβ–Iip22) serves as an *in vivo* substrate for Cat S to complete Ii proteolysis.

Unlike Cat B and Cat D, the ability of Cat S to cleave αβ–Iip10 complexes into αβ–CLIP *in vitro* held true *in vivo*. The bulk of Ii processing was stalled at the Iip10 stage in bone marrow-derived APCs isolated from Cat $S^{-/-}$ ($H\text{–}2^b$) mice (Nakagawa *et al.*, 1999; Shi *et al.*, 1999). To a lesser extent, αβ–p22 and αβ–p18 also accumulated in the absence of Cat S, indicating that, although not absolutely required, Cat S can mediate processing of these intermediates (Driessen *et al.*, 1999). Whereas αβ–p22 accumulated preferentially in early and late endosomes, αβ–p18 accumulated in lysosomal compartments (Driessen *et al.*, 1999). Despite the accumulation of αβ–p10 (A^b–Iip10) complexes in Cat $S^{-/-}$ APCs, αβ–peptide complexes were eventually detected at the cell surface, albeit with considerable delay (Shi *et al.*, 1999; Wolf Bryant *et al.*, submitted for publication). The defect in peptide-loading extended beyond kinetics to include the types of epitopes that could be presented by class II molecules in these mutant mice (Nakagawa *et al.*, 1999; Shi *et al.*, 1999).

Despite the defect observed in Ii proteolysis in Cat S-deficient B cells, T-cell selection in Cat $S^{-/-}$ mice was unaffected by the mutation, as normal numbers of $CD4^+$ T cells were found in peripheral lymphoid compartments (Nakagawa *et al.*, 1999; Shi *et al.*, 1999). $CD4^+$ T cells are positively selected as they migrate through the thymus by recognition of class II–peptide (self) complexes expressed on cortical thymic epithelial cells. The proteases required for the late stages of Ii processing are tissue specific. Whereas Cat S is required to convert αβ–Iip10 into αβ–CLIP in BM-derived B cells and DCs, Cat L is required for this cleavage in cTECs (Nakagawa *et al.*, 1998), thus explaining why T-cell selection is normal in Cat $S^{-/-}$ mice. Cat L is also expressed in macrophages along with Cat S. Indeed, the defect in Ii breakdown seen in Cat $S^{-/-}$ B cells was not as severe as that in Cat $S^{-/-}$ macrophages (Shi *et al.*, 2000). However, the Ii processing defect was no more pronounced in macrophages (alveolar and peritoneal) isolated from Cat S/L double-deficient mice than it was in the single, Cat $S^{-/-}$ macrophages (Shi *et al.*, 2000). Cat $S^{-/-}$ macrophages treated

with IFN-γ express Cat F, which can digest $\alpha\beta$–Iip10 into $\alpha\beta$–CLIP, at least *in vitro* (Shi *et al.*, 2000). If the rates of $\alpha\beta$–p10 conversion into $\alpha\beta$–peptide reflect the responsible enzymes' efficiency in generating $\alpha\beta$–CLIP (Wolf Bryant *et al.*, submitted for publication), then Cat S is still by far the most efficient and the preferred protease for generating $\alpha\beta$–CLIP even in macrophages, with Cat F running a close second.

The requirement for Cat S in generating $\alpha\beta$–CLIP complexes is not only tissue and cell-type specific, but also varies widely among class II alleles. The class II alleles I-A^q, I-A^k, I-A^u, and I-A^s are less dependent on Cat S for efficient Ii removal and subsequent peptide loading than is I-A^b (Nakagawa *et al.*, 1999; Villadangos *et al.*, 1997).

