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Insights into antigen processing gained by direct analysis of the naturally processed class I MHC associated peptide repertoire

Victor H. Engelhard*, Anthony G. Brickner, Angela L. Zarling

Carter Immunology Center and Department of Microbiology, School of Medicine, University of Virginia, P.O. Box 801386, Charlottesville, VA 22908, USA

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Abstract

MHC class I molecules are responsible for the presentation of antigenic peptides to CD8+ T lymphocytes. Based on their relatively promiscuous binding of peptides, these molecules display information derived from a large fraction of proteins that are made inside the cell. This review describes our characterization of the peptides comprising this repertoire, with particular attention given to their complexity and quantities, their post-translational modification, and the pathways leading to their expression.

Keywords: Antigen processing; Post-translational modification; Mass spectrometry; Peptides; Class I MHC molecules

1. Introduction

Within the last 10 years, research has led to a detailed understanding of how peptides derived from intracellular proteins become associated with class I MHC molecules. The basic cell biological mechanisms leading to peptide generation in the cytosol and import into the endoplasmic reticulum (ER) are the subjects of other reviews in this volume. In addition, the biochemical basis of peptide binding by class I MHC molecules has led to an understanding of how these molecules are able to bind to a broad range of ligands with high affinity and some degree of selectivity. This work enables reasonable inferences to be made about what peptide antigens are likely to be displayed on the surface of cells. Our own research has used mass spectrometry to directly analyze the peptide repertoire, as well as individual peptides, displayed on cells. In so doing, we have gained additional insights into the qualities of this repertoire as well as the mechanisms responsible for its generation.

2. Complexity and quantitation of peptides associated with class I MHC molecules

The analysis of MHC associated peptides by mass spectrometry involves the purification of MHC molecules, release of the peptides in acid, and in some cases, fractionation of the mixture by off-line HPLC. Whole peptide extracts or individual fractions are then separated on a high-resolution microcapillary HPLC column directly interfaced to an electrospray ionization device that creates peptide ions. These ions are distinguished by a mass spectrometer based on their mass to charge ratio (*m*/*z*). This combination thus leads to a two dimensional analytical separation of peptide mixtures based on hydrophobicity and *m*/*z*. Quantities of individual peptides may also be inferred from the magnitude of the ion current signal detected. By counting ions in representative HPLC fractions we estimated that several different class I and class II MHC isoforms present over 10,000 different peptidic species at the level of >1 fmol per 10^8 cells (Hunt et al., 1992; Huczko et al., 1993; Engelhard et al., 1993; Engelhard, 1994a,b; Lippolis et al., 2002). For class I MHC molecules, this complexity is similar on cells of B lymphoblastoid, melanoma, lung carcinoma, and ovarian carcinoma. We estimate that approximately 90% of these species are common to lymphoblastoid cells and melanoma, as might be expected from the fraction of proteins that are ubiquitously expressed. Based on 8×10^5 copies of the relevant class I molecule per B lymphoblastoid cell, individual

Abbreviations: ER, endoplasmic reticulum; *m/z*, mass to charge ratio; FTMS, Fourier transform mass spectrometer; BLCL, Epstein-Barr virus transformed B lymphoblastoid cell line; TAP, transporter associated with antigen processing

* Corresponding author. Tel: +1-804-924-2423; fax: +1-804-924-1221.
E-mail address: vhe@virginia.edu (V.H. Engelhard).

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peptides are represented at between 1 and 4000 complexes per cell.

Although these numbers establish the existence of a quantitatively and qualitatively complex peptide repertoire for any given MHC molecule, the number of peptides displayed is in fact significantly less than theoretically possible based on the use of simple binding motifs. Thus, an important issue concerns the factors that control which peptides are displayed and their quantities. Sequencing of individual peptides by mass spectrometry (Hunt et al., 1992) established greater variation in both binding motifs and peptide length than originally predicted from pooled Edman sequencing. These peptides nonetheless bound to the relevant MHC molecule with high affinity (Chen et al., 1994). Thus, far from offering an explanation for a constrained repertoire, these results established that the set of potential precursor peptides was even greater than originally thought. An important observation was that for a set of naturally processed peptides, the affinities for an MHC molecule were not directly related to cell surface density (Crotzer et al., 2000; Brickner et al., 2001; Pierce et al., 2001) (Fig. 1). This established that, in addition to the ability to bind to MHC molecules, peptide representation at the cell surface is controlled by other factors, such as precursor protein expression and stability, efficiency of peptide excision by proteases, and rate of peptide transport into the ER. Thus, high affinity MHC-binding peptides identified by motif scanning of proteins may not be presented endogenously (Chen et al., 1994; Crotzer et al., 2000; Brickner et al., 2001; Pierce et al., 2001), while low affinity peptides have been found to be important antigens (Cox et al., 1994; Skipper et al., 1996; Parkhurst et al., 1996). Further analysis of the role of the antigen processing system in controlling peptide expression is presented below.

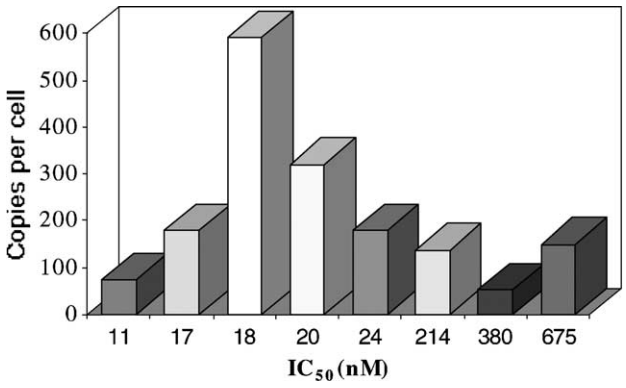

Fig. 1. Peptide surface density is not a direct function of peptide affinity for class I MHC molecules. Peptide yield data were derived from (Hunt et al., 1992) and affinity binding measurements from (Chen et al., 1994). The peptide sequences corresponding to each IC_{50} value are: 11, GLVPFLVSV; 17, LLDVPTAAV; 18, YLLPAIVHI; 20, TLWVDPYEV; 24, SLLPAIVEL; 214, SXPSGGXGV; 380, SXXVRAXEV; KXNEPVXXX. X represents Leu or Ile, which are not distinguishable by mass spectrometry. Synthetic peptides were thus synthesized using an equimolar mixture of these amino acids at those positions.

A question of substantial current interest is how the wide variation in surface densities of peptides that are expressed in association with MHC molecules affects their ability to be recognized by the immune system. The number of peptide-MHC complexes per target cell required for recognition and cytolysis by differentiated CTL has been shown to vary from several thousand to as few as one (Brower et al., 1994; Kageyama et al., 1995; Sykulev et al., 1995; Malarkannan et al., 1996; Sykulev et al., 1996; Mendoza et al., 1997; Crotzer et al., 2000). Although it has not been studied systematically, there appears to be a general correlation between peptide antigen density at the cell surface and the avidity of specific T cell lines (Cox et al., 1994; Skipper et al., 1996; Crotzer et al., 2000; Bullock et al., 2001). Several studies have shown a direct correlation between the in vivo immunogenicity of dominant and subdominant T cell epitopes and their cell surface densities (Tsomides et al., 1994; Restifo et al., 1995; Levitsky et al., 1996; Anton et al., 1997; Gallimore et al., 1998), while other studies have shown exactly the opposite (Vijh and Pamer, 1997; van Els et al., 2000). In particular, we quantitated the display of a set of Epstein-Barr virus derived peptides displayed by different HLA-B27 subtypes, and demonstrated that the more immunodominant species for each subtype were presented at lower density (Crotzer et al., 2000). In fact, the most dominant species for all subtypes was displayed at ~one copy per cell. The reasons for the disparities among these studies are not yet clear. While differences in TCR repertoire may have an influence, we and others have shown that higher doses of peptide antigen can reduce the magnitude of an immune response in vivo (van Els et al., 2000; Bullock et al., 2000) and lead to activation induced cell death of high avidity CTL in vitro (Alexander-Miller et al., 1996). This may be further influenced by the use of immunogens that establish persistent infections, which have also been associated with activation induced cell death (Moskophidis et al., 1993; Carmichael et al., 1993; Rehermann et al., 1995; Rehermann et al., 1996; Oxenius et al., 1998). A more systematic examination of how antigen cell surface density affects the immune response is clearly warranted.

