Multiple proteases process viral antigens for presentation by MHC class I molecules to $CD8^+$ T lymphocytes.

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Abstract

Recognition by $CD8^+$ cytotoxic T lymphocytes of any intracellular viral protein requires its initial cytosolic proteolytic processing, the translocation of processed peptides to the endoplasmic reticulum via the transporters associated with antigen processing, TAP, and their binding to nascent MHC class I molecules that then present the antigenic peptides at the infected cell surface. From initial assumptions that the multicatalytic and ubiquitous proteasome is the only protease capable of fully generating peptide ligands for MHC class I molecules, the last few years have seen the identification of a number of alternative proteases that contribute to endogenous antigen processing. Trimming by non-proteasomal proteases of precursor peptides produced by proteasomes is now a well-established fact. In addition, proteases that can process antigens in a fully proteasome-independent fashion have also been identified. The final level of presentation of many viral epitopes is probably the result of interplay between different proteolytic activities. This expands the number of tissues and physiological and pathological situations compatible with antigen presentation, as well as the universe of pathogen-derived sequences available for recognition by $CD8^+$ T lymphocytes.

The classical antigen processing and presentation pathway

After infection of an organism by a pathogen, the immune system is faced with the tasks of identifying and curing or eliminating the infected cells and the circulating pathogen. Recognition of foreign, soluble, repetitive particles, such as most pathogens are, is a task that is mostly carried out by soluble effectors, mainly antibodies. In collaboration with phagocytic cells, they contribute to their effective removal from the organism. Discrimination between healthy and infected cells, identifying the foreign determinants among a background of self cellular components, is the task of immune cells. Antigen-specific lymphocytes screen the surface of infected cells, where not only

surface products but also intracellular components are displayed. Specialized molecules encoded by genes in the major histocompatible complex (MHC) are responsible for displaying the universe of cellular components for screening by the antigen-specific receptor of T lymphocytes, the T-cell receptor (TCR). It is the molecular details of this process that are the scope of this review, particularly in what pertains the recent evidence on the involvement of multiple cellular proteases in this process. As a consequence of recognition by specific T lymphocytes of pathogen-derived, MHC-presented antigens, the infected cells are either physically eliminated by cytotoxic T lymphocytes, or cured from the virus infection by cytokines released by these same T cells.

In an infected cell, macromolecular synthesis of cellular as well as pathogen-encoded components takes place. Proteins encoded by the pathogen are synthesized in the cell's ribosomes, and can thereafter localize to any subcellular compartment. As part of the mechanism leading to immunosurveillance by T lymphocytes, a fraction of all molecules of any protein synthesized within a cell, be it a normal cell component or a pathogen-derived product, is subject to partial proteolysis. Peptide products are transported by the transporters associated with antigen processing (TAP) to the endoplasmic reticulum (ER), where they assemble in a trimolecular complex with β_2-microglobulin and the heavy chain of MHC class I molecules. Assembly is facilitated by TAP and a number of chaperones, and allows for some peptide exchange to achieve optimal MHC class I loading. The complexes are transported to the plasma membrane, where they interact with the TCR of a T lymphocyte. The specificity of recognition is given by the specific interaction of a unique combination of a clonotypic TCR with a pathogen-derived peptide 8- to 11-aa-long presented by a MHC class I molecule (Rock and Goldberg 1999).

Antigen processing: proteolysis

Proteolysis is the first step in this so-called antigen processing and presentation pathway. Whereas all other steps have evolved to serve to the pathway, and as such, are only present in higher organisms that possess a highly developed antigen recognition system, proteolysis is a basic function of the cell. Generally speaking, proteases fall into two categories. Either they contribute to the acquisition of the final, mature form of a protein, or else they are responsible for its degradation when it is defective or no longer needed. Peptidases involved in maturation of other proteins are generally quite site- and substrate-specific, are localized to specific subcellular sites or tissues, and are tightly regulated. Degradative proteases, on the other hand, are necessarily less substrate-specific and are usually more ubiquitously distributed, but their action is, again, very carefully regulated.

By far, the most potent, multicatalytic, ubiquitous and abundant degradative protease is the cytosolic proteasome complex (Rock and Goldberg 1999;Kloetzel 2001). The proteasome complex is found in all domains of life. The primary function of the proteasome in all species is the degradation of misfolded proteins for amino acid recycling and the turnover of proteins whose function is no longer required. The immune system of higher organisms evolved to use some partial cleavage products of degraded proteins for feeding the MHC class I antigen processing pathway. In this way, this pathway can continuously and thoroughly sample a large number of peptide sequences synthesized in healthy and infected cells. In addition, an extra regulator, PA28, developed, as well as three IFN-γ-regulated catalytic subunits of the proteasome, two of which are MHC-encoded. When under conditions of inflammation these are synthesized and included in the proteasomes, the modified proteolytic activity of the resulting immunoproteasomes can again be used for the benefit of the processing and presentation pathway (Rock and Goldberg 1999;Kloetzel 2001), (review by Kloetzel, this issue). A substantial fraction of peptides derived from

proteasomal degradation and which ultimately associate with MHC class I molecules come from proteins that have recently been synthesized, and which probably fail to fold quickly and correctly. These are termed defective ribosomal products (Schubert et al. 2000).

However, the assumption that the proteasome provides all peptidic ligands for MHC class I molecules and TCR is a simplified view of the situation. There is a clear advantage on the presentation of peptides derived from newly-synthesized proteins in providing the immune system with a fast alert of an ongoing infection. But proteasomes also degrade formerly synthesized substrates, be it protein precursors, mature proteins, or deglycosylated glycoproteins transported back to the cytosol after having been inserted in the ER (Skipper et al. 1996;Bacik et al. 1997). It remains to be established for each antigenic protein the relative contribution of each form to the universe of peptides available for recognition by $CD8^+$ T lymphocytes.

On the other hand, evidence is mounting that other proteases also contribute to the pool of peptides derived from endogenously synthesized proteins and bound by MHC class I molecules (Table 1). Some of these proteases are dealt with in other articles of this issue. These alternative proteases fall into two major categories. The first class involves those proteases that have been implicated in cooperating with proteasomes. These are mainly amino-peptidases, which are postulated to trim longer peptide products generated by the proteasome. There are many reports providing evidence for amino-peptidase activity in the ER (Table 1), as well as details of some catalytic requirements (Serwold et al. 2001), but unequivocal identification of the enzyme(s) involved is still missing. Amino-peptidases cooperating with proteasomes have also been invoked in the cytosol. Identification of some candidate enzymes, such as puromycin-sensitive amino-peptidase and bleomycin-hydrolase (Stoltze et al. 2000), contrasts with the lack of information in the ER. An involvement of tripeptidyl-peptidase II in generating MHC class I ligands (Glas et al. 1998;Wang et al. 2000) could not be confirmed with a selected epitope (Princiotta et al. 2001). Leucin-amino-peptidase has

also been postulated (Beninga et al. 1998), because it is induced by IFNγ. A further protease activity that belongs to the metallopeptidase family but is not sensitive to amino-peptidase inhibitors has also been reported (Lopez et al. 2000). Endoproteases such as this one may function as trimming enzymes in sequential pathways with the proteasome. It is an interesting possibility, that is not excluded by the current data, that, in some instances, proteasomes are actually trimming the precursor peptides generated by the action of another protease in the full-length protein substrate. As compared to the full-length protein, a truncated version might be differentially susceptible to proteasome action, or even give rise to different subsets of peptides, as postulated (Cascio et al. 2001), and as described for SV40 large T antigen (Schirmbeck and Reimann 1994).

Recent evidence in the MHC class II field supports the idea that two critical aspects in antigen processing are the first endoproteolytic cleavage and the reduction of disulphide bonds in folded proteins, because either can uncover previously hidden enzymatic cleavage sites (Sercarz 2002;Maric et al. 2001). The parallel in the MHC class I situation would be that an initiating protease may affect the subsequent processing by downstream proteases of epitopes located in distant regions of the antigen. The latter might well be the proteasome in the case of cytosolic processing, or further secretory pathway proteases in the case of TAP-independent mechanisms. Tissue-specific expression of non-proteasomal proteases involved in antigen processing may lead to differential accessibility to proteasomes of pro-proteins and thus to differential epitope presentation in different cell types.

The second category of proteases includes those proteases that are capable of processing antigens fully independently of the proteasome (Table 1). These include four different proteolytic activities. The first one to be described was the signal-peptidase, whose often hydrophobic products can bind to nascent MHC class I molecules of certain allele specificities (Henderson et al. 1992;Wei and Cresswell 1992). These products, which also require cooperation from trimming enzymes (Wei

and Cresswell 1992), are sometimes liberated to the lumen of the ER, and sometimes to the cytosol, giving a TAP-independent and TAP-dependent presentation to $CD8^+$ T cells, respectively. Cystein-proteases that can process peptides of intermediate size (19-aa) that are then presented via TAP have been reported (Lopez and Del Val 1997). It is still open whether these enzymes represent trimming proteases for unfrequent long peptides (Kisselev et al. 1999) produced by the proteasome or whether they can also process longer proteins. Description of an unidentified, but extremely efficient, endoprotease that processes the Jaw1 protein that is presented also independently of TAP followed (Snyder et al. 1998;Snyder et al. 1997). Shortly thereafter, antigen processing by the trans-Golgi protease furin was reported (Gil-Torregrosa et al. 1998;Gil-Torregrosa et al. 2000). Furin is a member of the family of subtilisin-like pro-protein convertases that cleave at clusters of basic residues. The results define a proteasome-independent, TAP-independent antigen processing pathway located in the secretory route with a central role for proteolytic maturation mediated by furin, involving removal of some 50-aa-long carboxy-terminal fragments, and leading to antigen presentation from epitopes located at this part of the molecules. As with other proteases, furin profits from trimming enzymes. Notably, in this case carboxy-peptidases as well as amino-peptidases are invoked. The strong evidence, at least in the rat (Powis et al. 1996), against the presence of carboxy-peptidases in the regular site of MHC class I complex assembly, the ER, points to more distal sites in the secretory pathway for antigen processing and trimming in the furin-mediated pathway. Interestingly, the specificity of the unknown protease that cleaves Jaw1 constructs is compatible with that of furin, both recognizing multibasic stretches of residues.

Antigen presentation: from proteolysis to recognition by the TCR

Following production of intermediate or final peptides, these have to have access to peptide-receptive MHC class I molecules. As the primary site for complex assembly is the ER, where nascent class I molecules are folding, a specific transport system

evolved to translocate peptides from the cytosol to the ER. The TAP transporters have some sequences preferences, as well as peptide size limitations, that quite accurately match those of the molecules they serve, the MHC class I molecules (Uebel and Tampe 1999). Interaction of TAP with MHC class I molecules via tapasin, as well as cooperation provided by chaperones such as calnexin and calreticulin, ensure that transported peptides have the chance to bind to folding MHC class I molecules. There is increasing evidence that the stable assembly of class I molecules involves an exchange of peptides before the complexes are released from the ER. TAP serves as a retention mechanism to ensure that peptide optimisation occurs (Lewis et al. 1996). Once a complex has formed that has sufficient affinity to maintain the folded state of MHC class I heavy chain, peptide, and $\beta_2$microglobulin, it may exit the ER. As affinity can improve, a further chance for peptide binding of suboptimally loaded molecules has been identified in the Golgi (Day et al. 1995). Furthermore, as some MHC class I molecules such as H-2L^d can leave the ER without peptide, this provides them with a chance to bind peptides later in the secretory route (Hansen et al. 2000). MHC class I complexes then reach the plasma membrane, where they are recognized specifically by the TCR and the CD8 molecules of a cytotoxic T lymphocyte. Peptides may still dissociate from MHC at this point. As they are usually not replaced, this leads to irreversible unfolding of the MHC class I molecule, followed by internalization and degradation.