D. Proteolytic Requirements for DM-Mediated Peptide Loading

The removal of CLIP from the peptide-binding groove of most class II alleles and subsequent loading with antigenic peptides is catalyzed by the accessory molecule DM (Alfonso and Karlsson, 2000; Busch *et al.*, 2000). In DM-deficient animals, 99.8% of I-A^b molecules are occupied with CLIP (Fung-Leung *et al.*, 1996; Martin *et al.*, 1996; Miyazaki *et al.*, 1996). The dependency on DM for CLIP–peptide exchange is allele specific. A physical interaction between DM and class II molecules is required for exchange of CLIP for antigenic peptides. The lateral aspects of DM bind to a region near the N terminus of the class II peptide-binding cleft (Doebele *et al.*, 2000; Guerra *et al.*, 1998; Mosyak *et al.*, 1998). Kinetic analysis of DM-mediated CLIP–peptide exchange (Vogt *et al.*, 1996) suggests that interaction with DM imparts an "open-transition" state to the class II peptide-binding cleft that favors CLIP release. The crystal structure of I-A^d–CLIP suggests that the breakage of a few hydrogen bonds between the N terminus of the peptide-binding groove and CLIP would suffice to mediate CLIP's release (Mosyak *et al.*, 1998). Loss of a single H bond at position 81 of the I-A^d β-chain, or of two H bonds at β82, is sufficient to render I-A^d incapable of stable interaction with CLIP (and the antigenic peptide OVA) (Bryant *et al.*, 1999). The changes in class II structure imposed by the β81 and β82 mutations may exemplify the types of alterations mediated by DM interaction that enable CLIP–peptide exchange. What are the minimal conformational requirements for DM's *in vivo* substrate(s)?

No study to date has identified the *in vivo* substrate for DM. DM does not interact with class II molecules associated with intact Ii, and thus Ii proteolysis is a prerequisite for DM function. The identity of DM's substrate cannot be found in the $DM^{-/-}$ mice alone. $DM^{-/-}$ mice contain a functionally complete repertoire of endocytic proteases, and thus the degradation of I-A^b-associated Ii proceeds to completion regardless of the DM mutation. Therefore, the accumulation of A^b–CLIP complexes in $DM^{-/-}$ APCs merely emphasizes the

requirement for DM in liberating the peptide-binding groove of I-A^b at some stage during Ii removal. It is not known whether DM can interact with Ii breakdown intermediates upstream of CLIP *in vivo*. *In vitro*, DM could exchange the Ii breakdown intermediates p22 and p10 in complex with the human class II molecule, HLA-DR1, in addition to CLIP (Denzin and Cresswell, 1995; Denzin *et al.*, 1996). However, the accumulation of $\alpha\beta$–Iip10 complexes in Cat $S^{-/-}$ APCs together with the delayed appearance of $\alpha\beta$–peptide complexes on the cell surface (Driessen *et al.*, 1999; Nakagawa *et al.*, 1999; Shi *et al.*, 1999) suggests that DM does not prefer either p22 or p10 as its substrate, but rather a more terminal Ii processing intermediate, such as CLIP. Thus, the appearance of $\alpha\beta$–peptide complexes on the surface of Cat $S^{-/-}$ APCs, if DM dependent, could result from one of two mechanisms. First, a yet unknown enzyme distinct from Cat S that can generate the $\alpha\beta$–CLIP substrate used by DM may be present in B cells. Alternatively, DM may exchange Iip10 for antigenic peptides or some processing intermediate between Iip10 and CLIP, albeit considerably less efficiently.

IV. Proteolytic Control of Vesicle Biogenesis and Class II Trafficking through the Endocytic Pathway

A. Targeting Class II Molecules to Endocytic Compartments

Successful delivery of MHC class II molecules to compartments of the endosomal/lysosomal pathway relies mainly on their prior association with Ii (Bakke and Dobberstein, 1990; Lotteau *et al.*, 1990). Ii contains two leucine-based signals in its cytoplasmic tail that confer this specific targeting function (Bremnes *et al.*, 1994; Odorizzi *et al.*, 1994). The cytosolic factors that interact with Ii's tail and thus direct these trafficking events have yet to be identified. The μ-chain of the adaptor protein complexes AP1 and AP2, which provide specificity for clathrin assembly and recognize sorting motifs in the cytoplasmic domains of membrane proteins, can interact with Ii's cytoplasmic tail *in vitro* (Hofmann *et al.*, 1999). Whether such interactions occur in living cells remains to be demonstrated.

Ii also affects the "architecture" of the endocytic pathway, an attribute that may play a role in antigen processing and/or presentation. The expression of high levels of Ii induces the formation of large endocytic compartments called macrosomes (Pieters *et al.*, 1993; Romagnoli *et al.*, 1993). Macrosome formation correlates with a delay in transport of proteins from early endosomes to late endosomes/lysosomes (Gorvel *et al.*, 1995; Romagnoli *et al.*, 1993). Langerhans cells are the only APCs found to contain such large acidic vesicles. The presence of macrosomes in these APCs correlates with a high rate of Ii synthesis (Kampgen *et al.*, 1991). Moreover, the formation of macrosomes is depend-