Continued improvements in methodology and instrumentation allow a more comprehensive analysis of the peptide repertoire using smaller amounts of sample material. It is now possible to accumulate up to 5000 peptide fragmentation spectra in a single run using an ion-trap mass spectrometer (Shabanowitz et al., 1999). These spectra are searched against a database of either translated cDNAs or genomic DNA (Yates et al., 1995) and confirmed by manual interpretation and/or comparison with the appropriate synthetic peptide. Using this “high throughput sequencing” approach we have identified 700 peptides displayed by HLA-DR*0401 on the surface of Epstein-Barr virus transformed B lymphoblastoid cell lines (BLCL) (Lippolis et al., 2002) and over 900 peptides displayed by HLA-A*0201 (unpublished). In contrast, the Fourier transform mass spectrometer (FTMS) gives very high precision mass information

(±0.01 amu, as opposed to the more typical ±1.5 amu under the conditions used for the ion-trap) with extremely high sensitivity (2–10 amol) detection. This enables many more peptides to be distinguished in complex samples, and also allows precise quantitation of peptides sequenced using the ion-trap mass spectrometer (Crotzer et al., 2000; Lippolis et al., 2002). Used together, these instruments make it possible to identify differences in peptides between two samples and sequence them—a true "differential peptide display" technique (Luckey et al., 2001; Flyer et al., 2002).

3. Definition of antigen processing pathways for class I associated peptides

The peptides presented by class I MHC molecules are typically generated from proteins that are degraded by proteases in the cytosol, one of which is the proteasome. These degradation products are transported into the lumen of the ER via the transporter associated with antigen processing (TAP), loaded onto newly synthesized MHC class I molecules, and trafficked to the cell surface (reviewed in Pamer and Cresswell, 1998). The identities of several hundred different class I associated peptides and their precursor proteins are consistent with this processing pathway. However, we have also characterized alternate pathways of processing, and provided more information on the involvement of components of the "conventional" pathway in controlling the displayed repertoire.

3.1. The role of TAP and ER proteolysis in the display of class I associated peptides

The first description of an alternate pathway for presentation of class I associated peptides came from the mass spectrometric analysis of peptides presented by HLA-A*0201 on TAP T2 cells. While most class I MHC molecules are expressed at extremely low levels on T2 cells, HLA-A*0201 is expressed at about 30% of wild-type levels. These HLA-A*0201 molecules were occupied with bound peptide to an extent comparable to that in wild-type cells (Henderson et al., 1992). However, the peptides extracted from T2-associated HLA-A*0201 molecules were dominated by a small number of major species that were derived from the signal sequence domains of membrane bound or translocated proteins (Henderson et al., 1992; Wei and Cresswell, 1992) (Fig. 2). This suggested that the ability of these peptides to circumvent the TAP defect is due to their entry into the ER during co-translational protein translocation. However, these peptides are also found in extracts of normal cells, indicating that this pathway operates even in the presence of TAP (Henderson et al., 1992). Antigenic peptides derived from signal sequences that are presented by class I MHC molecules in a TAP-independent manner have since been reported (Henderson et al., 1993; Wolfel et al., 2000). Taken together, these results identify a second pathway for the generation and association of peptides with class I MHC molecules that operates in normal cells, and that can give rise to epitopes recognizable by T cells during the course of a normal immune response.

It is of interest that the presented peptides are only fragments of the intact signal sequences, and that they are derived from both the amino and carboxyl terminal ends (Fig. 2). This suggests the existence of both amino and carboxyl peptidase activities, and perhaps endopeptidase activities located in the lumen of the ER. The involvement of aminopeptidase activity in the ER in antigen processing has been well established (Snyder et al., 1994; Elliott et al., 1995) and has recently been associated with several proteins (Serwold et al., 2001; Menoret et al., 2001; Komlosh et al., 2001), while carboxypeptidase activity has been considered to be limited (Snyder et al., 1994). However, the importance of carboxypeptidase and/or endopeptidase activity is also indicated by the TAP independent presentation of epitopes derived from internal segments of proteins synthesized in the ER (Hammond et al., 1993; Lee et al., 1996; Wood and Elliott, 1998). One possible source of these activities is the signal peptidase itself, which has been shown to cleave

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                -31        -21        -11         -1
IP-30     MDSRHTFAPAAMTLSPLLLFLPPLLLLLDVPTAAVQA  SPLQA
                                      LLLDVPTAAVQA
                                      LLLDVPTAAV
                                      LLDVPTAAV
                                     -11         -1
Calreticulin                        MLLSVPLLLGLLGLAVA  EPAVY
                                    MLLSVPLLLG
                -31        -21        -11         -1
SSRα             MRLLPRLLLLLLLVFPATVLFRGGPRGSLAVA  QDTLE
                                        VLFRGGPRGSLAVA
                           -21        -11         -1
HLA-E                        MVDGTLLLLLSEALALTQTWA  GSHSL
                             MVDGTLLLL
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Fig. 2. Relationship of peptides presented by HLA-A*0201 in T2 cells to full length signal sequences from which they are derived.

signal peptides into smaller fragments (Weihofen et al., 2000; Lemberg et al., 2001) and to generate peptides derived from conventional class I MHC signal sequences that are presentable by HLA-E (Lemberg et al., 2001). However, this pathway has so far only been associated with release of such fragments into the cytosol in a form that requires further proteolytic trimming and transport back into the ER by TAP. Nonetheless, presentation of several peptide epitopes derived from signal sequences is dependent on TAP function (Aldrich et al., 1994; Hombach et al., 1995; Braud et al., 1998) and carboxyl terminal trimming in the cytosol (Bai et al., 2000). Collectively, these results demonstrate that proteolytic activity in the ER is not confined to aminopeptidases, but may be limited in its ability to act on a broad range of substrates.

3.2. Involvement of proteasomes in antigen processing and presentation by class I MHC molecules

Although proteasomes have been suggested to be the major source of peptides presented by class I MHC molecules, much of this evidence is indirect. We showed that proteasome digestion in vitro destroyed a known epitope derived from the influenza M1 protein, while blockade of proteasome function in vivo augmented it presentation (Luckey et al., 1998). This suggested that a non-proteasomal protease was responsible for production of this epitope under normal circumstances. Similarly, while saturating concentrations of three different inhibitors of proteasome activity dramatically diminished the re-expression of some class I MHC alleles after acid stripping of viable cells, they had a minimal effect on re-expression of several others (Luckey et al., 2001). FTMS derived two dimensional displays of the peptides presented by MHC molecules in the presence and absence of inhibitors showed that diminished MHC re-expression was associated with a substantial change in the peptide repertoire, while unimpaired re-expression was not (Luckey et al., 2001). High level re-expression among different MHC alleles was correlated with a preference for binding to peptides with basic carboxyl terminal residues. However, by high throughput sequencing of peptides derived from HLA-B*2705, which binds to peptides with either basic, aromatic, or hydrophobic residues, we found that peptides with each of these termini were represented similarly. Thus, although the spectrum of available peptides was altered, the proteolytic activity expressed in the presence of proteasome inhibitors still generated a diverse repertoire of peptides for MHC-binding. These results suggest that the composition of the repertoire and the level of expression of individual peptides is determined by the interplay of epitope creating and epitope destroying activities. They also suggest that proteolytic activities other than then proteasome contribute significantly to the generation of the peptide repertoire for at least some class I MHC molecules.

3.3. The impact of antigen processing pathways on the display of peptides related to minor histocompatibility antigens

Minor histocompatibility antigens (mHAgs) are endogenously synthesized peptides whose MHC-restricted expression differs between individuals, enabling them to be recognized by alloreactive T cells in the context of MHC alleles that are shared by the responder and stimulator (Wallny and Rammensee, 1990; Simpson and Roopenian, 1997). We have identified seven human mHAgs, as well as their allelic variants and the proteins per genes from which they derive (den Haan et al., 1995; Wang et al., 1995; den Haan et al., 1996; den Haan et al., 1998; Pierce et al., 1999; Brickner et al., 2001; Pierce et al., 2001; and unpublished). These studies have demonstrated that mHAg disparities between individuals are usually due to one to two amino acid substitutions in an MHC restricted peptide. However, our work also demonstrates that immunological discrimination of mHAgs arises from multiple mechanisms, and that the class I MHC antigen processing pathway plays an important role.

In the case of the human mHAgs HY-B7 (Wang et al., 1995), HY-A1 (Pierce et al., 1999), and HA-1 (den Haan et al., 1998), the mHAg-negative alleles encode polymorphic peptides that bind well to the relevant MHC molecule, but are highly discriminated by T cells. The existence of these mHAgs would thus, seem to be dependent on the presence within any individual of TCR with an appropriate fine specificity to distinguish mHAg-expressing cells from their negative counterparts. However, direct analysis of endogenous peptide expression revealed that, while the negative allelic counterpart of the HY-A1 epitope was expressed at the cell surface, the counterpart to HA-1 was not, despite having a reasonable binding affinity. This result was the first indication that mHAg-related sequence differences might lead to a difference in interaction with components of class I antigen processing pathways.