However, several specific routes have been reported that can by-pass the requirement for TAP, and can nevertheless deliver peptides to receptive MHC class I molecules. These include (i) leader peptides released by signal peptidase in the ER (Henderson et al. 1992;Wei and Cresswell 1992), (ii) the ectodomains of some transmembrane glycoproteins that appear to be cleaved in the ER (Snyder et al. 1998;Hammond et al. 1993;Snyder et al. 1997;Elliott et al. 1995), (iii) peptides released from maturing proteins by furin in the trans-Golgi network (Gil-Torregrosa et al. 1998;Gil-Torregrosa et al. 2000), and (iv) peptides derived from hydrophobic segments

of transmembrane proteins, that after cleavage by the proteasome can freely difuse into the ER due to their hydrophobic nature (Lautscham et al. 2001).

A further alternative level of antigen presentation by MHC class I molecules involves exogenous antigens. This is the subject of another review in this issue (Reimann). Exogenous antigens that may enter this pathway are generally whole pathogens, soluble molecules, or debris from infected cells, as well as some interesting vaccine fomulations. Uptake of exogenous antigens that results in presentation by MHC class I molecules is restricted to professional APC, dendritic cells, and macrophages. While a good deal of detail has been unraveled in the recent years concerning the vesicular paths followed by these antigens, as well as the likely compartments involved, there is almost no information on the possible proteases involved. A particularly intriguing feature of this exogenous pathway is the very efficient chaperone-facilitated receptor-mediated delivery of antigens to dendritic cells, followed by generation of antigenic peptides. The picture that emerges is that two major processing pathways exist, one ending in the cytosol, and one in the endolysosomal system. In the first case, proteasomes and other cytosolic proteases would deal with exogenous antigens in the same way as they process antigens of endogenous origin. In the second case, the whole battery of endosomal and lysosomal proteases would contribute to antigen processing (Reimann and Kaufmann 1997). Binding may take place either to exiting or else to recycling MHC class I molecules either in the same cell or in neighbouring cells after peptide regurgitation (Gromme et al. 1999;Castellino et al. 2000;Yrlid et al. 2000).

A role for non-proteasomal proteases in antigen processing

As pointed out above, proteasomes have not evolved to serve for the antigen processing pathway. Indeed, only about one third of all peptides produced by mammalian as well as archeal proteasomes have sizes that permit their feeding into the antigen presentation pathway, mostly after a necessary trimming step (Kisselev et

al. 1999). In higher organisms, special catalytic and regulatory subunits of the proteasome exist that are induced after inflammation and conform the so-called immunoproteasome (Rock and Goldberg 1999;Kloetzel 2001). These particles release final peptides of a somewhat larger median size than proteasomes, and with enhanced efficiency (Cascio et al. 2001). Thus, they might in principle be more suited for producing MHC class I ligands. In fact, they are relevant in vivo, since mice that are genetically deficient in individual subunits of the immunoproteasome show partial antigen presentation function (Fehling et al. 1994;van Kaer et al. 1994), although this does not apply to all epitopes (Yewdell et al. 1994). Analogously to proteasomes, other proteases haven't evolved either to release products for antigen presentation by MHC. But it is clear that the cell might use any of these proteolytic activities to generate MHC ligands that can signal the presence of aberrant or foreign products to surveilling immune cells.

When comparing proteasomes with other proteases, a first difference that comes to one's mind is the stricter substrate specificity of non-proteasomal peptidases. However, while this is generally true, it needs qualification. On the one hand, most other cellular proteases share with the proteasome the ability to cut less efficiently at sites other than the optimal consensus site. On the other hand, if one would rate the proteasome as other proteolytic enzymes, it would still be considered as multicatalytic, since it is able to cleave at more than one consensus site. However, the proteasome does not cleave all possible bonds with high efficiency. On average, it cleaves one tenth of all bonds in a given molecule (Kisselev et al. 1999). A fact that makes the proteasome a multipotential proteolytic enzyme for the purpose of generating MHC class I ligands is the low abundance of peptides that is needed for presentation to the ultra-sensitive CTL. This can also apply to other proteases, with some limitations such as less abundance and less susceptible cleavage sites than the proteasome. Thus, very low efficiency cleavage by proteasomes and other proteases at multiple peptide

bonds can result in generation of low numbers of many different peptides from a given protein.

Proteases that act as primary enzymes to cut long proteins will be, however, less versatile than the proteasome in generating peptides of adequate size for binding to MHC class I molecules. It is probable that these alternative proteases are more dependent on further trimming enzymes than the proteasome. However, evidence is accumulating that the proteasome does need cooperation from trimming enzymes. Whereas the proteasome appears to be the only activity in cytosol and ER that can produce the carboxy termini of MHC-binding peptides, it profits from amino-peptidases to produce the correct amino termini.

Unlike other proteases, the proteasome degrades proteins processively without release of polypeptide intermediates. This reflects its function as a protein degradative protease, as opposed to other proteases involved in protein maturation. Different to other degradative proteases, which may proceed degradation to individual aa, the proteasome releases peptides with a minimum length, of about 4-5 aa (Kisselev et al. 1999). A minor but substantial proportion of the peptides liberated has 8 residues or more. It is this property that has become critical for its acquisition of the new function of providing ligands for MHC class I molecules in higher organisms. Whereas other degradative proteases that do not share this property to some extent would be marginally useful for antigen processing, proteases involved in normal protein maturation do not have any theoretical problem in contributing to generation of intermediates for antigen processing. For example, peptides released by signal-peptidase (Wei and Cresswell 1992;Henderson et al. 1992), by furin (Gil-Torregrosa et al. 1998), or by other pro-protein convertases excising pro-peptides from precursor proteins, are in principle very well suited to this end.

One property that the proteasome lacks is access to subcellular sites other than the cytosol. This is where we expect that proteases other than the proteasome are going to be more relevant. Particularly, in the secretory pathway, which is the easiest

place for peptide products to be able to bind to receptive MHC class I molecules. It is less likely that peptidic products released by proteases in distant organelles finally enter the MHC class I pathway.

Another unfavourable property of the proteasome is that is cannot cleave all bonds within a given protein. It is for example well established that changes in the sequences that flank an epitope can dramatically alter the efficiency of cleavage by the proteasome (Del Val et al. 1991;Ossendorp et al. 1996;Theobald et al. 1998;Beekman et al. 2000). As a consequence, the efficiency of antigen presentation drops to non-functional levels. It is of course expected that other proteases might also be sensitive to this effect, individually. But it is also evident that in those situations where the proteasome cannot process at a given site, other proteases might. Thus, the action of other proteases can complement the action of the proteasome. Actually, there are two examples of viral proteins that fully escape antigen processing by the classical proteasome pathway. An immediate-early cytomegalovirus (CMV) protein cannot be processed if phosphorylated (Gilbert et al. 1996), yet it represents an immunodominant specificity in patients (Kern et al. 1999), implicating its essentially efficient processing in vivo. The second example is the Epstein-Barr virus EBNA1 protein, which has a Gly-Ala repeat that prevents its processing by the proteasome (Blake et al. 1997). It is remarkable that nevertheless in naturally infected persons a strong $CD8^+$ T-cell response is generated to this antigen, which is efficiently presented after exogenous loading and processing by a TAP-independent pathway (Blake et al. 1997;Blake et al. 2000), necessarily involving processing by a non-proteasomal protease.

As pointed out above, one might argue that individual proteases might be more sensitive than the proteasome to changing sequences, and thus less efficient at antigen processing. However, the efficiency of the whole pathway is a summation of that of all individual steps. Proteasomes generate peptides in the cytosol. There, they are potentially subject to very intense proteolysis, yielding single amino acids, unless they are rescued by chaperones and by TAP for transport into the ER, a less

degradative compartment. The balance between useful trimming enzymes in the cytosol, chaperones that protect the peptide products (Paz et al. 1999), and total cytosolic degradation can be critical. It is probable that an intermediate product of the proteasome is fully degraded if not efficiently and rapidly transported to the ER.

In addition, the actual efficiency of peptide generation by the proteasome is tightly intertwined with the peptide sequence and length preferences of TAP. And, again, TAP have some sequence selectivity, and this is not fully matched with that of the proteasome (Uebel and Tampe 1999). The human proteins transport peptides with basic or hydrophobic residues at the C-terminus, and considerable selectivity is also exerted by the three amino-terminal residues. Peptides with the amino acid proline at position 2 are not transported by human TAP. Similarly, those with proline at position 3 cannot be transported by murine TAP. Several MHC class I molecules prefer proline at these positions as anchor sequences. Particularly, numerous pathogen-derived epitopes have proline at these positions (Rammensee et al. 1999). The source of peptides for these human and murine alleles can be twofold. First, they can be transported from the cytosol as precursor peptides by TAP, and then be trimmed in the ER by amino-peptidases that stop one amino acid before proline (Serwold et al. 2001). Alternatively, they may be generated in the secretory pathway and thus circumvent TAP selectivity. Interestingly, two of the epitopes that the trans-Golgi protease furin can produce contain such a proline motif at position 2 (Gil-Torregrosa et al. 1998;Gil-Torregrosa et al. 2000), and they are presented independently of TAP. In addition, TAP is sensitive to the effect of both flanking sequences and sequences within the epitope (Udaka et al. 1994;Uebel and Tampe 1999). Indeed, although peptide selection by TAP is adapted in general terms to the peptide preferences of MHC class I molecules, TAP probably contributes to epitope selection (Yellenshaw et al. 1997a;Uebel and Tampe 1999). Particularly relevant in the context we are discussing is that TAP appears not to have evolved to adapt its specificity of transport to the cleavage specificity of the proteasome, with the exception for the common preference for carboxy-terminal

hydrophobic residues (Uebel and Tampe 1999), and this not even for TAP from all species (Powis et al. 1996). For several individual epitopes, it is well documented that TAP cannot transport the optimal final size peptide (Fu et al. 1998;Knuehl et al. 2001). Instead, longer peptides are transported. These longer peptides need further trimming at the ER, otherwise they may never have the chance to bind MHC, which would severely compromise the efficacy of the proteasome for generating MHC and TCR ligands. In the ER, proteolytic enzymes that can trim precursor peptides have been described. However, the population of trimming enzymes available in the ER appears to be restricted to amino-peptidases (Paz et al. 1999;Bacik et al. 1994;Snyder et al. 1994;Mo et al. 1999;Shastri et al. 1995;Elliott et al. 1995;Powis et al. 1996). Indeed, any carboxy-terminal extension of epitopes usually does not result in productive antigen presentation (Snyder et al. 1994).

In some cases, it has been demonstrated that the final peptide has to be produced after translocation in the ER, showing the requirement for collaboration of secretory pathway proteases. An epitope derived from simian virus 40 large T antigen was poorly presented when expressed in the cytosol as a minimal determinant, probably because it was destroyed by proteasomes. ER delivery resulted in rescued presentation (Fu et al. 1998). In an in vitro system, the immunodominant epitope from murine CMV pp89 protein was only efficiently transported as an 11-mer by TAP, suggesting that the two N-terminal residues have to be removed in the ER (Knuehl et al. 2001).