ent on the trimerization of Ii (Gedde-Dahl *et al.*, 1997), a property that is required for proper delivery of $\alpha\beta$–Ii complexes to endosomal compartments (Arneson and Miller, 1995). Thus, it is likely that the "trimerized" cytoplasmic tail of Ii interacts with components of the molecular machinery involved in membrane traffic. To identify these components, we recently synthesized an affinity matrix in which the trimerization of Ii's cytoplasmic tail is forced chemically. This approach allowed us to identify hsc70 as one the cytosolic partners capable of interacting with Ii (Lagaudrière-Gesbert *et al.*, submitted for publication). Hsc70 was initially identified as the uncoating ATPase that dissociates clathrin triskelions from clathrin coated-vesicles (Schlossman *et al.*, 1984). Analysis of the clathrin-coated vesicle cycle in cells expressing ATPase-deficient hsc70 mutants suggested that this protein may also be required to chaperone cytosolic clathrin triskelions to allow their recruitement to coated pits (Newmyer and Schmid, 2001). The ability of Ii to recruit hsc70 activity might cause uncoating and thereby inhibit subsequent fission reactions essential for retrieval of vesicular constituents. This would shift the balance toward fusion and lead to an increase in size of the target organelle carrying Ii, as seen in COS cells expressing Ii. Indeed, expression of a dominant-negative version of hsc70 in Ii-transfected COS cells counteracted the ability of Ii to modify the endocytic pathway, demonstrating an interaction *in vivo* of Ii with hsc70 as part of the machinery of vesicular transport (Lagaudrière-Gesbert *et al.*, submitted for publication). While earlier studies postulated a role for hsc70 in peptide delivery to class II molecules (Panjwani *et al.*, 1999) by a yet unidentified mechanism, our recent data suggest that hsc70's role extends to maintenance of a properly organized endocytic pathway and, in this manner, contributes to MHC class II-restricted antigen presentation.

B. Is There a Connection between the Processing of Ii and the Architecture of the Endocytic Pathway?

Dendritic cells isolated from Cat $S^{-/-}$ mice have been exploited as a model to dissect the role of proteases in the trafficking and maturation of class II molecules along the endocytic route. Immunofluorescence and subcellular fractionation experiments demonstrated that in the absence of Cat S, class II molecules complexed to N-terminal fragments of Ii (mainly Iip10) accumulate in late-endocytic compartments of DCs (Driessen *et al.*, 1999). These findings were reminiscent of earlier studies conducted in leupeptin-treated B cells and immature BM-derived DCs, in which the retention of class II molecules in late-endocytic compartments correlated with the low activity of cysteine proteases in general (Brachet *et al.*, 1997) or of Cat S (Pierre and Mellman, 1998). Hence, Ii proteolysis must proceed in an ordered fashion to the end stage of CLIP to ensure proper trafficking of class II molecules through the endocytic route.

Furthermore, the cell biological studies referred to above proved that active Cat S is not restricted to late-endocytic compartments, but instead can be found all along the endocytic route of DCs, including early endosomes (Driessen *et al.*, 1999). The intracellular distribution of Cat S agrees with its unique ability to remain active at neutral pH. Thus, the conversion of $\alpha\beta$–Iip10 into $\alpha\beta$–CLIP could occur in a variety of endocytic compartments, enabling class II molecules to sample distinct pools of differentially compartmentalized antigenic peptides. Since, in addition to Iip10, the Ii intermediates p22 and p18 also accumulate in late compartments of Cat $S^{-/-}$ DC (Driessen *et al.*, 1999), those fragments of Ii may represent additional substrates for Cat S.

Cat S not only governs MHC class II trafficking via its role in Ii degradation, but also affects the architecture of endocytic vesicles themselves, independent of class II–Ii. DCs isolated from Cat $S^{-/-}$ mice exhibit increased levels of some late-endocytic markers, such as LAMP-1, when analyzed by confocal microscopy (Driessen *et al.*, 1999). This correlated with an increase in β-hexosaminidase activity in fractionated Cat $S^{-/-}$ DC (Driessen *et al.*, 1999). A more thorough morphological examination of endocytic vesicles in Cat $S^{-/-}$ cells was conducted using electron microscopy with striking results: compartments that stained for late-endocytic markers were considerably enlarged in Cat $S^{-/-}$ cells as compared to those in wildtype cells (P. Peters, personal communication). Since the cytoplasmic tail of Ii, responsible for binding hsc70, remains intact in Cat $S^{-/-}$ DCs (note that Iip18, Iip22 and Iip10 fragments retain this element), it is tempting to propose that the recruitment of the uncoating ATPase accounts for this abnormal morphology. The induction of enlarged late-endocytic vesicles in the absence of Cat S may parallel the formation of macrosomes observed in Ii-transfected COS cells, whose formation relies on the interaction of the Ii with hsc70. Either Cat S plays a direct role in controlling protein trafficking along the endocytic pathway or the enlarged endocytic compartments observed in Cat $S^{-/-}$ cells are merely the result of the accumulation of class II Iip10 complexes and recruitment of hsc70. The answer awaits the generation and characterization of Cat S × Ii double knockout mice.