Failure to present negative counterparts of mHAg peptides, despite their ability to bind to relevant MHC molecules in vitro, has now been observed in three additional systems: HA-8, HA-2, and HA-3. A striking observation in these cases is that after exogenous addition, the mHAg peptides and their negative counterparts are recognized similarly by mHAg specific T cells. Thus, these mHAg disparities exist because of differential antigen processing of immunologically similar peptides rather than differences in interaction with the relevant MHC molecule or T cell receptor. In the case of the HLA-A*0201 restricted mHAg HA-8, it was shown that minigene products encoding either the antigen or its allelic counterpart were recognized similarly if they were expressed directly in the ER, but not if they were expressed in the cytosol (Brickner et al., 2001). In vitro TAP transport experiments indicated that peptides containing the mHAg sequence were translocated more efficiently than those containing the negative counterpart. This deficiency was attributable to the substitution of an Arg at P1 in the HA-8

sequence with a Pro, which has previously been shown to have a detrimental effect on transport of other peptides by TAP (Momburg et al., 1994; van Endert et al., 1995; Uebel et al., 1997). In contrast, no difference was observed in the transport of peptides corresponding to HA-2 and its allelic homolog (Pierce et al., 2001). Since the substitution in this case is a Met for a Val at P9, this suggests that the lack of expression of the negative allelic counterpart is a consequence of differential proteolysis, resulting in either destruction or a failure to produce this species from a longer precursor. Analysis of the reasons for the lack of expression of the negative allelic counterpart of HA-3 are still under investigation.

Although the data set is still relatively small, it is interesting to note that the negative allelic counterparts of the majority of human mHAgs identified to date are not expressed because of their handling by the antigen processing system. Indeed, mHAg related polymorphisms that strongly interfere with peptide binding have been found relatively infrequently (Greenfield et al., 1996). While analysis of a large set of antigens is warranted, this may indicate an important role for antigen processing in the discrimination of these antigens. In this context, it will be interesting to determine if the failure to display the negative counterparts of mHAg in responding individuals enable a more robust T cell response.

4. Identification of post-translationally modified peptides presented by MHC molecules

The use of mass spectrometry for direct sequencing of MHC associated peptides also led to the surprising observation that many have undergone structural alteration. The first such modification detected was an asparagine deamidated to aspartic acid in an epitope derived from tyrosinase (Skipper et al., 1996). This result is discussed in more detail below in connection with the processing of this protein. We also have observed that several peptides containing cysteine residues have been altered by the attachment of another cysteine residue via a disulfide bond (Meadows et al., 1997; Kittlesen et al., 1998; Pierce et al., 1999). Using similar technology, Lopez de Castro and co-workers have characterized an HLA-B39 associated peptide with a modified N(G),N(G)-dimethyl-Arg residue (Yague et al., 2000). This common modification of RNA-binding proteins was estimated to be present on 1–2% of the peptides associated with this class I MHC molecule. For both deamidation and cysteinylation, the modification plays an important role in T cell recognition, and in some cases both modified and unmodified peptides are displayed on the same cell (Skipper et al., 1996; Meadows et al., 1997; Mosse et al., 1998; Kittlesen et al., 1998; Pierce et al., 1999). Thus, post-translational modifications increase the range of epitopes displayed from a single protein.

A post-translational modification of immense interest is phosphorylation on serine, threonine, and tyrosine. Due to deregulation of phosphorylation and dephosphorylation events associated with cellular proliferation and transformation, proteins from cancer cells are differentially phosphorylated by comparison with their normal cellular counterparts (Cantley et al., 1991; Sherr, 1996; Hunter, 1991). In addition, rapid turnover is an important mechanism for regulating the activity of many transcription factors, cell growth modulators, signal transducers and cell cycle proteins (Laney and Hochstrasser, 1999; Koepp et al., 1999). Degradation of these proteins is often regulated at the level of ubiquitination, which targets them for destruction by the 26S proteasome complex (reviewed in Bonifacino and Weissman, 1998; Ciechanover et al., 2000). Ubiquitination in turn, is often modulated by phosphorylation, which enables specific recognition by E3 ubiquitin ligases (Koepp et al., 1999; Ciechanover et al., 2000). Thus, peptides derived from differentially phosphorylated proteins are attractive candidates for MHC associated antigens that would be widely expressed on a broad array of tumors and associated with transformation and/or cellular proliferation.

Early work suggested that phosphorylated peptides could be presented by MHC class I molecules (Larson et al., 1992), and it was later shown that synthetic phosphorylated MHC class I peptide epitopes could be transported by TAP, bind to MHC class I molecules and induce specific CTL (Andersen et al., 1999). More recently, we demonstrated directly that naturally processed phosphorylated peptides are a component of the normal peptide repertoire displayed by eight different class I MHC molecules (Hogan et al., 1998; Zarling et al., 2000). Interestingly, many of the HLA-B alleles presented a larger number of phosphorylated peptides (43–60 peptides) than do any of the HLA-A alleles analyzed (9–15 peptides) (Zarling et al., 2000). We believe, this is due to the similarities between many protein kinases recognition motifs and elements of the peptide binding motifs of the HLA-B alleles examined. In keeping with this idea, the peptides presented by HLA-A molecules do show elements of kinase motifs, but these are not conserved elements involved in class I peptide binding. Phosphopeptide presentation is entirely dependent on TAP, suggesting that these peptides originate in the cytosol. Of the 20 phosphopeptides that have now been sequenced, seven match to proteins that are known kinases, kinase substrates, or phosphoproteins. However, only two of these phosphorylation sites were previously known. Thus, this analysis offers new information and potential insight into protein kinase cascades that control different cellular processes. Most of the peptides contain a phosphoserine, while only two contained a phosphothreonine and none contained phosphotyrosine. This finding is in accordance with the observation that phosphotyrosine normally represents 0.1% of the phosphoamino acid content of the cells (al-Obeidi et al., 1998). Collectively, these results indicate that the production of these phosphopeptides is a consequence of physiologically relevant phosphorylation and degradation processes occurring in the cytosol.

Our work also established that naturally processed phosphopeptides can induce and be recognized by specific

CD8+ T cells. These cells are specific for the phosphate moiety in the context of a specific amino acid and peptide sequence. The ability to generate phosphopeptide-specific T cells indicates that phosphopeptides presented by MHC molecules are protected from the action of phosphatases both in vitro and in vivo, and bodes well for the use of these peptides both as vaccines and to develop T cell reagents for use in adoptive immunotherapy. Consequently, we have begun to analyze extracts from carcinoma cell lines for class I MHC restricted phosphopeptides to determine whether there are species that are differentially displayed by transformed cells. Using the FTMS, each of the cancer lines was found to display several phosphopeptides that were not present on BLCL (unpublished). Their absence from BLCL suggests that they are not associated with phosphorylation events that are common to all proliferating cells. These peptides could be a result of either tissue specific gene expression or phosphorylation events that occur selectively in these cells, and are potentially associated with the transformation process. Interestingly, we have failed to detect any phosphopeptides in extracts of MHC class II molecules from BLCL, although CD4 T cell discrimination among differential phosphorylated forms of αB-crystallin has been previously reported (van Stipdonk et al., 1998). The paucity of class II MHC presented phosphopeptides suggests either that these peptides are not able to enter the endosome from the cytosol, although this does occur for cytosolically expressed proteins (Lich et al., 2000), or that the phosphorylation is not stable in the endosomal environment.

5. Tyrosinase as a model to understand the processing and presentation of membrane proteins

Since proteins that are membrane associated or secreted are co-translationally translocated into the ER from membrane-bound ribosomes, they should be protected from the action of cytoplasmic proteases. However, peptide epitopes do arise from such proteins. It was initially hypothesized that these arose from proteolysis of proteins that were aberrantly translated on cytoplasmic ribosomes (Townsend and Bodmer, 1989; Yewdell and Bennink, 1992). This could occur either as the result of incomplete translational blockade by signal sequences on cytosolic ribosomes, or the use of alternate start codons internal to the signal sequence (Lurquin et al., 1989; Boon et al., 1989; Sibille et al., 1990; Chomez et al., 1992; Scott et al., 1995; Wang et al., 1996; Bullock and Eisenlohr, 1996; Malarkannan et al., 1999). Indeed, aberrant translation products have more recently been proposed as a major source of class I associated epitopes from all proteins (Schubert et al., 2000). Alternatively, epitopes derived from membrane associated and secreted proteins might arise from processing in the ER, or through a more complex pathway.