On the other hand, the efficiency and sequence restrictions of alternative pathways also rely on those of their individual components. Indeed, individual alternative proteases, as pointed above, might be more sensitive to sequence restrictions, as they are, in principle, more site-specific than the proteasome, and do not have multiple catalytic activities. However, those in the secretory pathway have by-passed the TAP bottleneck. Furthermore, late in the secretory pathway, precursor products generated by these enzymes can have access to both amino- and carboxy-

terminal trimming enzymes. Concerning efficiency, proteases other than the proteasome have an advantage over the proteasome. Indeed, the efficiency of cleavage of a given bond by the proteasome comes close to a few percent of all cleaved molecules, precisely because of the proteasome's multicatalytic potency and property of rendering short peptides as final products. In contrast, the efficiency of cleavage at its consensus site by another protease comes closer to 100%. Efficiency of cleavage at non-consensus sites is probably similar to that of the proteasome. Thus, a combination of more efficient cleavage at certain sites, independence of TAP, and availability of a wider variety of trimming enzymes, might make the alternative proteases in the secretory route a significant complement to the role of the proteasome.

Liberation of defined peptides from the C-termini of proteins and minigene products targeted to the secretory pathway has been documented (Snyder et al. 1994). The evidence points to the combined action of endoproteases and amino-peptidases in the ER (Snyder et al. 1998). When instead of assaying individual epitopes, a more general look was taken at all possible ligands for a rat MHC class I allele, it was also concluded that the ER is essentially deficient in carboxy-terminal trimming (Powis et al. 1996). On the other hand, there is good but limited evidence of the involvement of endoproteases in antigen processing in the secretory pathway. The best examples so far involve the ER-resident signal peptidase and the trans-Golgi protease furin (Gil-Torregrosa et al. 1998;Gil-Torregrosa et al. 2000). Otherwise, endoprotease activity might not be as limited as initially thought, as several examples of processing of full-length proteins in the secretory pathway have been reported (Snyder et al. 1998;Hammond et al. 1993;Snyder et al. 1997), although the proteases involved have not been characterized.

Are there any disadvantages to alternative proteases joining in to produce a richer repertoire displayed? In principle not. Actually, the proteasome itself does change into the immunoproteasome after exposure of cells to an inflammatory

microenvironment. The only risk inherent to having more proteases producing epitopes is that they may also destroy potential epitopes, as has been shown for thimet oligopeptidase (Saric et al. 2001), puromycin-sensitive aminopeptidase and bleomycin hydrolase (Stoltze et al. 2000). But this has already been described for the couple proteasome/immunoproteasome: either of them, or both, may generate the epitope. In some cases, one of them cannot cleave off the right epitope or precursor peptide (Morel et al. 2000;Schultz et al. 2002), but also there are examples where either of them actually destroys the epitope (Anton et al. 1998;Luckey et al. 1998;Vinitsky et al. 1997;Schmidtke et al. 1998;Ossendorp et al. 1996). This limitation also applies to alternative proteases, but there are no reasons to think that they may be more deleterious than the proteasome or the immunoproteasome at destroying epitopes.

On the contrary, one clear advantage of having more proteases to produce MHC class I ligands is that the universe of possible peptides produced from a single protein sequence is enlarged, giving the $CD8^+$ T cells a larger chance of detecting the infected or diseased cell. After infection, the otherwise tightly-regulated steady-state proteolytic system of a cell may be unbalanced, and new proteolytic activities be uncovered, with the result that now more peptides might be produced. An interesting possibility is that proteasomes and alternative proteases may synergize in providing each other with suitable intermediate peptides, and altogether contributing to a higher diversity or abundance of potential MHC class I ligands. The final level of presentation of many pathogen-derived epitopes is probably the result of interplay between different proteolytic activities. Thus, having alternative proteases raises the collection of possible physiological, pathological, or cell-type situations where any protease system may contribute to producing peptides.

Last but not least, availability of proteases in secretory compartments that can process peptides and deliver them to MHC class I molecules covers the pathological situation created by certain tumours and certain viruses, mainly herpesviruses, whereby TAP peptide transport function is blocked (Hill et al. 1995;Hengel et al.

1997;Alcami and Koszinowski 2000). As the property of TAP to promote assembly of peptides with MHC class I molecules is retained, secretory route processing proteases would have the chance to contribute to presentation of at least some peptides from viral glycoproteins that may still signal the infected cell for destruction by immune $CD8^+$ T cells. Indeed, TAP-negative patients do not suffer from viral infections and have herpesvirus-specific CTL (Delasalle et al. 1997). In this context, and acknowledging that the immune response during infection of the mouse with herpes simplex virus is not the best model for the human immune response to these viruses, it is intriguing that herpesvirus glycoproteins are dominant antigens recognized by murine $CD8^+$ T lymphocytes (Hanke et al. 1991).

A physiological role for non-proteasomal proteases in antigen processing in vivo

TAP-negative cells have a very much decreased surface expression of MHC class I molecules. In TAP-negative mice or patients (van Kaer et al. 1992;Delasalle et al. 1997), where the classical pathway of antigen processing and presentation is knocked out, there are few mature $CD8^+$ T cells. These two observations would speak for a minimal contribution of alternative pathways to the generation of the bulk of MHC class I ligands. However, TAP-deficient patients can generate virus-specific CTL and, more significantly, they are not unusually susceptible to viral infections (Delasalle et al. 1997). This can be interpreted as a sufficient amount of MHC class I and TCR ligands being presented by TAP-independent mechanisms in these patients. This last conclusion is also supported by the increasing reports that block of the proteasome function with a specific inhibitor, lactacystin, still allows for the generation of many individual peptide epitopes (Vinitsky et al. 1997;Benham and Neefjes 1997;Anton et al. 1998;Yellenshaw et al. 1997b;Lopez et al. 2000), in contrast to earlier observations (Rock et al. 1994;Coux et al. 1996). In some cases, presentation of certain antigens was even enhanced in the presence of proteasome inhibitors (Anton et al. 1998;Vinitsky et al. 1997;Yellenshaw et al. 1997b;Luckey et al. 1998). More generally,

a large fraction of MHC class I alleles assemble with peptides and mature normally even in the presence of lactacystin (Benham et al. 1998;Vinitsky et al. 1997;Luckey et al. 2001). More recently, peptides produced after strong inhibition of proteasome function have been analyzed by mass-spectrometry (Luckey et al. 2001). The results reveal that a diverse array of peptides continues to be presented, encompassing the complete range of C- and N-termini. Thus, non-proteasomal proteases collectively have a broad spectrum of specificities, generate a significant fraction of all peptides associated with most MHC class I alleles, and probably produce peptides from a wide variety of cellular proteins from any subcellular site. In addition, interpretation of the effect of proteasome inhibitors on global antigen presentation or MHC class I stability may lead to an underestimation of the involvement of other proteases. Indeed, these experiments inhibit not only processing mediated exclusively by proteasomes, but also the increasingly reported sequential pathways (Table 1), where trimming enzymes cooperate with proteasomes in antigen processing. Collectively, these studies strongly suggest that both proteasomes and nonproteasomal proteases can independently generate class I-associated peptides.

Lactacystin data indicate that proteases other than the lactacystin-sensitive proteasomes can generate a significant number of peptides, although it is not even excluded that this potent inhibitor is still incompletely blocking the proteasome's multiple proteolytic activities (Fenteany and Schreiber 1998). One interpretation of the fact that TAP deficiency results in a more profound deficit in antigen presentation than proteasome blockade could be that all the alternative proteases are actually TAP-dependent, that is to say, cytoplasmic. Another explanation is that lack of TAP results in a more profound deficiency in assembly of the trimolecular complex of peptide, MHC class I heavy chain and $\beta_2$microglobulin, since it acts as a molecular scaffold for the final stage of this process (Solheim 1999;Lehner and Trowsdale 1998;Androlewicz 1999;Tan et al. 2002). If this is true, it means that peptides generated anywhere in the secretory pathway assemble mainly in the ER. This is well possible, as a retrograde

transport of vesicles from the distal to the proximal regions of the secretory pathway has been described to operate for all types of cargo that is not particularly resident in the proximal areas of this route (Cole et al. 1998). Evidence from our work with the trans-Golgi protease furin also seems to be compatible with this putative binding of peptides at the ER. Indeed, we have tested several viral epitopes for presentation by the furin pathway. To this end, they were placed downstream of furin consensus cleavage sites in a secretory protein context. These epitopes were chosen to cover presentation by several class I molecules, that represent extreme behaviours in the maturation characteristics of MHC class I molecules, ability to bind peptides, nature of the peptides bound, stability in the absence of peptides, etc. The molecules tested are L^d, D^d, and K^d. L^d binds rather hydrophobic peptides with weak affinity, and binds poorly to $\beta_2$microglobulin. As a result, L^d travels very slowly to the plasma membrane, where low expression is achieved, including some empty molecules that escape thus far due to suboptimal loading in the ER. Exchange of peptides by surface L^d molecules is documented (Hansen et al. 2000). It has actually been hypothesized that MHC class I molecules that share some of these properties might be particularly prone to acquire peptides generated in alternative sites of antigen processing (Hansen et al. 2000). K^d and D^d represent the opposite behaviour to L^d. All epitopes tested so far by us are indeed presented by the pathway involving furin (Gil-Torregrosa et al. 1998;Gil-Torregrosa et al. 2000) (unpublished observations). This happens regardless of the MHC class I molecule that binds the peptides. This is most simply explained by a conventional and common binding of all peptides to MHC class I molecules at the canonical site, the ER. Experiments are underway to confirm this hypothesis. The picture may become more complex because even at the ER distinct assembly pathways seem to exist, that require more or less accessory molecules, probably depending on the pool of suitable peptides for each MHC class I allelic molecule (Androlewicz 1999).

Newly-synthesized MHC class I molecules can exchange their bound peptides, even high-affinity ligands (Sijts and Pamer 1997). This happens in the ER (Lewis and Elliott 1998), as well as at a later point in the secretory pathway (Day et al. 1995). The latter quality control stage is probably facilitated by the fact that the mildly acidic pH of these compartments favours dissociation of some peptides while promoting the association of others (Stryhn et al. 1996). This may be used by peptides processed by furin, which is active in the trans-Golgi network and beyond. Preloaded MHC class I molecules in transit to the plasma membrane may exchange the most acid-labile subset of peptides for those produced in these distal regions of the exocytic route, either derived from endogenous routes, as discussed in this review, or those derived from the extracellular area by endocytosis, as discussed in other reports in this same issue (Reimann).

An interesting implication of these findings is that a further, paradoxical, function of TAP is to permit the exit from the ER of MHC class I molecules that are empty or suboptimally loaded with peptides, that is, those molecules which may be receptive for peptide binding at later, alternative locations in the secretory or endocytic pathways. Indeed, the rapid exit from the ER of empty molecules of an MHC class I mutant is surprisingly dependent on a TAP-derived supply of peptides (Lewis and Elliott 1998). Also, low efficiency of extra-cytosolic presentation of a bacterial-expressed antigen in TAP-deficient cells was found to parallel the concomitant decrease of peptide-receptive MHC class I molecules at the cell surface (Song and Harding 1996;Yewdell et al. 1999). In the absence of TAP expression, in TAP-deficient cells and organisms, MHC class I molecules are retained in the ER and eventually degraded, without having access to potential good ligands produced later in the exocytic pathway. In normal conditions, that is, in the presence of TAP, access to those later ligands should not be impaired. In summary, in addition to its transporter function, TAP is involved in MHC class I assembly with peptides, whatever their procedence, and TAP is needed for generation of peptide-receptive MHC class I molecules in vesicular compartments

other than the ER. Thus, studies with TAP-deficient cells or organisms, which lack all three TAP functions, while necessary for uncovering these alternative pathways, lead to an underestimation of the real contribution from TAP-independent pathways to the overall amount and diversity of epitopes available for T cell recognition.