V. The Role of Ii in Regulating the Proteolytic Activities of APCs

So far we have emphasized the degree to which the class II antigen presentation pathway exploits the normal housekeeping functions of a cell—specifically proteolysis—for its own purpose and success. From proper trafficking of class II complexes through endocytic vesicles to Ii breakdown and peptide acquisition, the functions of a cell's proteases are indispensable. However, the immune system may not be doing all the "taking" and no "giving." The proteolytic machinery of the cell may itself exploit components of the class II antigen presentation pathway for its own benefit. We now discuss recent data that

demonstrate a role for Ii in the regulation of the proteolytic environment of an individual APC.

A. The p41 Fragment Binds the Active Site of Cat L

In addition to their indistinguishable functions as chaperones for class II folding and intracellular trafficking (Peterson and Miller, 1992; Takaesu *et al.*, 1995, 1997), both the p31 and p41 isoforms of murine Ii can be converted into CLIP (Fineschi *et al.*, 1995; Takaesu *et al.*, 1995, 1997), presumably by Cat S. p31 and p41 are expressed in different ratios in the various types of APCs. Whereas p41 represents no more than 10% of the total pool of Ii in splenocytes, its expression levels are considerably higher in macrophages, DC, and Langerhans cells (Kampgen *et al.*, 1991; Koch and Harris, 1984; Pierre and Mellman, 1998; Pure *et al.*, 1990). The p41-specific 64-aa segment resembles a thyroglobulin type-1 domain, rich in cysteine residues (O'Sullivan *et al.*, 1987). This p41 segment was found noncovalently bound to the mature form of Cat L purified from human kidney (Ogrinc *et al.*, 1993). The crystal structure of Cat L in a complex with the p41 segment complex shows that this fragment occupies the Cat L active site (Guncar *et al.*, 1999). Moreover, *in vitro* studies demonstrate that the 65-aa segment of p41 inhibits Cat L enzymatic activity (Bevec *et al.*, 1996; Fineschi *et al.*, 1996; Turk *et al.*, 1999). What is the functional relevance of Cat L–p41 association *in vivo*?

B. p41 Is Required for Full Cat L Activity

As most lysosomal hydrolases, Cat L is synthesized in the ER as a proenzyme (Erickson, 1989; McGrath, 1999). The Cat L proregion consists of 96 amino acids that occupy its active-site cleft, maintaining the enzyme in an inactive state (Coulombe *et al.*, 1996). During export along the endocytic pathway, pro-Cat L undergoes several proteolytic cleavages to generate the Cat L single-chain (30 kDa) and two-chain mature forms, composed of a 25-kDa heavy chain linked to a 5-kDa light chain by disulfide bonds (Erickson, 1989; Ishidoh and Kominami, 1998; McGrath, 1999; Reilly *et al.*, 1989). Mutant mouse strains deficient for Ii, or expressing either p31 or p41 Ii, were developed to study the function of each isoform in antigen presentation (Takaesu *et al.*, 1995, 1997). We have used these animals to study the functional significance of the Cat L–p41 interaction *in vivo*. Contrary to expectations, Cat L expression and activity are strongly reduced in APCs isolated from Ii-deficient mice, as measured by immunoblotting and active-site labeling experiments (Lennon-Dumenil *et al.*, 2001). In agreement with the described Cat L–p41 interaction, the mature two-chain forms of Cat L depend on p41 (not p31) to be expressed at wildtype levels: levels of mature Cat L are considerably decreased in the endocytic compartments of cells that lack only the p41 isoform of Ii (Lennon-Dumenil *et al.*, 2001).

C. p41 Protects the Mature Forms of Cat L from Degradation by Surrounding Cysteine Proteases

Why are the levels of mature Cat L decreased in the absence of p41? This question was addressed by performing pulse–chase experiments in the presence of different protease inhibitors. These analyses showed that in the absence of p41, Cat L is degraded in acidic compartments by cysteine proteases (Lennon-Dumenil *et al.* submitted for publication). Thus, p41 appears to protect Cat L from premature destruction. p41 is therefore not merely an inhibitor of Cat L enzymatic activity, but serves as a chaperone to help maintain a pool of mature enzyme in late-endocytic compartments of antigen-presenting cells.