Tyrosinase, which is expressed in melanocytes and widely in human melanoma cells, is a particularly interesting protein by which to evaluate these issues. It is synthesized in the ER as a membrane associated glycoprotein with a 19aa signal sequence, a 454aa lumenal domain, a 26aa transmembrane domain, and a 30aa cytoplasmic tail. Properly folded tyrosinase is normally sorted to melanosomes, which are closely related to but distinct from late endosomes and lysosomes (Orlow, 1995; Raposo et al., 2001). A large number of tyrosinase-derived epitopes have been identified as targets for both MHC class I (Wolfel et al., 1994; Kang et al., 1995; Skipper et al., 1996; Brichard et al., 1996; Kawakami et al., 1998; Kittlesen et al., 1998; Morel et al., 1999) and class II (Topalian et al., 1996; Kobayashi et al., 1998a,b; Kierstead et al., 2001) MHC restricted T cells isolated from melanoma patients. Thus, these epitopes could potentially arise from tyrosinase molecules processed in the ER, the cytosol, or an endosomal compartment.

Presentation of the HLA-A*0201 restricted $Tyr_{1–9}$ epitope derived from the signal sequence has been shown to be both TAP and proteasome independent (Wolfel et al., 2000), in keeping with the mechanism for processing of other signal sequence derived peptides described above. Our analysis of the processing pathway of the HLA-A*0201 restricted $Tyr_{369–377}$[1] epitope was initially based on the observation that, while the sequence encoded by the tyrosinase gene was YMNGTMSQV, the only peptide derived from this sequence and displayed at the surface of tyrosinase expressing cells is YMDGTMSQV (Skipper et al., 1996; Mosse et al., 1998). Both peptides bind to HLA-A*0201 (Skipper et al., 1996) and are transported by TAP (Androlewicz, 1996; Wang et al., 1998) equivalently. The YMDGTMSQV peptide does not arise from non-enzymatic deamidation of the asparagine residue in YMNGTMSQV either inside the cell or after isolation (Skipper et al., 1996). N_{373} is constituitively glycosylated in tyrosinase (Ujvari et al., 2001) and the only well-established enzymatic deamidation of asparagine in eukaryotic cells occurs during protein degradation as a result of the removal of *N*-linked carbohydrates catalyzed by either peptide *N*-glycanase or glycoasparaginase. Consequently, we hypothesized that the generation of YMDGTMSQV occurred after synthesis and glycosylation of the intact protein in the ER (Skipper et al., 1996).

Potentially at odds with this hypothesis were additional observations that presentation of YMDGTMSQV in cells expressing full-length tyrosinase was dependent on both proteasome function (Luckey et al., 1998) and TAP activity (Mosse et al., 1998), indicating that either the epitope or a precursor was located in the cytosol. Indeed, in both the membrane and cytosolic fractions of cells treated with proteasome inhibitors, we detected two new tyrosinase species—one apparently full-length and not glycosylated and the other partially proteolyzed and fully glycosylated

[1] The sequence YMNGTMSQV from the tyrosinase protein was initially identified as residues 368–376 (Wolfel et al., 1994), and our laboratory used that numbering system in an earlier paper (Mosse et al., 1998). The correct numbering for the YMNGTMSQV peptide is 369–377.

(Mosse et al., 1998; Mosse et al., 2001). The appearance of these species in the cytosol was blocked by the glycosylation inhibitor tunicamycin (Mosse et al., 2001), indicating that they originated from glycosylated species in the ER, rather than from mistranslated species in the cytosol. These results are in accord with recent data from many other laboratories demonstrating that degradation of many proteins located in the ER is accomplished by reverse translocation into the cytosol and degradation by the proteasome (reviewed in Brodsky and McCracken, 1997). They are also in agreement with other work showing that the degradation of apparently misfolded wild-type or mutant forms of tyrosinase is blocked by inhibitors of proteasome activity (Halaban et al., 1997; Berson et al., 2000; Toyofuku et al., 2001). Based on these observations, we have proposed that presentation of YMDGTMSQV was due to quantitative glycosylation during synthesis of the full-length protein in the ER, followed by reverse translocation, deglycosylation accompanied by deamidation and proteolysis in the cytosol, and TAP mediated transport of the resulting peptide fragments into the ER for HLA-A*0201 binding (Fig. 3).

Additional evidence relevant to this model came from analysis of the requirements for presentation of the deamidated or genetically encoded forms of the epitope. Although YMNGTMSQV is not presented by cells expressing full-length tyrosinase at any level detectable with either T cells or mass spectrometry, this peptide is the only form presented by cells expressing a minigene encoding YMNGTMSQV together with an initiator Met (Mosse et al., 1998). Somewhat surprisingly, both YMNGTMSQV and YMDGTMSQV are expressed in cells expressing a tyrosinase fragment that lacks the first 143 residues, including the signal sequence, and terminates at 379, two residues to the carboxyl terminal side of the epitope. We hypothesized that YMNGTMSQV produced in the cytosol from a minigene was rapidly bound to HLA-A*0201 after TAP transport, protecting it from glycosylation at least in part and from deamidation altogether. Additionally, we proposed that expression of the cytosolic fragment of tyrosinase led to presentation of both forms because the optimal peptide derived from processing was protected as outlined above, while longer peptides transported by TAP were unable to bind to HLA-A*0201, allowing them to undergo glycosylation, deglycosylation and deamidation, and further proteolytic processing.

Further work is necessary to provide direct support for these mechanisms, as well as to directly demonstrate that deglycosylation is a prelude to deamidation. Indeed, while one study in another model system supported the concept that membrane protein derived epitopes derive from ER synthesized protein, accompanied by *N*-linked glycosylation and deamidation of membrane proteins as a source of "new" epitopes (Selby et al., 1999), another did not (Ferris et al., 1996). In addition, with the exception of the Tyr_{369} epitope presented by HLA-A*0201, the pathway(s) by which the other class I restricted epitopes from tyrosinase are presented is unknown. In particular, it is not clear whether the proteolysis of the mature protein in melanosomes or endosomes (Berson et al., 2000) enables presentation of any of these class I associated tyrosinase peptides, as has been demonstrated in other models (Reimann et al., 1994; Watts, 1997; Jondal et al., 1996; Gil-Torregrosa et al., 1998; Canaday et al., 1999; Campbell et al., 2000). Regardless of the exact pathways followed, an important concept supported by these studies is that subcellular targeting and compartmentalization of proteins can alter the patterns of displayed epitopes.

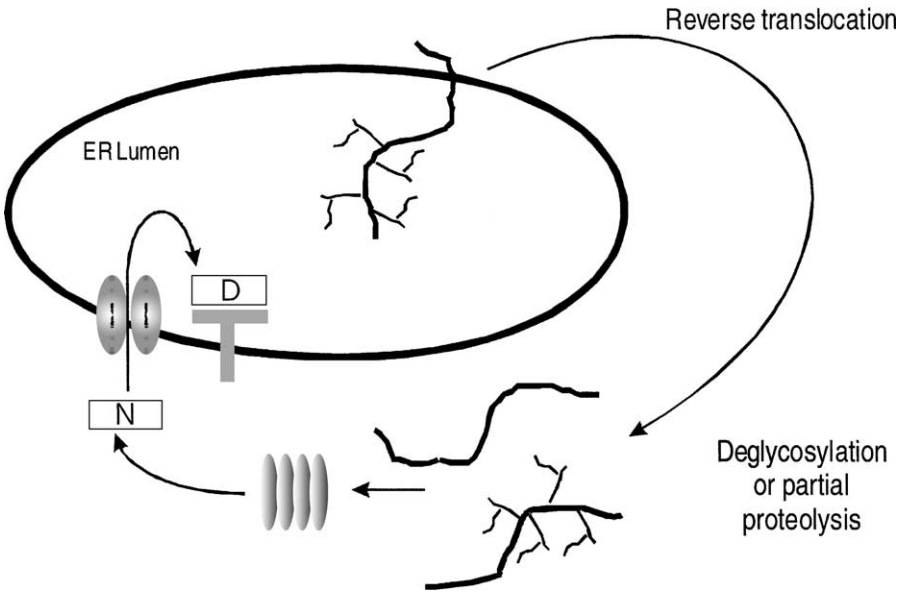

Fig. 3. Model for the processing, post-translational modification and presentation of the $Tyr_{369–377}$ epitope from tyrosinase.

6. Conclusions and future directions

The direct analysis of naturally processed peptides displayed by class I MHC molecules has offered several important insights into the characteristics and origin of this repertoire. Of particular interest is the wide variation in numbers of individual peptide complexes per cell, which has now been shown to be an important determinant of immunity. Additional work is necessary to ascertain how this aspect of antigen presentation can be manipulated to improve or diminish immune responses. In addition, the display of post-translationally modified peptides gives the potential for both increased antigenic diversity and to distinguish cells based on alterations in intracellular metabolism other than protein synthesis. However, much remains to be done in exploiting this potential for immunotherapeutic approaches to disease. Also, the modifications detected to date are still a relatively small fraction of those known to occur in cells, and it will be of interest to examine whether others are also presented in the context of class I or class II MHC associated peptides. Finally, this analysis has offered continuing insight into the diversity of mechanisms by which proteins in different compartments are degraded to generate class I MHC-binding peptides. Aside from an increased understanding of how basic cell biological mechanisms have been adapted by the immune system, we expect that this information will be an important consideration in designing new generations of vaccines.