Classical and alternative antigen processing pathways in the initiation of the immune response by professional antigen-presenting cells

Peptides presented by MHC class I molecules are in most cell types limited to those of endogenous origin, synthesized in the presenting cell. Functionally, this prevents lysis by CTL of neighbour cells that may have phagocytosed virally-infected or tumour cells, but are not infected or transformed themselves. There is, however, an exception to this safety limitation in the MHC class I pathway, which is antigen processing and presentation by professional antigen presenting cells (pAPC). This is an issue of outstanding interest nowadays. Professional APC, such as dendritic cells, are critical in initiating the immune response to pathogens (Heath and Carbone 2001;den Haan and Bevan 2001). They sample peripheral tissues capturing potential antigens, and transfer them to the lymph nodes, where naive T cells are found. In addition, while some intracellular pathogens infect such cells preserving their antigen processing and presentation abilities, for most pathogens either direct infection of dendritic cells does not occur, or infection results in abrogation of pAPC function. This is particularly evident for those viruses that have evolved strategies to evade antigen presentation (Alcami and Koszinowski 2000). In these two latter cases, initiation of the immune response relies on the cross-presentation ability of pAPC, that is, on their ability to take up antigens from surrounding infected cell debris and introduce them into their endogenous pathway of antigen processing for presentation by MHC class I molecules. Cross-priming follows, that is, antigen-specific stimulation of naive $CD8^+$ T cells by these cross-presenting dendritic cells. Essentially two major pathways have been identified (Thery and Amigorena 2001). One seems to be the classical pathway,

in that it is TAP-dependent, and most probably involves delivery of exogenous antigens from the phagosome to the cytosol (Kovacsovics-Bankowski and Rock 1995), by a dendritic-cell-selective membrane transport pathway involving the ER (Rodriguez et al. 1999). This would be followed by processing by proteasomes, transport by TAP, and loading onto nascent MHC class I molecules in the ER.

The second pathway that has been identified in pAPC is TAP-independent. This implies that the antigens do not gain access to the cytosol, and must rather travel from the endosomes to undefined compartments of the constitutive secretory pathway. As a consequence, they are processed necessarily by non-proteasomal and non-cytosolic proteases. Binding of final peptides may occur in the canonical location, the ER. It is not excluded, though, that they might intersect the pathway at later stages and replace sub-optimally bound peptides in a step that would optimize the affinity, and presumably the half-life, of peptide/MHC complexes at the plasma membrane. Indeed, peptide/MHC class I complex formation after cross-presentation by dendritic cells has been demonstrated at either site, the ER and the endosomes (Castellino et al. 2000) or the MHC class II compartments (Gromme et al. 1999;Yrlid et al. 2000). Which of these pathways, the classical TAP-dependent or the alternative TAP-independent one, is more relevant in different relevant pAPC is an open question, since it seems to vary for different viruses, antigenic determinants, delivery systems, and even protein contexts (Norbury et al. 2001;Sigal and Rock 2000). Of particular importance is elucidating the relative contributions of these pathways in vivo. Some examples of the relevance of the TAP-dependent pathway for tumour cells and some viruses (Huang et al. 1996;Sigal et al. 1999;Sigal and Rock 2000), and of the TAP-independent pathway for a soluble protein expressed by a virus and for epitopes from three other viruses (Norbury et al. 2001;Sigal and Rock 2000;Blake et al. 2000) have already been reported. As discussed above, the described relative inefficiency of the TAP-independent pathway (Sigal and Rock 2000) might rather reflect a limitation of the experimental models necessary to unveil it, which imply use of TAP-deficient systems. These are not only

deficient in peptide transport to the ER, but also in peptide/MHC class I complex formation in the ER and at later locations in the secretory pathway.

The same question of the relative contribution of either pathway is also relevant for any other cell type susceptible to any infectious agent. So far, alternative proteases or processing pathways have been qualitatively defined. However, their quantitative relevance in comparison with the classical proteasome pathway remains to be established. It is also at present open which is the physiological relevance of these novel pathways. Particularly, one might question whether potential alternative processing in pAPC would be of any relevance, were the products of this alternative processing not to exist in normal infected cells. Indeed, if antigen processing during cross-presentation yields a series of epitopes by these alternative pathways, and these epitopes are never presented during normal infection, the activation of $CD8^+$ T cells specific for these epitopes would be useless, as shown for particular bacterial or viral antigens (Shen et al. 1998;Blake et al. 1997). This is probably an innocuous side-effect of the need to have the widest variety of epitope-producing pathways in pAPC, to cover most of the possible processing pathways generating epitopes in any type of infected cell. Conversely, production of epitopes by the alternative pathway only in normal cells would pass undetected, as T cells reactive with them would never be activated by professional APC.

All three different processing routes tested so far for the 9pp89 epitope render the same final peptide as the proteasome pathway.

Although this is an issue that warrants clearance on its own, some results from our group provide a way around this paradox. We have described so far several alternative protease systems for antigen processing (Figure 1). Some of them are completely independent of the proteasome, which implies that the proteases involved are *bona fide* antigen processing enzymes. One of them is quite defined, inasmuch as the protease involved is fully characterized: the trans-Golgi protease furin. This pro-

protein convertase removes from precursor proteins a C-terminal fragment containing antigenic epitopes, than can be trimmed and presented by several MHC class I molecules (Gil-Torregrosa et al. 1998;Gil-Torregrosa et al. 2000). In the second system, uncharacterized cystein-proteases mediate proteasome-independent, ubiquitin-independent antigen processing of relatively short endogenously-synthesized peptides (Lopez and Del Val 1997). In a third system not fully characterized (M. Montoya, I. Casal, M. Del Val, unpublished observations) (not depicted in Figure 1), antigen processing occurs exogenously at the plasma membrane. One common epitope was tested in all three cases. After infection of BALB/c mice with murine CMV, the immunodominant determinant recognized by $CD8^+$ CTL has been shown to be 9pp89 (Del Val et al. 1988), which accounts for half of all activated CMV-specific CTL precursors (Reddehase and Koszinowski 1984). The epitope is a 9mer derived from the non-structural nuclear phosphoprotein pp89, which is expressed immediately after infection. Intrinsic features of 9pp89 itself, such as its strong association with the presenting molecule L^d, contribute to its capacity to induce $CD8^+$-mediated protective immunity when expressed in isolation from other CMV sequences in rVV (Jonjic et al. 1988). Naturally processed peptides were isolated from infected cells that were treated or chosen to allow antigen processing by each of these pathways selectively, or by the classical, proteasome pathway. When they were analyzed by HPLC and detected with specific CTL, the antigenic peptides were found in all three cases to have the same chromatographic properties among them, and also to be undistinguishable from the peptide endogenously produced by proteasomes.

We assume in some of the cases (Gil-Torregrosa et al. 2000) (D. López, M. Montoya, unpublished observations), and in others have direct proof of it (Gil-Torregrosa et al. 1998), that antigen processing by each of these three pathways is the result of the concerted action of several proteases, one giving the most critical, probably the initial, cut, and others essential for trimming. We do not expect that the finality of all these systems is to produce the same and only optimal 9mer peptide. We

rather believe that several peptides are generated by each pathway, and recall the central role of MHC class I molecules. These molecules are essential either in selecting the peptide with highest affinity among a pool of available products (Ojcius et al. 1994), or in directing the final trimming of precursors (Brouwenstijn et al. 2001;Komlosh et al. 2001), competing with the exopeptidases for access to the core peptide. We postulate that this is true in general terms for major high-affinity immunodominant antigens, although the relative abundance of several epitopes from the same pathogen may differ depending on the predominant processing pathway. Thus, classical and alternative processing pathways may operate to a different extent in different antigen processing cells, be it infected pAPC, pAPC mediating cross-presentation, or infected parenchymal cells, but essentially the same peptide would be selected by MHC class I molecules form the pool of products of each pathway. An exception is represented by epitopes derived from EBV EBNA1 protein (Blake et al. 1997) or from nonsecreted proteins from intracellular bacteria (Shen et al. 1998), that are only produced by pAPC during cross-presentation, but not during infection, and that have therefore little relevance in terms of controlling infection. This probably represents the necessarily redundant and sometimes superfluous effort of pAPC to process and display as many epitopes as might be produced later in any type of infected parenchymal cell. Although some pathways and subcellular localizations, as well as different forms of antigen delivery, will certainly produce distinct sets of peptides (the remarkable example of HBsAg is reviewed by Reimann, this issue), we expect these peptide collections to be rather overlapping. Thus, in general terms, there should not be any major conflict between the universe of peptides inducing activation of naive T cells and those displayed at infected cells that have to be recognized and cured or eliminated. In any case, availability of more antigen processing pathways can only be for good, contributing to expanding the repertoire of pathogen-derived MHC ligands for recognition by the TCR.

Conclusions

From initial assumptions that the multicatalytic and ubiquitous proteasome is the only protease capable of fully generating peptide ligands for MHC class I molecules, the last few years have seen the identification of a surprisingly large number of alternative proteases that contribute to endogenous antigen processing. Trimming by non-proteasomal proteases of precursor peptides produced by proteasomes is now a well-established fact. More importantly, proteases that can process antigens in a fully proteasome-independent fashion have also been identified. While in still too many cases the evidence for these proteolytic activities is still only indirect, the next few years will probably see the ascription of these activities to individual enzymes. We will learn about the possibly redundant and sometimes complementary action of different proteases. This will represent a big leap in understanding their mechanism of action and in unravelling the detailed and complex pathways of antigen processing by the concerted action of multiple proteases. As a consequence, more will be known of the available mechanisms to present pathogen-derived sequences to $CD8^+$ T lymphocytes. Establishing the relevance of each pathway in vivo in different parenchymal or professional APC will contribute to the rational design of new vaccines to combat infectious diseases.

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Figure legend

Figure 1. Novel routes of viral antigen processing described by the authors.

The trans-Golgi network pro-protein convertase furin acts on secretory pathway precursor proteins with stretches of basic residues. It liberates a C-terminal fragment containing antigenic epitopes, that can be trimmed by amino- and by carboxy-peptidases and then presented by several MHC class I molecules (Gil-Torregrosa et al. 1998;Gil-Torregrosa et al. 2000). This pathway is TAP- and proteasome-independent. In the second route, cystein-proteases mediate proteasome-independent, ubiquitin-independent, TAP-dependent antigen processing of relatively short endogenously-synthesized peptides (Lopez and Del Val 1997). These two pathways can process the immunodominant CMV pp89 9mer epitope (Del Val et al. 1988) from suitable constructs, and allow presentation by the MHC class I molecule L^d to specific $CD8^+$ CTL. The subcellular localization of these cystein-proteases, as well as that of metallo-proteases, is at present unknown. The metallo-proteases process the full-length antigenic glycoprotein ENV from HIV in cooperation with the proteasome, following a TAP-dependent sequential pathway (Lopez et al. 2000). P stands for peptidase.