Prevention of Cat L destruction by p41 might be linked to the ability of p41 to bind the active site of Cat L (Guncar *et al.*, 1999). This interaction is reminiscent of the interaction of Cat L with its propeptide, which not only maintains the enzyme in an inactive state but also assists its folding and stabilizes its conformation (Jerala *et al.*, 1998; McGrath, 1999). As pro-Cat L traverses endocytic compartments, the attendant drop in pH induces conformational changes in the propeptide, which then detaches from the Cat L active site and is cleaved to generate the 30-kDa single-chain form of the enzyme (Jerala *et al.*, 1998). Even though removal of the Cat L propeptide is necessary for enzymatic activation, it may destabilize the tertiary structure of the enzyme sufficiently to allow partial unfolding and subsequent degradation. In late-endocytic compartments p41 may function as a Cat L chaperone, not unlike the propeptide in compartments with neutral pH. This would allow the cell to maintain a pool of (latent) mature Cat L in late-endocytic compartments that is protected from destruction by hydrolases.

Our data demonstrate that leupeptin-sensitive cysteine proteases control the turnover of Cat L in late-endocytic compartments by partially degrading its mature active forms (Lennon-Dumenil *et al.*, 2001). Which enzymes are involved in this process? The levels of 24-kDa Cat L are increased in BM macrophages from Cat S and Cat B knockout mice (AML, unpublished data). In agreement with this observation, recent data published by Honey *et al.* (2000) showed increased Cat L activity in cells lacking Cat S. In addition to Cat S and Cat B, Cat L could regulate its own levels of activity by self-degradation. In this context, p41 would exert a protective effect by preventing self-destruction of mature Cat L.

VI. Antigen Processing

The presentation of antigen requires that it first be fragmented by proteases into smaller peptides that can fit easily into the peptide-binding groove of an MHC molecule. MHC class II molecules bind and present peptides derived

from antigens that gain access to the endocytic pathway by endocytosis, phagocytosis, or both (Watts, 1997; Wolf and Ploegh, 1995). Antigens taken up by APCs traverse the endocytic route via vesicles of decreasing pH. The gradual exposure of antigen to increased protease activity ensures that the antigen is not completely destroyed but is instead broken down into class II-presentable peptides.

Many if not all of the endocytic compartments may host the loading of peptides onto class II $\alpha\beta$-dimers. Given the fact that each endocytic vesicle is likely to vary in its proteolytic content coupled to the different population of antigens housed in each, class II molecules are ensured access to a diverse pool of antigenic peptides. Moreover, the location at which class II molecules become receptive to peptide loading may determine the types of epitopes presented to T cells. In contrast to our detailed understanding of Ii processing, the proteolytic events that lead to the generation of class II-presentable peptides from intact antigen remain somewhat enigmatic. Nonetheless, the generation of these T-cell epitopes will no doubt require specific cleavages during the course of antigen destruction. Whereas the proteolytic cleavages required for efficient Ii removal from class II molecules occur during the late stages of Ii breakdown (i.e., Iip10 into CLIP), the specific cleavages required to ensure the generation of immunogenic epitopes from intact antigens may instead be needed during the early stages of antigen processing (see below).

A. Antigen Acquisition

Antigen acquisition is the first step in MHC class II-restricted antigen presentation. The mechanisms by which acquisition occurs are diverse. These mechanisms are regulated and can be highly specific. The mode of antigen uptake by an APC depends on the source of the antigen, the type of APC, and the activation state of the APC. The method of internalization utilized dictates the endocytic compartment in which the antigen will be targeted. The mechanisms of antigen uptake can be either specific or nonspecific (Bakke and Nordeng, 1999).

Nonspecific uptake involves fluid-phase endocytosis of extracellular fluid. The primary, nonspecific mechanism used by DCs to internalize exogenous antigens is macropinocytosis, in which large volumes of extracellular fluid are engulfed and the macrosolutes captured are concentrated in class II-positive endocytic compartments (Cella *et al.*, 1997; Sallusto *et al.*, 1995). Macropinocytosis is linked to membrane-ruffling activity rather than uptake mediated by clathrin-coated pits. Only immature DCs can internalize antigen whereas mature DCs rather function in the presentation of the resulting peptides on the cell surface via class II molecules (Thery and Amigorena, 2001).