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References

al-Obeidi, F.A., Wu, J.J., Lam, K.S., 1998. Protein tyrosine kinases: structure, substrate specificity, and drug discovery. Biopolymers 47, 197–223.

Aldrich, C.J., DeCloux, A., Woods, A.S., Cotter, R.J., Soloski, M.J., Forman, J., 1994. Identification of a TAP-dependent leader peptide recognized by alloreactive T cells specific for a class Ib antigen. Cell 79, 649–658.

Alexander-Miller, M.A., Leggatt, G.R., Berzofsky, J.A., 1996. Selective expansion of high- or low-avidity cytotoxic T lymphocytes and efficacy for adoptive immunotherapy. Proc. Natl. Acad. Sci. U.S.A. 93, 4102–4107.

Andersen, M.H., Bonfill, J.E., Neisig, A., Arsequell, G., Sondergaard, I., Neefjes, J., Zeuthen, J., Elliot, T., Haurum, J.S., 1999. Phosphorylated peptides can be transported by TAP molecules, presented by class I MHC molecules, and recognized by phosphopeptide-specific CTL. J. Immunol. 163, 3812–3818.

Androlewicz, M.J., 1996. An *N*-glycosylated tyrosinase epitope associates with newly synthesized MHC class I molecules in melanoma cells. Hum. Immunol. 51, 81–88.

Anton, L.C., Yewdell, J.W., Bennink, J.R., 1997. MHC class I-associated peptides produced from endogenous gene products with vastly different efficiencies. J. Immunol. 158, 2535–2542.

Bai, A., Aldrich, C.J., Forman, J., 2000. Factors controlling the trafficking and processing of a leader-derived peptide presented by Qa-1. J Immunol. 165, 7025–7034.

Berson, J.F., Frank, D.W., Calvo, P.A., Bieler, B.M., Marks, M.S., 2000. A common temperature-sensitive allelic form of human tyrosinase is retained in the endoplasmic reticulum at the non-permissive temperature. J. Biol. Chem. 275, 12281–12289.

Bonifacino, J.S., Weissman, A.M., 1998. Ubiquitin and the control of protein fate in the secretory and endocytic pathways. Annu. Rev. Cell Dev. Biol. 14, 19–57.

Boon, T., Van Pel, A., De Plaen, E., Chomez, P., Lurquin, C., Szikora, J.P., Sibille, C., Mariame, B., Van den Eynde, B., Lethe, B., et al., 1989. Genes coding for T cell-defined tum transplantation antigens: point mutations, antigenic peptides, and subgenic expression. Cold Spring Harbor Symp. Quant. Biol. 54 (Pt. 1), 587–596.

Braud, V.M., Allan, D.S., Wilson, D., McMichael, A.J., 1998. TAP- and tapasin-dependent HLA-E surface expression correlates with the binding of an MHC class I leader peptide. Curr. Biol. 8, 1–10.

Brichard, V.G., Herman, J., Van Pel, A., Wildmann, C., Gaugler, B., Wolfel, T., Boon, T., Lethe, B., 1996. A tyrosinase non-apeptide presented by HLA-B44 is recognized on a human melanoma by autologous cytolytic T lymphocytes. Eur. J. Immunol. 26, 224–230.

Brickner, A.G., Warren, E.H., Caldwell, J.A., Akatsuka, Y., Golovina, T.N., Zarling, A.L., Shabanowitz, J., Eisenlohr, L.C., Hunt, D.F., Engelhard, V.H., Riddell, S.R., 2001. The immunogenicity of a new human minor histocompatibility antigen results from differential antigen processing. J. Exp. Med. 193, 195–206.

Brodsky, J.L., McCracken, A.A., 1997. ER-associated and proteasome-mediated protein degradation—how two topologically restricted events came together. Trends Cell Biol. 7, 151–156.

Brower, R.C., England, R., Takeshita, T., Kozlowski, S., Margulies, D.H., Berzofsky, J.A., DeLisi, C., 1994. Minimal requirements for peptide mediated activation of CD8+ CTL. Mol. Immunol. 31, 1285–1293.

Bullock, T.N., Mullins, D.W., Colella, T.A., Engelhard, V.H., 2001. Manipulation of avidity to improve effectiveness of adoptively transferred CD8+ T cells for melanoma immunotherapy in human MHC class I-transgenic mice. J. Immunol. 167, 5824–5831.

Bullock, T.N.J., Colella, T.A., Engelhard, V.H., 2000. The density of peptides displayed by dendritic cells affects immune responses to human tyrosinase and gp100 in HLA-A2 transgenic mice. J. Immunol. 164, 2354–2361.

Bullock, T.N.J., Eisenlohr, L.C., 1996. Ribosomal scanning past the primary initiation codon as a mechanism for expression of CTL epitopes encoded in alternative reading frames. J. Exp. Med. 184, 1319–1329.

Campbell, D.J., Serwold, T., Shastri, N., 2000. Bacterial proteins can be processed by macrophages in a transporter associated with antigen processing-independent, cysteine protease-dependent manner for presentation by MHC class I molecules. J. Immunol. 164, 168–175.

Canaday, D.H., Ziebold, C., Noss, E.H., Chervenak, K.A., Harding, C.V., Boom, W.H., 1999. Activation of human CD8+ alpha beta TCR+ cells by *Mycobacterium tuberculosis* via an alternate class I MHC antigen-processing pathway. J. Immunol. 162, 372–379.

Cantley, L.C., Auger, K.R., Carpenter, C.B., Duckworth, B., Graziani, A., Kapeller, R., Soltoff, S., 1991. Oncogenes and signal transduction. Cell 64, 281–302.

Carmichael, A., Jin, X., Sissons, P., Borysiewicz, L., 1993. Quantitative analysis of the human immunodeficiency virus type 1 (HIV-1)-specific cytotoxic T lymphocyte (CTL) response at different stages of HIV-1 infection: differential CTL responses to HIV-1 and Epstein-Barr virus in late disease. J. Exp. Med. 177, 249–256.

Chen, Y., Sidney, J., Southwood, S., Cox, A.L., Sakaguchi, K., Henderson, R., Appella, E., Hunt, D.F., Sette, A., Engelhard, V.H., 1994. Naturally processed peptides longer than nine amino acid residues bind to the class I MHC molecule HLA-A2.1 with high affinity and in different conformations. J. Immunol. 152, 2874–2881.

Chomez, P., De Plaen, E., Van Pel, A., de Smet, C., Szikora, J.P., Lurquin, C., Lebacq-Verheyden, A.M., Boon, T., 1992. Efficient expression of tum-antigen P91A by transfected subgenic fragments. Immunogenetics 35, 241–252.

Ciechanover, A., Orian, A., Schwartz, A.L., 2000. Ubiquitin-mediated proteolysis: biological regulation via destruction. Bioessays 22, 442.

Cox, A.L., Skipper, J.C., Chen, Y., Henderson, R.A., Darrow, T.L., Shabanowitz, J., Engelhard, V.H., Hunt, D.F., Slingluff, C.L., 1994. Identification of a peptide recognized by five melanoma-specific human cytotoxic T cell lines. Science 264, 716–719.

Crotzer, V.L., Christian, R.E., Brooks, J.M., Shabanowitz, J., Settlage, R.E., Marto, J.A., White, F.M., Rickinson, A.B., Hunt, D.F., Engelhard, V.H., 2000. Immunodominance among EBV-derived epitopes restricted by HLA-B27 does not correlate with epitope abundance in EBV-transformed B-lymphoblastoid cell lines. J. Immunol. 164, 6120–6129.

den Haan, J.M., Bontrop, R.E., Pool, J., Sherman, N., Blokland, E., Engelhard, V.H., Hunt, D.F., Goulmy, E., 1996. Conservation of minor histocompatibility antigens between human and non-human primates. Eur. J. Immunol. 26, 2680–2685.

den Haan, J.M., Meadows, L., Wang, W., Pool, J., Blokland, E., Bishop, T.L., Reinhardus, C., Shabanowitz, J., Offringa, R., Hunt, D.F., Engelhard, V.H., Goulmy, E., 1998. The minor histocompatibility antigen HA-1: a diallelic gene with a single amino acid polymorphism. Science 279, 1054–1057.

den Haan, J.M., Sherman, N.E., Blokland, E., Huczko, E., Koning, F., Drijfhout, J.W., Skipper, J.C., Shabanowitz, J., Hunt, D.F., Engelhard, V.H., Goulmy, E., 1995. Identification of a graft versus host disease-associated human minor histocompatibility antigen. Science 268, 1476–1480.