Multiple proteases process viral antigens for presentation by MHC class I molecules to $CD8^+$ T lymphocytes.

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Abstract

Recognition by $CD8^+$ cytotoxic T lymphocytes of any intracellular viral protein requires its initial cytosolic proteolytic processing, the translocation of processed peptides to the endoplasmic reticulum via the transporters associated with antigen processing, TAP, and their binding to nascent MHC class I molecules that then present the antigenic peptides at the infected cell surface. From initial assumptions that the multicatalytic and ubiquitous proteasome is the only protease capable of fully generating peptide ligands for MHC class I molecules, the last few years have seen the identification of a number of alternative proteases that contribute to endogenous antigen processing. Trimming by non-proteasomal proteases of precursor peptides produced by proteasomes is now a well-established fact. In addition, proteases that can process antigens in a fully proteasome-independent fashion have also been identified. The final level of presentation of many viral epitopes is probably the result of interplay between different proteolytic activities. This expands the number of tissues and physiological and pathological situations compatible with antigen presentation, as well as the universe of pathogen-derived sequences available for recognition by $CD8^+$ T lymphocytes.

The classical antigen processing and presentation pathway

After infection of an organism by a pathogen, the immune system is faced with the tasks of identifying and curing or eliminating the infected cells and the circulating pathogen. Recognition of foreign, soluble, repetitive particles, such as most pathogens are, is a task that is mostly carried out by soluble effectors, mainly antibodies. In collaboration with phagocytic cells, they contribute to their effective removal from the organism. Discrimination between healthy and infected cells, identifying the foreign determinants among a background of self cellular components, is the task of immune cells. Antigen-specific lymphocytes screen the surface of infected cells, where not only

surface products but also intracellular components are displayed. Specialized molecules encoded by genes in the major histocompatible complex (MHC) are responsible for displaying the universe of cellular components for screening by the antigen-specific receptor of T lymphocytes, the T-cell receptor (TCR). It is the molecular details of this process that are the scope of this review, particularly in what pertains the recent evidence on the involvement of multiple cellular proteases in this process. As a consequence of recognition by specific T lymphocytes of pathogen-derived, MHC-presented antigens, the infected cells are either physically eliminated by cytotoxic T lymphocytes, or cured from the virus infection by cytokines released by these same T cells.

In an infected cell, macromolecular synthesis of cellular as well as pathogen-encoded components takes place. Proteins encoded by the pathogen are synthesized in the cell's ribosomes, and can thereafter localize to any subcellular compartment. As part of the mechanism leading to immunosurveillance by T lymphocytes, a fraction of all molecules of any protein synthesized within a cell, be it a normal cell component or a pathogen-derived product, is subject to partial proteolysis. Peptide products are transported by the transporters associated with antigen processing (TAP) to the endoplasmic reticulum (ER), where they assemble in a trimolecular complex with β_2-microglobulin and the heavy chain of MHC class I molecules. Assembly is facilitated by TAP and a number of chaperones, and allows for some peptide exchange to achieve optimal MHC class I loading. The complexes are transported to the plasma membrane, where they interact with the TCR of a T lymphocyte. The specificity of recognition is given by the specific interaction of a unique combination of a clonotypic TCR with a pathogen-derived peptide 8- to 11-aa-long presented by a MHC class I molecule (Rock and Goldberg 1999).

Antigen processing: proteolysis

Proteolysis is the first step in this so-called antigen processing and presentation pathway. Whereas all other steps have evolved to serve to the pathway, and as such, are only present in higher organisms that possess a highly developed antigen recognition system, proteolysis is a basic function of the cell. Generally speaking, proteases fall into two categories. Either they contribute to the acquisition of the final, mature form of a protein, or else they are responsible for its degradation when it is defective or no longer needed. Peptidases involved in maturation of other proteins are generally quite site- and substrate-specific, are localized to specific subcellular sites or tissues, and are tightly regulated. Degradative proteases, on the other hand, are necessarily less substrate-specific and are usually more ubiquitously distributed, but their action is, again, very carefully regulated.

By far, the most potent, multicatalytic, ubiquitous and abundant degradative protease is the cytosolic proteasome complex (Rock and Goldberg 1999;Kloetzel 2001). The proteasome complex is found in all domains of life. The primary function of the proteasome in all species is the degradation of misfolded proteins for amino acid recycling and the turnover of proteins whose function is no longer required. The immune system of higher organisms evolved to use some partial cleavage products of degraded proteins for feeding the MHC class I antigen processing pathway. In this way, this pathway can continuously and thoroughly sample a large number of peptide sequences synthesized in healthy and infected cells. In addition, an extra regulator, PA28, developed, as well as three IFN-γ-regulated catalytic subunits of the proteasome, two of which are MHC-encoded. When under conditions of inflammation these are synthesized and included in the proteasomes, the modified proteolytic activity of the resulting immunoproteasomes can again be used for the benefit of the processing and presentation pathway (Rock and Goldberg 1999;Kloetzel 2001), (review by Kloetzel, this issue). A substantial fraction of peptides derived from

proteasomal degradation and which ultimately associate with MHC class I molecules come from proteins that have recently been synthesized, and which probably fail to fold quickly and correctly. These are termed defective ribosomal products (Schubert et al. 2000).

However, the assumption that the proteasome provides all peptidic ligands for MHC class I molecules and TCR is a simplified view of the situation. There is a clear advantage on the presentation of peptides derived from newly-synthesized proteins in providing the immune system with a fast alert of an ongoing infection. But proteasomes also degrade formerly synthesized substrates, be it protein precursors, mature proteins, or deglycosylated glycoproteins transported back to the cytosol after having been inserted in the ER (Skipper et al. 1996;Bacik et al. 1997). It remains to be established for each antigenic protein the relative contribution of each form to the universe of peptides available for recognition by $CD8^+$ T lymphocytes.

On the other hand, evidence is mounting that other proteases also contribute to the pool of peptides derived from endogenously synthesized proteins and bound by MHC class I molecules (Table 1). Some of these proteases are dealt with in other articles of this issue. These alternative proteases fall into two major categories. The first class involves those proteases that have been implicated in cooperating with proteasomes. These are mainly amino-peptidases, which are postulated to trim longer peptide products generated by the proteasome. There are many reports providing evidence for amino-peptidase activity in the ER (Table 1), as well as details of some catalytic requirements (Serwold et al. 2001), but unequivocal identification of the enzyme(s) involved is still missing. Amino-peptidases cooperating with proteasomes have also been invoked in the cytosol. Identification of some candidate enzymes, such as puromycin-sensitive amino-peptidase and bleomycin-hydrolase (Stoltze et al. 2000), contrasts with the lack of information in the ER. An involvement of tripeptidyl-peptidase II in generating MHC class I ligands (Glas et al. 1998;Wang et al. 2000) could not be confirmed with a selected epitope (Princiotta et al. 2001). Leucin-amino-peptidase has

also been postulated (Beninga et al. 1998), because it is induced by IFNγ. A further protease activity that belongs to the metallopeptidase family but is not sensitive to amino-peptidase inhibitors has also been reported (Lopez et al. 2000). Endoproteases such as this one may function as trimming enzymes in sequential pathways with the proteasome. It is an interesting possibility, that is not excluded by the current data, that, in some instances, proteasomes are actually trimming the precursor peptides generated by the action of another protease in the full-length protein substrate. As compared to the full-length protein, a truncated version might be differentially susceptible to proteasome action, or even give rise to different subsets of peptides, as postulated (Cascio et al. 2001), and as described for SV40 large T antigen (Schirmbeck and Reimann 1994).

Recent evidence in the MHC class II field supports the idea that two critical aspects in antigen processing are the first endoproteolytic cleavage and the reduction of disulphide bonds in folded proteins, because either can uncover previously hidden enzymatic cleavage sites (Sercarz 2002;Maric et al. 2001). The parallel in the MHC class I situation would be that an initiating protease may affect the subsequent processing by downstream proteases of epitopes located in distant regions of the antigen. The latter might well be the proteasome in the case of cytosolic processing, or further secretory pathway proteases in the case of TAP-independent mechanisms. Tissue-specific expression of non-proteasomal proteases involved in antigen processing may lead to differential accessibility to proteasomes of pro-proteins and thus to differential epitope presentation in different cell types.

The second category of proteases includes those proteases that are capable of processing antigens fully independently of the proteasome (Table 1). These include four different proteolytic activities. The first one to be described was the signal-peptidase, whose often hydrophobic products can bind to nascent MHC class I molecules of certain allele specificities (Henderson et al. 1992;Wei and Cresswell 1992). These products, which also require cooperation from trimming enzymes (Wei

and Cresswell 1992), are sometimes liberated to the lumen of the ER, and sometimes to the cytosol, giving a TAP-independent and TAP-dependent presentation to $CD8^+$ T cells, respectively. Cystein-proteases that can process peptides of intermediate size (19-aa) that are then presented via TAP have been reported (Lopez and Del Val 1997). It is still open whether these enzymes represent trimming proteases for unfrequent long peptides (Kisselev et al. 1999) produced by the proteasome or whether they can also process longer proteins. Description of an unidentified, but extremely efficient, endoprotease that processes the Jaw1 protein that is presented also independently of TAP followed (Snyder et al. 1998;Snyder et al. 1997). Shortly thereafter, antigen processing by the trans-Golgi protease furin was reported (Gil-Torregrosa et al. 1998;Gil-Torregrosa et al. 2000). Furin is a member of the family of subtilisin-like pro-protein convertases that cleave at clusters of basic residues. The results define a proteasome-independent, TAP-independent antigen processing pathway located in the secretory route with a central role for proteolytic maturation mediated by furin, involving removal of some 50-aa-long carboxy-terminal fragments, and leading to antigen presentation from epitopes located at this part of the molecules. As with other proteases, furin profits from trimming enzymes. Notably, in this case carboxy-peptidases as well as amino-peptidases are invoked. The strong evidence, at least in the rat (Powis et al. 1996), against the presence of carboxy-peptidases in the regular site of MHC class I complex assembly, the ER, points to more distal sites in the secretory pathway for antigen processing and trimming in the furin-mediated pathway. Interestingly, the specificity of the unknown protease that cleaves Jaw1 constructs is compatible with that of furin, both recognizing multibasic stretches of residues.

Antigen presentation: from proteolysis to recognition by the TCR

Following production of intermediate or final peptides, these have to have access to peptide-receptive MHC class I molecules. As the primary site for complex assembly is the ER, where nascent class I molecules are folding, a specific transport system

evolved to translocate peptides from the cytosol to the ER. The TAP transporters have some sequences preferences, as well as peptide size limitations, that quite accurately match those of the molecules they serve, the MHC class I molecules (Uebel and Tampe 1999). Interaction of TAP with MHC class I molecules via tapasin, as well as cooperation provided by chaperones such as calnexin and calreticulin, ensure that transported peptides have the chance to bind to folding MHC class I molecules. There is increasing evidence that the stable assembly of class I molecules involves an exchange of peptides before the complexes are released from the ER. TAP serves as a retention mechanism to ensure that peptide optimisation occurs (Lewis et al. 1996). Once a complex has formed that has sufficient affinity to maintain the folded state of MHC class I heavy chain, peptide, and $\beta_2$microglobulin, it may exit the ER. As affinity can improve, a further chance for peptide binding of suboptimally loaded molecules has been identified in the Golgi (Day et al. 1995). Furthermore, as some MHC class I molecules such as H-2L^d can leave the ER without peptide, this provides them with a chance to bind peptides later in the secretory route (Hansen et al. 2000). MHC class I complexes then reach the plasma membrane, where they are recognized specifically by the TCR and the CD8 molecules of a cytotoxic T lymphocyte. Peptides may still dissociate from MHC at this point. As they are usually not replaced, this leads to irreversible unfolding of the MHC class I molecule, followed by internalization and degradation.