Specific mechanisms of antigen uptake involve endocytosis and phagocytosis mediated by receptors expressed on the surface of APCs (Bakke and Nordeng,

1999; van Bergen *et al.*, 1999; Watts, 1997). Each type of professional APC—B cells, macrophages, and DCs—expresses distinct receptors that mediate the internalization of exogenous antigens (summarized in Table II). These receptors include the B-cell receptor (BCR, B cells only), Fc receptors (B cells, macrophages, and DCs), and members of the macrophage mannose receptor (MMR) C-type lectin family (macrophages and DCs). These receptors serve as scaffolds to concentrate the antigen they internalize in peptide-loading compartments. The result is an immune response with enhanced sensitivity. In fact, receptor-mediated endocytosis of antigen enables the immune system to respond to 10^3 to 10^4 lower antigen concentrations as compared to fluid-phase uptake of antigen. Indeed, allergen presentation to T cells is 100- to 1000-fold more effective if the allergen has been targeted via allergen-specific IgE and FcεRI on APCs (Maurer *et al.*, 1995, 1998). As atopic individuals have significantly higher levels of FcεRI on the cell surface of several APCs (Maurer *et al.*, 1994), this is a good example of a mechanism that lowers the individual's

TABLE II
MODES OF ANTIGEN UPTAKE BY DISTRICT APC TYPES

APC type	Ag-uptake mechanism	Receptors
B cell	Receptor-mediated endocytosis	BCR
		FcRs
Dendritic cell	Macropinocytosis	None
	Phagocytosis (nonspecific)	None
	Receptor-mediated phagocytosis	FcγRs
		Complement receptors
	Receptor-mediated endocytosis	Complement receptors
		FcγRs, FcεRs
		ILT3
		C-type lectins (DEC-205,MMR)
		Heat shock protein receptors (CD91)
		Integrins ($\alpha v\beta 5$ for apoptotic corps)
	Restored macropinocytosis on activated DCs	None
Macrophage	Receptor-mediated phagocytosis	FcγRs
		Complement receptors
		CD14
		Toll-like receptors
	Receptor-mediated endocytosis	FcγRs
		C-type lectins (MMR)
		ILT3
		Integrins ($\alpha v\beta 3$)
	Macropinocytosis	None

threshold to develop allergen-specific T-cell responses. In addition, phagocytosis of microorganisms and other particles by macrophages and DCs is most efficient when mediated by specific receptors, such as Fcγ receptors, C3R, or CD14 (Gregory, 2000). Interaction between these receptors and their ligands leads to actin polymerization and phagosome formation. In most instances, the phagosome develops into a phagolysosome by fusing with early/late endosomes and lysosomes of the host cell, and thus its contents become part of the "normal" endocytic route traveled by class II molecules. The phagosomes of the intracellular bacteria *Legionella* and *Mycobacterium*, however, exhibit limited fusion with their host cells' lysosomes (Ojcius *et al.*, 1996). This may represent a means by which these pathogens evade the immune response.

Unlike macrophages and DCs, B cells exhibit low rates of pinocytosis and phagocytosis. To compensate, they possess an antigen-specific receptor known as the BCR (Siemasko and Clark, 2001). The specificity of the BCR allows it to recognize rare and low-affinity antigens. Antigens capable of cross-linking the BCR are preferentially captured, as BCR aggregation enhances its internalization. The BCR is composed of membrane Ig, noncovalently associated with an Igα–Igβ heterodimer. The membrane portion recognizes and binds specific antigen, while the heterodimer contains the immunoreceptor tyrosine-based activation motif that initiates the signaling cascades necessary for internalization and targeting of the antigen to endocytic compartments. BCR–antigen complexes are internalized via clathrin-coated pits into early endosomes and then sorted into various endocytic compartments containing class II molecules. The sorting of Ag to early endosomes depends on signals located in the cytoplasmic tail of Igβ, whereas delivery of antigen to late-endocytic vesicles enriched for class II molecules requires the phosphorylation of tyrosine residues in the tail of Igα (Bakke and Nordeng, 1999; Siemasko and Clark, 2001).

The FcγR receptor is utilized by most professional APCs to facilitate the enodyctosis or phagocytosis of immune complexes (Amigorena and Bonnerot, 199a,b). Several types of Fc receptors have been characterized that can be distinguished in part by their cytoplasmic tail sorting signals. These signals determine to which intracellular compartment the ligand bound by the Fc receptor will be delivered (Bakke and Nordeng, 1999).