Elliott, T., Willis, A., Cerundolo, V., Townsend, A., 1995. Processing of major histocompatibility class I-restricted antigens in the endoplasmic reticulum. J. Exp. Med. 181, 1481–1491.

Engelhard, V.H., 1994a. Structure of peptides associated with class I and class II MHC molecules. Annu. Rev. Immunol. 12, 181–207.

Engelhard, V.H., 1994b. Structure of peptides associated with MHC class I molecules. Curr. Opin. Immunol. 6, 13–23.

Engelhard, V.H., Appella, E., Benjamin, D.C., Bodnar, W.M., Cox, A.L., Chen, Y., Henderson, R.A., Huczko, E.L., Michel, H., Sakaguchi, K., Shabanowitz, J., Sevilir, N., Slingluff, C.L., Hunt, D.F., 1993. Mass spectrometric analysis of peptides associated with the human class I MHC molecules HLA-A2.1 and HLA-B7 and identification of structural features that determine binding. Chem. Immunol. 57, 39–62.

Ferris, R.L., Buck, C., Hammond, S.A., Woods, A.S., Cotter, R.J., Takiguchi, M., Igarashi, Y., Ichikawa, Y., Siliciano, R.F., 1996. Class I-restricted presentation of an HIV-1 gp41 epitope containing an *N*-linked glycosylation site—implications for the mechanism of processing of viral envelope proteins. J. Immunol. 156, 834–840.

Flyer, D.C., Ramakrishna, V., Miller, C., Meyers, H., Stenz, M., Root, K., Fluornoy, C., Engelhard, V.H., Canaday, D.H., Ross, M.M., Hunt, D.F., Shabanowitz, J., White, F.M., 2002. Identification of CD8+ T cell *Mycobacterium tuberculosis* epitopes within the Rv0341 gene product by mass spectrometry, unpublished work.

Gallimore, A., Dumrese, T., Hengartner, H., Zinkernagel, R.M., Rammensee, H.G., 1998. Protective immunity does not correlate with the hierarchy of virus-specific cytotoxic T cell responses to naturally processed peptides. J. Exp. Med. 187, 1647–1657.

Gil-Torregrosa, B.C., Raul, C., Del Val, M., 1998. Major histocompatibility complex class I viral antigen processing in the secretory pathway defined by the trans-golgi network protease furin. J. Exp. Med. 188, 1105–1116.

Greenfield, A., Scott, D., Pennisi, D., Ehrmann, I., Ellis, P., Cooper, L., Simpson, E., Koopman, P., 1996. An H-YD^b epitope is encoded by a novel mouse Y chromosome gene. Nat. Genet. 14, 474–478.

Halaban, R., Cheng, E., Zhang, Y., Moellmann, G., Hanlon, D., Michalak, M., Setaluri, V., Hebert, D.N., 1997. Aberrant retention of tyrosinase in the endoplasmic reticulum mediates accelerated degradation of the enzyme and contributes to the dedifferentiated phenotype of amelanotic melanoma cells. Proc. Natl. Acad. Sci. U.S.A. 94, 6210–6215.

Hammond, S.A., Bollinger, R.C., Toberty, T.W., Siliciano, R.F., 1993. Transport-independent processing of HIV-1 envelope protein for recognition by CD8+ T cells. Nature 364, 158–161.

Henderson, R.A., Cox, A.L., Sakaguchi, K., Appella, E., Shabanowitz, J., Hunt, D.F., Engelhard, V.H., 1993. Direct identification of an endogenous peptide recognized by multiple HLA-A2.1 specific cytotoxic T cells. Proc. Natl. Acad. Sci. U.S.A. 90, 10275–10279.

Henderson, R.A., Michel, H., Sakaguchi, K., Shabanowitz, J., Appella, E., Hunt, D.F., Engelhard, V.H., 1992. HLA-A2.1 associated peptides from a mutant cell line: a second pathway of antigen presentation. Science 255, 1264–1266.

Hogan, K.T., Eisinger, D.P., Cupp III, S.B., Lekstrom, K.J., Deacon, D.D., Shabanowitz, J., Hunt, D.F., Engelhard, V.H., Slingluff Jr., C.L., Ross, M.M., 1998. The peptide recognized by HLA-A68.2-restricted, squamous cell carcinoma of the lung-specific cytotoxic T lymphocytes is derived from a mutated elongation factor 2 gene. Cancer Res. 58, 5144–5150.

Hombach, J., Pircher, H., Tonegawa, S., Zinkernagel, R.M., 1995. Strictly transporter of antigen presentation (TAP)-dependent presentation of an immunodominant cytotoxic T lymphocyte epitope in the signal sequence of a virus protein. J. Exp. Med. 182, 1615–1619.

Huczko, E.L., Bodnar, W.M., Benjamin, D., Sakaguchi, K., Zhu, N.Z., Shabanowitz, J., Henderson, R.A., Appella, E., Hunt, D.F., Engelhard, V.H., 1993. Characteristics of endogenous peptides eluted from the class I MHC molecule HLA-B7 determined by mass spectrometry and computer modeling. J. Immunol. 151, 2572–2587.

Hunt, D.F., Henderson, R.A., Shabanowitz, J., Sakaguchi, K., Michel, H., Sevilir, N., Cox, A., Appella, E., Engelhard, V.H., 1992. Characterization of peptides bound to the class I MHC molecule HLA-A2.1 by mass spectrometry. Science 255, 1261–1263.

Hunter, T., 1991. Cooperation between oncogenes. Cell 64, 249–270.

Jondal, M., Schirmbeck, R., Reimann, J., 1996. MHC class I-restricted CTL responses to exogenous antigens. Immunity 5, 295–302.

Kageyama, S., Tsomides, T.J., Sykulev, Y., Eisen, H.N., 1995. Variations in the number of peptide-MHC class I complexes required to activate cytotoxic T cell responses. J. Immunol. 154, 567–576.

Kang, X., Kawakami, Y., el-Gamil, M., Wang, R., Sakaguchi, K., Yannelli, J.R., Appella, E., Rosenberg, S.A., Robbins, P.F., 1995. Identification of a tyrosinase epitope recognized by HLA-A24-restricted, tumor-infiltrating lymphocytes. J. Immunol. 155, 1343–1348.

Kawakami, Y., Robbins, P.F., Wang, X., Tupesis, J.P., Parkhurst, M.R., Kang, X., Sakaguchi, K., Appella, E., Rosenberg, S.A., 1998. Identification of new melanoma epitopes on melanosomal proteins recognized by tumor infiltrating T lymphocytes restricted by HLA-A1,-A2, and -A3 alleles. J. Immunol. 161, 6985–6992.

Kierstead, L.S., Ranieri, E., Olson, W., Brusic, V., Sidney, J., Sette, A., Kasamon, Y.L., Slingluff Jr., C.L., Kirkwood, J.M., Storkus, W.J., 2001. gp100/pmel17 and tyrosinase encode multiple epitopes recognized by Th1-type CD4+ T cells. Br. J. Cancer 85, 1738–1745.

Kittlesen, D.J., Thompson, L.W., Gulden, P.H., Skipper, J.C., Colella, T.A., Shabanowitz, J.A., Hunt, D.F., Engelhard, V.H., Slingluff Jr., C.L., 1998. Human melanoma patients recognize an HLA-A1-restricted CTL epitope from tyrosinase containing two cysteine residues: implications for tumor vaccine development. J. Immunol. 160, 2099–2106.

Kobayashi, H., Kokubo, T., Sato, K., Kimura, S., Asano, K., Takahashi, H., Iizuka, H., Miyokawa, N., Katagiri, M., 1998a. CD4+ T cells from peripheral blood of a melanoma patient recognize peptides derived from non-mutated tyrosinase. Cancer Res. 58, 296–301.

Kobayashi, H., Kokubo, T., Takahashi, M., Sato, K., Miyokawa, N., Kimura, S., Kinouchi, R., Katagiri, M., 1998b. Tyrosinase epitope recognized by an HLA-DR-restricted T cell line from a Vogt-Koyanagi-Harada disease patient. Immunogenetics 47, 398–403.

Koepp, D.M., Harper, J.W., Elledge, S.J., 1999. How the cyclin became a cyclin: regulated proteolysis in the cell cycle. Cell 97, 431–434.

Komlosh, A., Momburg, F., Weinschenk, T., Emmerich, N., Schild, H., Nadav, E., Shaked, I., Reiss, Y., 2001. A role for a novel luminal endoplasmic reticulum aminopeptidase in final trimming of 26S proteasome-generated major histocompatability complex class I antigenic peptides. J. Biol. Chem. 276, 30050–30056.

Laney, J.D., Hochstrasser, M., 1999. Substrate targeting in the ubiquitin system. Cell 97, 427–430.