However, several specific routes have been reported that can by-pass the requirement for TAP, and can nevertheless deliver peptides to receptive MHC class I molecules. These include (i) leader peptides released by signal peptidase in the ER (Henderson et al. 1992;Wei and Cresswell 1992), (ii) the ectodomains of some transmembrane glycoproteins that appear to be cleaved in the ER (Snyder et al. 1998;Hammond et al. 1993;Snyder et al. 1997;Elliott et al. 1995), (iii) peptides released from maturing proteins by furin in the trans-Golgi network (Gil-Torregrosa et al. 1998;Gil-Torregrosa et al. 2000), and (iv) peptides derived from hydrophobic segments

of transmembrane proteins, that after cleavage by the proteasome can freely difuse into the ER due to their hydrophobic nature (Lautscham et al. 2001).

A further alternative level of antigen presentation by MHC class I molecules involves exogenous antigens. This is the subject of another review in this issue (Reimann). Exogenous antigens that may enter this pathway are generally whole pathogens, soluble molecules, or debris from infected cells, as well as some interesting vaccine fomulations. Uptake of exogenous antigens that results in presentation by MHC class I molecules is restricted to professional APC, dendritic cells, and macrophages. While a good deal of detail has been unraveled in the recent years concerning the vesicular paths followed by these antigens, as well as the likely compartments involved, there is almost no information on the possible proteases involved. A particularly intriguing feature of this exogenous pathway is the very efficient chaperone-facilitated receptor-mediated delivery of antigens to dendritic cells, followed by generation of antigenic peptides. The picture that emerges is that two major processing pathways exist, one ending in the cytosol, and one in the endolysosomal system. In the first case, proteasomes and other cytosolic proteases would deal with exogenous antigens in the same way as they process antigens of endogenous origin. In the second case, the whole battery of endosomal and lysosomal proteases would contribute to antigen processing (Reimann and Kaufmann 1997). Binding may take place either to exiting or else to recycling MHC class I molecules either in the same cell or in neighbouring cells after peptide regurgitation (Gromme et al. 1999;Castellino et al. 2000;Yrlid et al. 2000).

A role for non-proteasomal proteases in antigen processing

As pointed out above, proteasomes have not evolved to serve for the antigen processing pathway. Indeed, only about one third of all peptides produced by mammalian as well as archeal proteasomes have sizes that permit their feeding into the antigen presentation pathway, mostly after a necessary trimming step (Kisselev et

al. 1999). In higher organisms, special catalytic and regulatory subunits of the proteasome exist that are induced after inflammation and conform the so-called immunoproteasome (Rock and Goldberg 1999;Kloetzel 2001). These particles release final peptides of a somewhat larger median size than proteasomes, and with enhanced efficiency (Cascio et al. 2001). Thus, they might in principle be more suited for producing MHC class I ligands. In fact, they are relevant in vivo, since mice that are genetically deficient in individual subunits of the immunoproteasome show partial antigen presentation function (Fehling et al. 1994;van Kaer et al. 1994), although this does not apply to all epitopes (Yewdell et al. 1994). Analogously to proteasomes, other proteases haven't evolved either to release products for antigen presentation by MHC. But it is clear that the cell might use any of these proteolytic activities to generate MHC ligands that can signal the presence of aberrant or foreign products to surveilling immune cells.

When comparing proteasomes with other proteases, a first difference that comes to one's mind is the stricter substrate specificity of non-proteasomal peptidases. However, while this is generally true, it needs qualification. On the one hand, most other cellular proteases share with the proteasome the ability to cut less efficiently at sites other than the optimal consensus site. On the other hand, if one would rate the proteasome as other proteolytic enzymes, it would still be considered as multicatalytic, since it is able to cleave at more than one consensus site. However, the proteasome does not cleave all possible bonds with high efficiency. On average, it cleaves one tenth of all bonds in a given molecule (Kisselev et al. 1999). A fact that makes the proteasome a multipotential proteolytic enzyme for the purpose of generating MHC class I ligands is the low abundance of peptides that is needed for presentation to the ultra-sensitive CTL. This can also apply to other proteases, with some limitations such as less abundance and less susceptible cleavage sites than the proteasome. Thus, very low efficiency cleavage by proteasomes and other proteases at multiple peptide

bonds can result in generation of low numbers of many different peptides from a given protein.

Proteases that act as primary enzymes to cut long proteins will be, however, less versatile than the proteasome in generating peptides of adequate size for binding to MHC class I molecules. It is probable that these alternative proteases are more dependent on further trimming enzymes than the proteasome. However, evidence is accumulating that the proteasome does need cooperation from trimming enzymes. Whereas the proteasome appears to be the only activity in cytosol and ER that can produce the carboxy termini of MHC-binding peptides, it profits from amino-peptidases to produce the correct amino termini.

Unlike other proteases, the proteasome degrades proteins processively without release of polypeptide intermediates. This reflects its function as a protein degradative protease, as opposed to other proteases involved in protein maturation. Different to other degradative proteases, which may proceed degradation to individual aa, the proteasome releases peptides with a minimum length, of about 4-5 aa (Kisselev et al. 1999). A minor but substantial proportion of the peptides liberated has 8 residues or more. It is this property that has become critical for its acquisition of the new function of providing ligands for MHC class I molecules in higher organisms. Whereas other degradative proteases that do not share this property to some extent would be marginally useful for antigen processing, proteases involved in normal protein maturation do not have any theoretical problem in contributing to generation of intermediates for antigen processing. For example, peptides released by signal-peptidase (Wei and Cresswell 1992;Henderson et al. 1992), by furin (Gil-Torregrosa et al. 1998), or by other pro-protein convertases excising pro-peptides from precursor proteins, are in principle very well suited to this end.

One property that the proteasome lacks is access to subcellular sites other than the cytosol. This is where we expect that proteases other than the proteasome are going to be more relevant. Particularly, in the secretory pathway, which is the easiest

place for peptide products to be able to bind to receptive MHC class I molecules. It is less likely that peptidic products released by proteases in distant organelles finally enter the MHC class I pathway.

Another unfavourable property of the proteasome is that is cannot cleave all bonds within a given protein. It is for example well established that changes in the sequences that flank an epitope can dramatically alter the efficiency of cleavage by the proteasome (Del Val et al. 1991;Ossendorp et al. 1996;Theobald et al. 1998;Beekman et al. 2000). As a consequence, the efficiency of antigen presentation drops to non-functional levels. It is of course expected that other proteases might also be sensitive to this effect, individually. But it is also evident that in those situations where the proteasome cannot process at a given site, other proteases might. Thus, the action of other proteases can complement the action of the proteasome. Actually, there are two examples of viral proteins that fully escape antigen processing by the classical proteasome pathway. An immediate-early cytomegalovirus (CMV) protein cannot be processed if phosphorylated (Gilbert et al. 1996), yet it represents an immunodominant specificity in patients (Kern et al. 1999), implicating its essentially efficient processing in vivo. The second example is the Epstein-Barr virus EBNA1 protein, which has a Gly-Ala repeat that prevents its processing by the proteasome (Blake et al. 1997). It is remarkable that nevertheless in naturally infected persons a strong $CD8^+$ T-cell response is generated to this antigen, which is efficiently presented after exogenous loading and processing by a TAP-independent pathway (Blake et al. 1997;Blake et al. 2000), necessarily involving processing by a non-proteasomal protease.

As pointed out above, one might argue that individual proteases might be more sensitive than the proteasome to changing sequences, and thus less efficient at antigen processing. However, the efficiency of the whole pathway is a summation of that of all individual steps. Proteasomes generate peptides in the cytosol. There, they are potentially subject to very intense proteolysis, yielding single amino acids, unless they are rescued by chaperones and by TAP for transport into the ER, a less

degradative compartment. The balance between useful trimming enzymes in the cytosol, chaperones that protect the peptide products (Paz et al. 1999), and total cytosolic degradation can be critical. It is probable that an intermediate product of the proteasome is fully degraded if not efficiently and rapidly transported to the ER.

In addition, the actual efficiency of peptide generation by the proteasome is tightly intertwined with the peptide sequence and length preferences of TAP. And, again, TAP have some sequence selectivity, and this is not fully matched with that of the proteasome (Uebel and Tampe 1999). The human proteins transport peptides with basic or hydrophobic residues at the C-terminus, and considerable selectivity is also exerted by the three amino-terminal residues. Peptides with the amino acid proline at position 2 are not transported by human TAP. Similarly, those with proline at position 3 cannot be transported by murine TAP. Several MHC class I molecules prefer proline at these positions as anchor sequences. Particularly, numerous pathogen-derived epitopes have proline at these positions (Rammensee et al. 1999). The source of peptides for these human and murine alleles can be twofold. First, they can be transported from the cytosol as precursor peptides by TAP, and then be trimmed in the ER by amino-peptidases that stop one amino acid before proline (Serwold et al. 2001). Alternatively, they may be generated in the secretory pathway and thus circumvent TAP selectivity. Interestingly, two of the epitopes that the trans-Golgi protease furin can produce contain such a proline motif at position 2 (Gil-Torregrosa et al. 1998;Gil-Torregrosa et al. 2000), and they are presented independently of TAP. In addition, TAP is sensitive to the effect of both flanking sequences and sequences within the epitope (Udaka et al. 1994;Uebel and Tampe 1999). Indeed, although peptide selection by TAP is adapted in general terms to the peptide preferences of MHC class I molecules, TAP probably contributes to epitope selection (Yellenshaw et al. 1997a;Uebel and Tampe 1999). Particularly relevant in the context we are discussing is that TAP appears not to have evolved to adapt its specificity of transport to the cleavage specificity of the proteasome, with the exception for the common preference for carboxy-terminal

hydrophobic residues (Uebel and Tampe 1999), and this not even for TAP from all species (Powis et al. 1996). For several individual epitopes, it is well documented that TAP cannot transport the optimal final size peptide (Fu et al. 1998;Knuehl et al. 2001). Instead, longer peptides are transported. These longer peptides need further trimming at the ER, otherwise they may never have the chance to bind MHC, which would severely compromise the efficacy of the proteasome for generating MHC and TCR ligands. In the ER, proteolytic enzymes that can trim precursor peptides have been described. However, the population of trimming enzymes available in the ER appears to be restricted to amino-peptidases (Paz et al. 1999;Bacik et al. 1994;Snyder et al. 1994;Mo et al. 1999;Shastri et al. 1995;Elliott et al. 1995;Powis et al. 1996). Indeed, any carboxy-terminal extension of epitopes usually does not result in productive antigen presentation (Snyder et al. 1994).

In some cases, it has been demonstrated that the final peptide has to be produced after translocation in the ER, showing the requirement for collaboration of secretory pathway proteases. An epitope derived from simian virus 40 large T antigen was poorly presented when expressed in the cytosol as a minimal determinant, probably because it was destroyed by proteasomes. ER delivery resulted in rescued presentation (Fu et al. 1998). In an in vitro system, the immunodominant epitope from murine CMV pp89 protein was only efficiently transported as an 11-mer by TAP, suggesting that the two N-terminal residues have to be removed in the ER (Knuehl et al. 2001).