Macrophages and DCs take up mannosylated and/or fucosylated antigens via mannose receptors. Upon endocytosis, the manose receptor–ligand complex quickly uncouples and the mannose receptor is recycled back to the cell surface intact for round after round of antigen capture and internalization. The macrophage mannose receptor primarily recycles through peripheral, early endosomes (Engering *et al.*, 1997; Tan *et al.*, 1997), although some entry into late endosomes has been observed (Prigozy *et al.*, 1997). Bone marrow-derived

DCs express another receptor that belongs to the MMR family, designated DEC-205 (Jiang *et al.*, 1995; Mahnke *et al.*, 2000). In contrast to MMR, DEC-205 delivers antigens to late endosomes and lysosomes rich in MHC class II molecules (Mahnke *et al.*, 2000). Acidic clusters in the cytoplasmic tail of DEC-205 are responsible for its intracellular sorting functions. The targeting functions of DEC-205 ensure that antigen and class II molecules intersect.

B. Marking the Antigen for Degradation

Antigen degradation starts with the exposure of the native protein to low pH in a reducing environment to dissociate the antigen from its receptor and destabilize its native structure. Disulfide bounds must be reduced to unfold the protein and improve access of proteolytic enzymes (Fig. 1A). Reduction of disulfide bonds can be a rate-limiting step in antigen degradation in endocytic compartments (Collins *et al.*, 1991; Jensen, 1991, 1993). The lysosomal thiol reductase (GILT) is an enzyme active at low pH that is capable of catalyzing disulfide bond reduction both *in vivo* and *in vitro*. GILT is expressed constitutively in antigen-presenting cells and is induced by IFN-γ in other cell types, suggesting a role in antigen processing (Arunachalam *et al.*, 2000; Phan *et al.*, 2000).

C. Antigen Degradation by Endocytic Proteases

The redundancy in cleavage specificity between the endocytic proteases has made identifying the enzymes required to generate specific T-cell epitopes difficult. The MHC class II peptide-binding cleft is open at both ends and can therefore accommodate peptides of various lengths, ranging from 12 residues to as many as 30 residues, with a preferred size of ~15 residues. The characteristic peptide bound by class II consists of a core sequence with ragged N and C termini. This suggests that more than one enzyme is responsible for the proteolysis of antigen, involving an initial cleavage by endopeptidases followed by sequential trimming of the ends by amino- and carboxy-peptidases (Watts, 1997). Whether a specific protease dedicated to the generation of class II-presentable peptides from a single antigen is required is still a matter of debate. Recently, AEP was shown to initiate the proteolytic degradation of the carboxy-terminal domain of the tetanus toxin antigen (TTCF) in B lymphoblasts (Antoniou *et al.*, 2000; Manoury *et al.*, 1998). Cleavage of TTCF at a single site by AEP was shown to be critical for the generation and presentation of a variety of T-cell epitopes, even those distant from the cleavage site (Antoniou *et al.*, 2000). Thus, a single cleavage by AEP was proposed to "unlock" the native TTCF structure, facilitating its subsequent degradation into presentable peptides by undefined proteases (Antoniou *et al.*, 2000). Given the strict specificity of AEP, it is unlikely that the bulk of antigens endure the same proteolytic fate as TTCF.

Most of what we know concerning antigen degradation comes from studies performed *in vitro*, which, as shown for Ii processing, do not necessarily mirror what takes place *in vivo*. *In vitro*, cathepsins B, D, and E can generate T-cell epitopes when incubated with intact antigens (Bennett *et al.*, 1992; Rodriguez and Diment, 1992, 1995; van Noort and Jacobs, 1994; Vidard *et al.*, 1992). *In vivo*, a modest shift in the efficiency of presentation of some antigenic determinants was seen in APCs isolated from Cat B- and Cat D-deficient animals (Deussing *et al.*, 1998). Nonetheless, the overall capacity of Cat $B^{-/-}$ or Cat $D^{-/-}$ APCs to process and present antigens via class II molecules was unaffected. Even Cat $S^{-/-}$ APC were still capable of normal antigen presentation for most epitopes tested (Nakagawa *et al.*, 1999; Shi *et al.*, 1999). It is still possible that the presence or absence of one of these major cathepsins might be pivotal for the generation of immunogenic peptides from an invading pathogen or in other disease states. Cat B is important for the degradation of peptides, proteins, toxins, and even cell surface receptors that enter the cell via endocytosis or phagocytosis (Authier *et al.*, 1999; Mort and Buttle, 1997; Zhang *et al.*, 2000). In addition, the activity of Cat S was shown to be critical for the presentation of a pathogenic, arthritis-inducing, collagen-derived epitope in $I\text{-}A^q$ mice (Nakagawa *et al.*, 1999). Functional studies using a specific inhibitor of Cat E showed that this enzyme is essential for the processing of ovalbumin in murine A20 cells (Bennett *et al.*, 1992). Furthermore, the regulation of Cat E during human B-cell activation indicates Cat E plays an important role in antigen degradation in these cells (Sealy *et al.*, 1996).