Larson, J.K., Otvos Jr., L., Ertl, H.C., 1992. Post-translational side chain modification of a viral epitope results in diminished recognition by specific T cells. J. Virol. 66, 3996–4002.

Lee, S.P., Thomas, W.A., Blake, N.W., Rickinson, A.B., 1996. Transporter (TAP)-independent processing of a multiple membrane-spanning protein, the Epstein-Barr virus latent membrane protein 2. Eur. J. Immunol. 26, 1875–1883.

Lemberg, M.K., Bland, F.A., Weihofen, A., Braud, V.M., Martoglio, B., 2001. Intramembrane proteolysis of signal peptides: an essential step in the generation of HLA-E epitopes. J. Immunol. 167, 6441–6446.

Levitsky, V., Zhang, Q.J., Levitskaya, J., Masucci, M.G., 1996. The life span of major histocompatibility complex-peptide complexes influences the efficiency of presentation and immunogenicity of two class I-restricted cytotoxic T lymphocyte epitopes in the Epstein-Barr virus nuclear antigen 4a. J. Exp. Med. 183, 915–926.

Lich, J.D., Elliott, J.F., Blum, J.S., 2000. Cytoplasmic processing is a prerequisite for presentation of an endogenous antigen by major histocompatibility complex class II proteins. J. Exp. Med. 191, 1513–1524.

Lippolis, J.D., White, F.M., Marto, J.A., Luckey, C.J., Shabanowitz, J., Hunt, D.F., Engelhard, V.H., 2002. Analysis of MHC class II antigen processing by quantitation of peptides that constitute nested sets, unpublished work.

Luckey, C.J., King, G.M., Marto, J.A., Venketeswaran, S., Maier, B.F., Crotzer, V.L., Colella, T.A., Shabanowitz, J., Hunt, D.F., Engelhard, V.H., 1998. Proteasomes can either generate or destroy MHC class I epitopes: evidence for non-proteasomal epitope generation in the cytosol. J. Immunol. 161, 112–121.

Luckey, C.J., Marto, J.A., Partridge, M., Hall, E., White, F.M., Lippolis, J.D., Shabanowitz, J., Hunt, D.F., Engelhard, V.H., 2001. Differences in the expression of human class I MHC alleles and their associated peptides in the presence of proteasome inhibitors. J. Immunol. 167, 1212–1221.

Lurquin, C., Van Pel, A., Mariame, B., De Plaen, E., Szikora, J.P., Janssens, C., Reddehase, M.J., Lejeune, J., Boon, T., 1989. Structure of the gene of tum-transplantation antigen P91A: the mutated exon encodes a peptide recognized with Ld by cytolytic T cells. Cell 58, 293–303.

Malarkannan, S., Gonzalez, F., Nguyen, V., Adair, G., Shastri, N., 1996. Alloreactive CD8+ T cells can recognize unusual, rare, and unique processed peptide per MHC complexes. J. Immunol. 157, 4464–4473.

Malarkannan, S., Horng, T., Shih, P.P., Schwab, S., Shastri, N., 1999. Presentation of out-of-frame peptide-MHC class I complexes by a novel translation initiation mechanism. Immunity 10, 681–690.

Meadows, L.R., Wang, W., den Haan, J.M., Blokland, E., Reinhardus, C., Drijfhout, J.W., Shabanowitz, J., Pierce, R., Agulnik, A., Bishop, C.E., Hunt, D.F., Goulmy, E., Engelhard, V.H., 1997. The HLA-A*0201-restricted HY antigen contains a post-translationally modified cysteine that significantly affects T cell recognition. Immunity 6, 273–281.

Mendoza, L., Paz, P., Zuberi, A.R., Christianson, G., Roopenian, D.C., Shastri, N., 1997. Minors held by majors. The H13 minor histocompatibility locus defined as a peptide per MHC class I complex. Immunity 7, 461–472.

Menoret, A., Li, Z., Niswonger, M.L., Altmeyer, A., Srivastava, P.K., 2001. An endoplasmic reticulum protein implicated in chaperoning peptides to major histocompatibility class I is an aminopeptidase. J. Biol. Chem. 276, 33313–33318.

Momburg, F., Roelse, J., Howard, J.C., Butcher, G.W., Hammerling, G.J., Neefjes, J.J., 1994. Selectivity of MHC-encoded peptide transporters from human, mouse and rat. Nature 367, 648–651.

Morel, S., Ooms, A., Van Pel, A., Wolfel, T., Brichard, V.G., van der Bruggen, P., Van den Eynde, B., Degiovanni, G., 1999. A tyrosinase peptide presented by HLA-B35 is recognized on a human melanoma by autologous cytotoxic T lymphocytes. Int. J. Cancer 83, 755–759.

Moskophidis, D., Lechner, F., Pircher, H., Zinkernagel, R.M., 1993. Virus persistence in acutely infected immunocompetent mice by exhaustion of antiviral cytotoxic effector T cells. Nature 362, 758–761.

Mosse, C.A., Hsu, W., Engelhard, V.H., 2001. Tyrosinase degradation via two pathways during reverse translocation to the cytosol. Biochem. Biophys. Res. Commun. 285, 313–319.

Mosse, C.A., Meadows, L., Luckey, C.J., Kittlesen, D.J., Huczko, E.L., Slingluff Jr., C.L., Shabanowitz, J., Hunt, D.F., Engelhard, V.H., 1998. The class I antigen processing pathway for the membrane protein tyrosinase involves translation in the endoplasmic reticulum and processing in the cytosol. J. Exp. Med. 187, 37–48.

Orlow, S.J., 1995. Melanosomes are specialized members of the lysosomal lineage of organelles. J. Invest. Dermatol. 105, 3–7.

Oxenius, A., Zinkernagel, R.M., Hengartner, H., 1998. Comparison of activation versus induction of unresponsiveness of virus-specific CD4+ and CD8+ T cells upon acute versus persistent viral infection. Immunity 9, 449–457.

Pamer, E., Cresswell, P., 1998. Mechanisms of MHC class I—restricted antigen processing. Annu. Rev. Immunol. 16, 323–358.

Parkhurst, M.R., Salgaller, M.L., Southwood, S., Robbins, P.F., Sette, A., Rosenberg, S.A., Kawakami, Y., 1996. Improved induction of melanoma-reactive CTL with peptides from the melanoma antigen gp100 modified at HLA-A*0201-binding residues. J. Immunol. 157, 2539–2548.

Pierce, R.A., Field, E.D., den Haan, J.M., Caldwell, J.A., White, F.M., Marto, J.A., Wang, W., Frost, L.M., Blokland, E., Reinhardus, C., Shabanowitz, J., Hunt, D.F., Goulmy, E., Engelhard, V.H., 1999. Cutting edge: the HLA-A*0101-restricted HY minor histocompatibility antigen originates from DFFRY and contains a cysteinylated cysteine residue as identified by a novel mass spectrometric technique. J. Immunol. 163, 6360–6364.

Pierce, R.A., Field, E.D., Mutis, T., Golovina, T.N., Kap-Herr, C., Wilke, M., Pool, J., Shabanowitz, J., Pettenati, M.J., Eisenlohr, L.C., Hunt, D.F., Goulmy, E., Engelhard, V.H., 2001. The HA-2 minor histocompatibility antigen is derived from a diallelic gene encoding a novel human class I myosin protein. J. Immunol. 167, 3223–3230.

Raposo, G., Tenza, D., Murphy, D.M., Berson, J.F., Marks, M.S., 2001. Distinct protein sorting and localization to premeanosomes, melanosomes, and lysosomes in pigmented melanocytic cells. J. Cell Biol. 152, 809–823.

Rehermann, B., Chang, K.M., McHutchison, J.G., Kokka, R., Houghton, M., Chisari, F.V., 1996. Quantitative analysis of the peripheral blood cytotoxic T lymphocyte response in patients with chronic hepatitis C virus infection. J. Clin. Invest. 98, 1432–1440.

Rehermann, B., Fowler, P., Sidney, J., Person, J., Redeker, A., Brown, M., Moss, B., Sette, A., Chisari, F.V., 1995. The cytotoxic T lymphocyte response to multiple hepatitis B virus polymerase epitopes during and after acute viral hepatitis. J. Exp. Med. 181, 1047–1058.

Reimann, J., Bohm, W., Schirmbeck, R., 1994. Alternative processing pathways for MHC class I-restricted epitope presentation to CD8+ cytotoxic T lymphocytes. Bio. Chem. Hoppe-Seyler 375, 731–736.

Restifo, N.P., Bacik, I., Irvine, K.R., Yewdell, J.W., McCabe, B.J., Anderson, R.W., Eisenlohr, L.C., Rosenberg, S.A., Bennink, J.R., 1995. Antigen processing in vivo and the elicitation of primary CTL responses. J. Immunol. 154, 4414–4422.