On the other hand, the efficiency and sequence restrictions of alternative pathways also rely on those of their individual components. Indeed, individual alternative proteases, as pointed above, might be more sensitive to sequence restrictions, as they are, in principle, more site-specific than the proteasome, and do not have multiple catalytic activities. However, those in the secretory pathway have by-passed the TAP bottleneck. Furthermore, late in the secretory pathway, precursor products generated by these enzymes can have access to both amino- and carboxy-

terminal trimming enzymes. Concerning efficiency, proteases other than the proteasome have an advantage over the proteasome. Indeed, the efficiency of cleavage of a given bond by the proteasome comes close to a few percent of all cleaved molecules, precisely because of the proteasome's multicatalytic potency and property of rendering short peptides as final products. In contrast, the efficiency of cleavage at its consensus site by another protease comes closer to 100%. Efficiency of cleavage at non-consensus sites is probably similar to that of the proteasome. Thus, a combination of more efficient cleavage at certain sites, independence of TAP, and availability of a wider variety of trimming enzymes, might make the alternative proteases in the secretory route a significant complement to the role of the proteasome.

Liberation of defined peptides from the C-termini of proteins and minigene products targeted to the secretory pathway has been documented (Snyder et al. 1994). The evidence points to the combined action of endoproteases and amino-peptidases in the ER (Snyder et al. 1998). When instead of assaying individual epitopes, a more general look was taken at all possible ligands for a rat MHC class I allele, it was also concluded that the ER is essentially deficient in carboxy-terminal trimming (Powis et al. 1996). On the other hand, there is good but limited evidence of the involvement of endoproteases in antigen processing in the secretory pathway. The best examples so far involve the ER-resident signal peptidase and the trans-Golgi protease furin (Gil-Torregrosa et al. 1998;Gil-Torregrosa et al. 2000). Otherwise, endoprotease activity might not be as limited as initially thought, as several examples of processing of full-length proteins in the secretory pathway have been reported (Snyder et al. 1998;Hammond et al. 1993;Snyder et al. 1997), although the proteases involved have not been characterized.

Are there any disadvantages to alternative proteases joining in to produce a richer repertoire displayed? In principle not. Actually, the proteasome itself does change into the immunoproteasome after exposure of cells to an inflammatory

microenvironment. The only risk inherent to having more proteases producing epitopes is that they may also destroy potential epitopes, as has been shown for thimet oligopeptidase (Saric et al. 2001), puromycin-sensitive aminopeptidase and bleomycin hydrolase (Stoltze et al. 2000). But this has already been described for the couple proteasome/immunoproteasome: either of them, or both, may generate the epitope. In some cases, one of them cannot cleave off the right epitope or precursor peptide (Morel et al. 2000;Schultz et al. 2002), but also there are examples where either of them actually destroys the epitope (Anton et al. 1998;Luckey et al. 1998;Vinitsky et al. 1997;Schmidtke et al. 1998;Ossendorp et al. 1996). This limitation also applies to alternative proteases, but there are no reasons to think that they may be more deleterious than the proteasome or the immunoproteasome at destroying epitopes.

On the contrary, one clear advantage of having more proteases to produce MHC class I ligands is that the universe of possible peptides produced from a single protein sequence is enlarged, giving the $CD8^+$ T cells a larger chance of detecting the infected or diseased cell. After infection, the otherwise tightly-regulated steady-state proteolytic system of a cell may be unbalanced, and new proteolytic activities be uncovered, with the result that now more peptides might be produced. An interesting possibility is that proteasomes and alternative proteases may synergize in providing each other with suitable intermediate peptides, and altogether contributing to a higher diversity or abundance of potential MHC class I ligands. The final level of presentation of many pathogen-derived epitopes is probably the result of interplay between different proteolytic activities. Thus, having alternative proteases raises the collection of possible physiological, pathological, or cell-type situations where any protease system may contribute to producing peptides.

Last but not least, availability of proteases in secretory compartments that can process peptides and deliver them to MHC class I molecules covers the pathological situation created by certain tumours and certain viruses, mainly herpesviruses, whereby TAP peptide transport function is blocked (Hill et al. 1995;Hengel et al.

1997;Alcami and Koszinowski 2000). As the property of TAP to promote assembly of peptides with MHC class I molecules is retained, secretory route processing proteases would have the chance to contribute to presentation of at least some peptides from viral glycoproteins that may still signal the infected cell for destruction by immune $CD8^+$ T cells. Indeed, TAP-negative patients do not suffer from viral infections and have herpesvirus-specific CTL (Delasalle et al. 1997). In this context, and acknowledging that the immune response during infection of the mouse with herpes simplex virus is not the best model for the human immune response to these viruses, it is intriguing that herpesvirus glycoproteins are dominant antigens recognized by murine $CD8^+$ T lymphocytes (Hanke et al. 1991).

A physiological role for non-proteasomal proteases in antigen processing in vivo

TAP-negative cells have a very much decreased surface expression of MHC class I molecules. In TAP-negative mice or patients (van Kaer et al. 1992;Delasalle et al. 1997), where the classical pathway of antigen processing and presentation is knocked out, there are few mature $CD8^+$ T cells. These two observations would speak for a minimal contribution of alternative pathways to the generation of the bulk of MHC class I ligands. However, TAP-deficient patients can generate virus-specific CTL and, more significantly, they are not unusually susceptible to viral infections (Delasalle et al. 1997). This can be interpreted as a sufficient amount of MHC class I and TCR ligands being presented by TAP-independent mechanisms in these patients. This last conclusion is also supported by the increasing reports that block of the proteasome function with a specific inhibitor, lactacystin, still allows for the generation of many individual peptide epitopes (Vinitsky et al. 1997;Benham and Neefjes 1997;Anton et al. 1998;Yellenshaw et al. 1997b;Lopez et al. 2000), in contrast to earlier observations (Rock et al. 1994;Coux et al. 1996). In some cases, presentation of certain antigens was even enhanced in the presence of proteasome inhibitors (Anton et al. 1998;Vinitsky et al. 1997;Yellenshaw et al. 1997b;Luckey et al. 1998). More generally,

a large fraction of MHC class I alleles assemble with peptides and mature normally even in the presence of lactacystin (Benham et al. 1998;Vinitsky et al. 1997;Luckey et al. 2001). More recently, peptides produced after strong inhibition of proteasome function have been analyzed by mass-spectrometry (Luckey et al. 2001). The results reveal that a diverse array of peptides continues to be presented, encompassing the complete range of C- and N-termini. Thus, non-proteasomal proteases collectively have a broad spectrum of specificities, generate a significant fraction of all peptides associated with most MHC class I alleles, and probably produce peptides from a wide variety of cellular proteins from any subcellular site. In addition, interpretation of the effect of proteasome inhibitors on global antigen presentation or MHC class I stability may lead to an underestimation of the involvement of other proteases. Indeed, these experiments inhibit not only processing mediated exclusively by proteasomes, but also the increasingly reported sequential pathways (Table 1), where trimming enzymes cooperate with proteasomes in antigen processing. Collectively, these studies strongly suggest that both proteasomes and nonproteasomal proteases can independently generate class I-associated peptides.

Lactacystin data indicate that proteases other than the lactacystin-sensitive proteasomes can generate a significant number of peptides, although it is not even excluded that this potent inhibitor is still incompletely blocking the proteasome's multiple proteolytic activities (Fenteany and Schreiber 1998). One interpretation of the fact that TAP deficiency results in a more profound deficit in antigen presentation than proteasome blockade could be that all the alternative proteases are actually TAP-dependent, that is to say, cytoplasmic. Another explanation is that lack of TAP results in a more profound deficiency in assembly of the trimolecular complex of peptide, MHC class I heavy chain and $\beta_2$microglobulin, since it acts as a molecular scaffold for the final stage of this process (Solheim 1999;Lehner and Trowsdale 1998;Androlewicz 1999;Tan et al. 2002). If this is true, it means that peptides generated anywhere in the secretory pathway assemble mainly in the ER. This is well possible, as a retrograde

transport of vesicles from the distal to the proximal regions of the secretory pathway has been described to operate for all types of cargo that is not particularly resident in the proximal areas of this route (Cole et al. 1998). Evidence from our work with the trans-Golgi protease furin also seems to be compatible with this putative binding of peptides at the ER. Indeed, we have tested several viral epitopes for presentation by the furin pathway. To this end, they were placed downstream of furin consensus cleavage sites in a secretory protein context. These epitopes were chosen to cover presentation by several class I molecules, that represent extreme behaviours in the maturation characteristics of MHC class I molecules, ability to bind peptides, nature of the peptides bound, stability in the absence of peptides, etc. The molecules tested are L^d, D^d, and K^d. L^d binds rather hydrophobic peptides with weak affinity, and binds poorly to $\beta_2$microglobulin. As a result, L^d travels very slowly to the plasma membrane, where low expression is achieved, including some empty molecules that escape thus far due to suboptimal loading in the ER. Exchange of peptides by surface L^d molecules is documented (Hansen et al. 2000). It has actually been hypothesized that MHC class I molecules that share some of these properties might be particularly prone to acquire peptides generated in alternative sites of antigen processing (Hansen et al. 2000). K^d and D^d represent the opposite behaviour to L^d. All epitopes tested so far by us are indeed presented by the pathway involving furin (Gil-Torregrosa et al. 1998;Gil-Torregrosa et al. 2000) (unpublished observations). This happens regardless of the MHC class I molecule that binds the peptides. This is most simply explained by a conventional and common binding of all peptides to MHC class I molecules at the canonical site, the ER. Experiments are underway to confirm this hypothesis. The picture may become more complex because even at the ER distinct assembly pathways seem to exist, that require more or less accessory molecules, probably depending on the pool of suitable peptides for each MHC class I allelic molecule (Androlewicz 1999).

Newly-synthesized MHC class I molecules can exchange their bound peptides, even high-affinity ligands (Sijts and Pamer 1997). This happens in the ER (Lewis and Elliott 1998), as well as at a later point in the secretory pathway (Day et al. 1995). The latter quality control stage is probably facilitated by the fact that the mildly acidic pH of these compartments favours dissociation of some peptides while promoting the association of others (Stryhn et al. 1996). This may be used by peptides processed by furin, which is active in the trans-Golgi network and beyond. Preloaded MHC class I molecules in transit to the plasma membrane may exchange the most acid-labile subset of peptides for those produced in these distal regions of the exocytic route, either derived from endogenous routes, as discussed in this review, or those derived from the extracellular area by endocytosis, as discussed in other reports in this same issue (Reimann).

An interesting implication of these findings is that a further, paradoxical, function of TAP is to permit the exit from the ER of MHC class I molecules that are empty or suboptimally loaded with peptides, that is, those molecules which may be receptive for peptide binding at later, alternative locations in the secretory or endocytic pathways. Indeed, the rapid exit from the ER of empty molecules of an MHC class I mutant is surprisingly dependent on a TAP-derived supply of peptides (Lewis and Elliott 1998). Also, low efficiency of extra-cytosolic presentation of a bacterial-expressed antigen in TAP-deficient cells was found to parallel the concomitant decrease of peptide-receptive MHC class I molecules at the cell surface (Song and Harding 1996;Yewdell et al. 1999). In the absence of TAP expression, in TAP-deficient cells and organisms, MHC class I molecules are retained in the ER and eventually degraded, without having access to potential good ligands produced later in the exocytic pathway. In normal conditions, that is, in the presence of TAP, access to those later ligands should not be impaired. In summary, in addition to its transporter function, TAP is involved in MHC class I assembly with peptides, whatever their procedence, and TAP is needed for generation of peptide-receptive MHC class I molecules in vesicular compartments

other than the ER. Thus, studies with TAP-deficient cells or organisms, which lack all three TAP functions, while necessary for uncovering these alternative pathways, lead to an underestimation of the real contribution from TAP-independent pathways to the overall amount and diversity of epitopes available for T cell recognition.