Little or no evidence exists for a role of Cat D and Cat L in antigen degradation *in vivo*. It is possible that Cat L's ability to degrade extracellular matrix proteins might be important in pathologic conditions where peptides from such proteins are presented. Indeed, the detection of a small population of "empty" class II complexes at the cell surface of DCs raises the possibility that processing outside the endocytic pathway could also play a role in class II-restricted antigen (Santambrogio *et al.*, 1999a, b). In addition, extracellular proteases may further trim antigens that occupy the cleft of class II molecules already at the cell surface and thus assist in generating the final epitope seen by T cells. Such a role has been postulated for aminopeptidase N (APN, CD13) (Larsen *et al.*, 1996). APN was capable of digesting the NH_2-terminal end of a long peptide bound in the cleft of a class II molecule.

Perhaps more pronounced defects in antigen degradation would be observed in APCs where more than one of the essential cathepsins is absent. Studies with human DCs and high concentrations of the active-site inhibitor LHVS (at concentrations that inhibit Cat S, Cat B, and Cat L) indicate that Cat S and Cat B act concertedly to degrade antigen (Fiebiger *et al.*, 2001). To avoid the limitations inherent in using pharmacological inhibitors and cell lysates, Driessen *et al.* conducted a study in which the contributions of cathepsins S, B, L,

and D in the degradation of antigen could be assessed *in vivo* (Driessen *et al.*, 2001). The fate of a radiolabeled immune complex, ^{125}I-labeled F(ab′)$_2$, internalized by the FcγR was followed in bone marrow-derived APCs isolated from the different cathepsin mutant mice. Nonspecifically radiolabeled ^{125}I-labeledF(ab′)$_2$ was degraded rather slowly by proteases into relatively stable peptide intermediates that could be resolved and visualized by SDS–PAGE and autoradiography. The results showed that both Cat B and Cat S mediated the bulk of ^{125}I-labeled F(ab′)$_2$ degradation, via independent pathways, while Cat L and Cat D were dispensable (Driessen *et al.*, 2001).

Regardless of the proteases involved, antigen unfolding and subsequent proteolysis must be balanced in such a way that the T-cell epitopes destined for presentation by class II molecules are not themselves destroyed in the process. The open ends of the MHC class II binding groove do allow binding of longer protein fragments, with the T-cell epitope lying in the core of the groove. Thus, the antigen may initially be digested into fragments somewhat longer. than the final immunogenic peptide. Once in the peptide-binding groove of class II, that which is tightly enclosed by the α-helices of the cleft would be protected from further degradation, while the ends of the fragment hanging outside the cleft would be accessible to further trimming by endo- and exopeptidases (i.e., Cat H and Cat B). Thus, the final antigenic peptide seen by T cells may not be fully processed until after it is loaded onto class II.

VII. Concluding Remarks

The processing events that lead to peptide-loaded class II molecules can be defined in molecular terms because of decades of work in the field of lysosomal biology. The classification of essential proteases, the definition of their specificity and the design of specific inhibitors predate the immunologist's appreciation of these efforts. As class II-restricted antigen presentation is completely dependent on lysosomal proteolysis, it is a subject that offers itself naturally to the blending of these two fields of study. For example, by drawing on the research of lysosomal biologists, immunologists were able to determine the key events of Ii proteolysis. It is clear from this review that the picture is still not complete. The proteases involved in the generation of the Iip10 intermediate are still unknown. Moreover, the specific proteolytic requirements for the generation of T-cell epitopes from intact antigens remain unclear. It is possible that the proteolytic digestion of antigen requires the action(s) of other components of the endocytic pathway, such as chaperones and the thiol reductase GILT. A more complete picture of the molecular and cellular requirements for the initiation of an antigen-specific immune response will depend on continued interactions between the fields of lysosomal biology and antigen presentation.

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