Classical and alternative antigen processing pathways in the initiation of the immune response by professional antigen-presenting cells

Peptides presented by MHC class I molecules are in most cell types limited to those of endogenous origin, synthesized in the presenting cell. Functionally, this prevents lysis by CTL of neighbour cells that may have phagocytosed virally-infected or tumour cells, but are not infected or transformed themselves. There is, however, an exception to this safety limitation in the MHC class I pathway, which is antigen processing and presentation by professional antigen presenting cells (pAPC). This is an issue of outstanding interest nowadays. Professional APC, such as dendritic cells, are critical in initiating the immune response to pathogens (Heath and Carbone 2001;den Haan and Bevan 2001). They sample peripheral tissues capturing potential antigens, and transfer them to the lymph nodes, where naive T cells are found. In addition, while some intracellular pathogens infect such cells preserving their antigen processing and presentation abilities, for most pathogens either direct infection of dendritic cells does not occur, or infection results in abrogation of pAPC function. This is particularly evident for those viruses that have evolved strategies to evade antigen presentation (Alcami and Koszinowski 2000). In these two latter cases, initiation of the immune response relies on the cross-presentation ability of pAPC, that is, on their ability to take up antigens from surrounding infected cell debris and introduce them into their endogenous pathway of antigen processing for presentation by MHC class I molecules. Cross-priming follows, that is, antigen-specific stimulation of naive $CD8^+$ T cells by these cross-presenting dendritic cells. Essentially two major pathways have been identified (Thery and Amigorena 2001). One seems to be the classical pathway,

in that it is TAP-dependent, and most probably involves delivery of exogenous antigens from the phagosome to the cytosol (Kovacsovics-Bankowski and Rock 1995), by a dendritic-cell-selective membrane transport pathway involving the ER (Rodriguez et al. 1999). This would be followed by processing by proteasomes, transport by TAP, and loading onto nascent MHC class I molecules in the ER.

The second pathway that has been identified in pAPC is TAP-independent. This implies that the antigens do not gain access to the cytosol, and must rather travel from the endosomes to undefined compartments of the constitutive secretory pathway. As a consequence, they are processed necessarily by non-proteasomal and non-cytosolic proteases. Binding of final peptides may occur in the canonical location, the ER. It is not excluded, though, that they might intersect the pathway at later stages and replace sub-optimally bound peptides in a step that would optimize the affinity, and presumably the half-life, of peptide/MHC complexes at the plasma membrane. Indeed, peptide/MHC class I complex formation after cross-presentation by dendritic cells has been demonstrated at either site, the ER and the endosomes (Castellino et al. 2000) or the MHC class II compartments (Gromme et al. 1999;Yrlid et al. 2000). Which of these pathways, the classical TAP-dependent or the alternative TAP-independent one, is more relevant in different relevant pAPC is an open question, since it seems to vary for different viruses, antigenic determinants, delivery systems, and even protein contexts (Norbury et al. 2001;Sigal and Rock 2000). Of particular importance is elucidating the relative contributions of these pathways in vivo. Some examples of the relevance of the TAP-dependent pathway for tumour cells and some viruses (Huang et al. 1996;Sigal et al. 1999;Sigal and Rock 2000), and of the TAP-independent pathway for a soluble protein expressed by a virus and for epitopes from three other viruses (Norbury et al. 2001;Sigal and Rock 2000;Blake et al. 2000) have already been reported. As discussed above, the described relative inefficiency of the TAP-independent pathway (Sigal and Rock 2000) might rather reflect a limitation of the experimental models necessary to unveil it, which imply use of TAP-deficient systems. These are not only

deficient in peptide transport to the ER, but also in peptide/MHC class I complex formation in the ER and at later locations in the secretory pathway.

The same question of the relative contribution of either pathway is also relevant for any other cell type susceptible to any infectious agent. So far, alternative proteases or processing pathways have been qualitatively defined. However, their quantitative relevance in comparison with the classical proteasome pathway remains to be established. It is also at present open which is the physiological relevance of these novel pathways. Particularly, one might question whether potential alternative processing in pAPC would be of any relevance, were the products of this alternative processing not to exist in normal infected cells. Indeed, if antigen processing during cross-presentation yields a series of epitopes by these alternative pathways, and these epitopes are never presented during normal infection, the activation of $CD8^+$ T cells specific for these epitopes would be useless, as shown for particular bacterial or viral antigens (Shen et al. 1998;Blake et al. 1997). This is probably an innocuous side-effect of the need to have the widest variety of epitope-producing pathways in pAPC, to cover most of the possible processing pathways generating epitopes in any type of infected cell. Conversely, production of epitopes by the alternative pathway only in normal cells would pass undetected, as T cells reactive with them would never be activated by professional APC.

All three different processing routes tested so far for the 9pp89 epitope render the same final peptide as the proteasome pathway.

Although this is an issue that warrants clearance on its own, some results from our group provide a way around this paradox. We have described so far several alternative protease systems for antigen processing (Figure 1). Some of them are completely independent of the proteasome, which implies that the proteases involved are *bona fide* antigen processing enzymes. One of them is quite defined, inasmuch as the protease involved is fully characterized: the trans-Golgi protease furin. This pro-

protein convertase removes from precursor proteins a C-terminal fragment containing antigenic epitopes, than can be trimmed and presented by several MHC class I molecules (Gil-Torregrosa et al. 1998;Gil-Torregrosa et al. 2000). In the second system, uncharacterized cystein-proteases mediate proteasome-independent, ubiquitin-independent antigen processing of relatively short endogenously-synthesized peptides (Lopez and Del Val 1997). In a third system not fully characterized (M. Montoya, I. Casal, M. Del Val, unpublished observations) (not depicted in Figure 1), antigen processing occurs exogenously at the plasma membrane. One common epitope was tested in all three cases. After infection of BALB/c mice with murine CMV, the immunodominant determinant recognized by $CD8^+$ CTL has been shown to be 9pp89 (Del Val et al. 1988), which accounts for half of all activated CMV-specific CTL precursors (Reddehase and Koszinowski 1984). The epitope is a 9mer derived from the non-structural nuclear phosphoprotein pp89, which is expressed immediately after infection. Intrinsic features of 9pp89 itself, such as its strong association with the presenting molecule L^d, contribute to its capacity to induce $CD8^+$-mediated protective immunity when expressed in isolation from other CMV sequences in rVV (Jonjic et al. 1988). Naturally processed peptides were isolated from infected cells that were treated or chosen to allow antigen processing by each of these pathways selectively, or by the classical, proteasome pathway. When they were analyzed by HPLC and detected with specific CTL, the antigenic peptides were found in all three cases to have the same chromatographic properties among them, and also to be undistinguishable from the peptide endogenously produced by proteasomes.

We assume in some of the cases (Gil-Torregrosa et al. 2000) (D. López, M. Montoya, unpublished observations), and in others have direct proof of it (Gil-Torregrosa et al. 1998), that antigen processing by each of these three pathways is the result of the concerted action of several proteases, one giving the most critical, probably the initial, cut, and others essential for trimming. We do not expect that the finality of all these systems is to produce the same and only optimal 9mer peptide. We

rather believe that several peptides are generated by each pathway, and recall the central role of MHC class I molecules. These molecules are essential either in selecting the peptide with highest affinity among a pool of available products (Ojcius et al. 1994), or in directing the final trimming of precursors (Brouwenstijn et al. 2001;Komlosh et al. 2001), competing with the exopeptidases for access to the core peptide. We postulate that this is true in general terms for major high-affinity immunodominant antigens, although the relative abundance of several epitopes from the same pathogen may differ depending on the predominant processing pathway. Thus, classical and alternative processing pathways may operate to a different extent in different antigen processing cells, be it infected pAPC, pAPC mediating cross-presentation, or infected parenchymal cells, but essentially the same peptide would be selected by MHC class I molecules form the pool of products of each pathway. An exception is represented by epitopes derived from EBV EBNA1 protein (Blake et al. 1997) or from nonsecreted proteins from intracellular bacteria (Shen et al. 1998), that are only produced by pAPC during cross-presentation, but not during infection, and that have therefore little relevance in terms of controlling infection. This probably represents the necessarily redundant and sometimes superfluous effort of pAPC to process and display as many epitopes as might be produced later in any type of infected parenchymal cell. Although some pathways and subcellular localizations, as well as different forms of antigen delivery, will certainly produce distinct sets of peptides (the remarkable example of HBsAg is reviewed by Reimann, this issue), we expect these peptide collections to be rather overlapping. Thus, in general terms, there should not be any major conflict between the universe of peptides inducing activation of naive T cells and those displayed at infected cells that have to be recognized and cured or eliminated. In any case, availability of more antigen processing pathways can only be for good, contributing to expanding the repertoire of pathogen-derived MHC ligands for recognition by the TCR.

Conclusions

From initial assumptions that the multicatalytic and ubiquitous proteasome is the only protease capable of fully generating peptide ligands for MHC class I molecules, the last few years have seen the identification of a surprisingly large number of alternative proteases that contribute to endogenous antigen processing. Trimming by non-proteasomal proteases of precursor peptides produced by proteasomes is now a well-established fact. More importantly, proteases that can process antigens in a fully proteasome-independent fashion have also been identified. While in still too many cases the evidence for these proteolytic activities is still only indirect, the next few years will probably see the ascription of these activities to individual enzymes. We will learn about the possibly redundant and sometimes complementary action of different proteases. This will represent a big leap in understanding their mechanism of action and in unravelling the detailed and complex pathways of antigen processing by the concerted action of multiple proteases. As a consequence, more will be known of the available mechanisms to present pathogen-derived sequences to $CD8^+$ T lymphocytes. Establishing the relevance of each pathway in vivo in different parenchymal or professional APC will contribute to the rational design of new vaccines to combat infectious diseases.

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Figure legend

Figure 1. Novel routes of viral antigen processing described by the authors.

The trans-Golgi network pro-protein convertase furin acts on secretory pathway precursor proteins with stretches of basic residues. It liberates a C-terminal fragment containing antigenic epitopes, that can be trimmed by amino- and by carboxy-peptidases and then presented by several MHC class I molecules (Gil-Torregrosa et al. 1998;Gil-Torregrosa et al. 2000). This pathway is TAP- and proteasome-independent. In the second route, cystein-proteases mediate proteasome-independent, ubiquitin-independent, TAP-dependent antigen processing of relatively short endogenously-synthesized peptides (Lopez and Del Val 1997). These two pathways can process the immunodominant CMV pp89 9mer epitope (Del Val et al. 1988) from suitable constructs, and allow presentation by the MHC class I molecule L^d to specific $CD8^+$ CTL. The subcellular localization of these cystein-proteases, as well as that of metallo-proteases, is at present unknown. The metallo-proteases process the full-length antigenic glycoprotein ENV from HIV in cooperation with the proteasome, following a TAP-dependent sequential pathway (Lopez et al. 2000). P stands for peptidase.