Schubert, U., Anton, L.C., Gibbs, J., Norbury, C.C., Yewdell, J.W., Bennink, J.R., 2000. Rapid degradation of a large fraction of newly synthesized proteins by proteasomes. Nature 404, 770–774.

Scott, D.M., Ehrmann, I.E., Ellis, P.S., Simpson, E., Agulnik, A.I., Bishop, C.E., Mitchell, M.J., 1995. Identification of a mouse male-specific transplantation antigen, H-Y. Nature 376, 695–698.

Selby, M., Erickson, A., Dong, C., Cooper, S., Parham, P., Houghton, M., Walker, C.M., 1999. Hepatitis C virus envelope glycoprotein E1 originates in the endoplasmic reticulum and requires cytoplasmic processing for presentation by class I MHC molecules. J. Immunol. 162, 669–676.

Serwold, T., Gaw, S., Shastri, N., 2001. ER aminopeptidases generate a unique pool of peptides for MHC class I molecules. Nat. Immunol. 2, 644–651.

Shabanowitz, J., Settlage, R.E., Marto, J.A., Christian, R.E., White, F.M., Russo, P.S., Martin, S.E., Hunt, D.F., 1999. Sequencing the primordial soup. In: Burlingame, A.L., Carr, S.A., Baldwin, M.A. (Eds.), Mass Spectrometry in Biology and Medicine. Humana Press, Towata, NJ.

Sherr, C.J., 1996. Cancer cell cycles. Science 274, 1672–1677.

Sibille, C., Chomez, P., Wildmann, C., Van Pel, A., De Plaen, E., Maryanski, J.L., de Bergeyck, V., Boon, T., 1990. Structure of the gene of tum-transplantation antigen P198: a point mutation generates a new antigenic peptide. J. Exp. Med. 172, 35–45.

Simpson, E., Roopenian, D.C., 1997. Minor histocompatibility antigens. Curr. Opin. Immunol. 9, 655–661.

Skipper, J.C.A., Hendrickson, R.C., Gulden, P.H., Brichard, V., Van Pel, A., Chen, Y., Shabanowitz, J., Wolfel, T., Slingluff, C.L., Boon, T., Hunt, D.F., Engelhard, V.H., 1996. An HLA-A2 restricted tyrosinase antigen on melanoma cells results from post-translational modification and suggests a novel processing pathway for membrane proteins. J. Exp. Med. 183, 527–534.

Snyder, H.L., Yewdell, J.W., Bennink, J.R., 1994. Trimming of antigenic peptides in an early secretory compartment. J. Exp. Med. 180, 2389–2394.

Sykulev, Y., Cohen, R.J., Eisen, H.N., 1995. The law of mass action governs antigen-stimulated cytolytic activity of CD8+ cytotoxic T lymphocytes. Proc. Natl. Acad. Sci. U.S.A. 92, 11990–11992.

Sykulev, Y., Joo, M., Vturina, I., Tsomides, T.J., Eisen, H.N., 1996. Evidence that a single peptide-MHC complex on a target cell can elicit a cytolytic T cell response. Immunity 4, 565–571.

Topalian, S.L., Gonzales, M.I., Parkhurst, M., Li, Y.F., Southwood, S., Sette, A., Rosenberg, S.A., Robbins, P.F., 1996. Melanoma-specific CD4+ T cells recognize non-mutated HLA-DR-restricted tyrosinase epitopes. J. Exp. Med. 183, 1965–1971.

Townsend, A., Bodmer, H., 1989. Antigen recognition by class I-restricted T lymphocytes. Annu. Rev. Immunol. 7, 601–624.

Toyofuku, K., Wada, I., Spritz, R.A., Hearing, V.J., 2001. The molecular basis of oculocutaneous albinism type 1 (OCA1); sorting failure and degradation of mutant tyrosinases results in a lack of pigmentation. Biochem. J. 355, 259–269.

Tsomides, T.J., Aldovini, A., Johnson, R.P., Walker, B.D., Young, R.A., Eisen, H.N., 1994. Naturally processed viral peptides recognized by cytotoxic T lymphocytes on cells chronically infected by human immunodeficiency virus type 1. J. Exp. Med. 180, 1283–1293.

Uebel, S., Kraas, W., Kienle, S., Wiesmuller, K.-H., Jung, G., Tampe, R., 1997. Recognition principle of the TAP transporter disclosed by combinatorial peptide libraries. Proc. Natl. Acad. Sci. U.S.A. 94, 8976–8981.

Ujvari, A., Aron, R., Eisenhaure, T., Cheng, E., Parag, H.A., Smicun, Y., Halaban, R., Hebert, D.N., 2001. Translation rate of human tyrosinase determines its *N*-linked glycosylation level. J. Biol. Chem. 276, 5924–5931.

van Els, C.A., Herberts, C.A., van der Heeft, E., Poelen, M.C., van Gaans-van den Brink, J., van der Kooi, A., Hoogerhout, P., Jan, T., Meiring, H.D., de Jong, A.P., 2000. A single naturally processed measles virus peptide fully dominates the HLA-A*0201-associated peptide display and is mutated at its anchor position in persistent viral strains. Eur. J. Immunol. 30, 1172–1181.

van Endert, P.M., Riganelli, D., Greco, G., Fleischhauer, K., Sidney, J., Sette, A., Bach, J.-F., 1995. The peptide-binding motif for the human transporter associated with antigen processing. J. Exp. Med. 182, 1883–1895.

van Stipdonk, M.J., Willems, A.A., Amor, S., Persoon-Dean, C., Travers, P.J., Boog, C.J., van Noort, J.M., 1998. T cells discriminate between differentially phosphorylated forms of αB-crystallin, a major central nervous system myelin antigen. Int. Immunol. 10, 943–950.

Vijh, S., Pamer, E.G., 1997. Immunodominant and subdominant CTL responses to listeria monocytogenes infection. J. Immunol. 158, 3366–3371.

Wallny, H.J., Rammensee, H.G., 1990. Identification of classical minor histocompatibility antigen as cell-derived peptide. Nature 343, 275–278.

Wang, R.F., Parkhurst, M.R., Kawakami, Y., Robbins, P.F., Rosenberg, S.A., 1996. Utilization of an alternative open reading frame of a normal gene in generating a novel human cancer antigen. J. Exp. Med. 183, 1131–1140.

Wang, W., Meadows, L.R., den Haan, J.M., Sherman, N.E., Chen, Y., Blokland, E., Shabanowitz, J., Agulnik, A., Hendrickson, R.C., Bishop, C.E., Hunt, D.F., Goulmy, E., Engelhard, V.H., 1995. Human H-Y: a male-specific histocompatibility antigen derived from the SMCY protein. Science 269, 1588–1590.

Wang, Y., Guttoh, D.S., Androlewicz, M.J., 1998. TAP prefers to transport melanoma antigenic peptides which are longer than the optimal T cell epitope: evidence for further processing in the endoplasmic reticulum. Melanoma Res. 8, 345–353.

Watts, C., 1997. Capture and processing of exogenous antigens for presentation on MHC molecules. Annu. Rev. Immunol. 15, 821–850.

Wei, M.L., Cresswell, P., 1992. HLA-A2 molecules in an antigen-processing mutant cell contain signal sequence-derived peptides. Nature 356, 443–446.

Weihofen, A., Lemberg, M.K., Ploegh, H.L., Bogyo, M., Martoglio, B., 2000. Release of signal peptide fragments into the cytosol requires cleavage in the transmembrane region by a protease activity that is specifically blocked by a novel cysteine protease inhibitor. J Biol. Chem. 275, 30951–30956.

Wolfel, C., Drexler, I., Van Pel, A., Thres, T., Leister, N., Herr, W., Sutter, G., Huber, C., Wolfel, T., 2000. Transporter (TAP)-and proteasome-independent presentation of a melanoma-associated tyrosinase epitope. Int. J. Cancer 88, 432–438.

Wolfel, T., Van Pel, A., Brichard, V., Schneider, J., Seliger, B., zum Buschenfelde, K.H.M., Boon, T., 1994. Two tyrosinase non-apeptides recognized on HLA-A2 melanomas by autologous cytolytic T lymphocytes. Eur. J. Immunol. 24, 759–764.

Wood, P., Elliott, T., 1998. Glycan-regulated antigen processing of a protein in the endoplasmic reticulum can uncover cryptic cytotoxic T cell epitopes. J. Exp. Med. 188, 773–778.

Yague, J., Vazquez, J., Lopez de Castro, J.A., 2000. A post-translational modification of nuclear proteins, N(G), N(G)-dimethyl-Arg, found in a natural HLA class I peptide ligand. Protein Sci. 9, 2210–2217.

Yates Jr., J.R., Eng, J.K., McCormack, A.L., 1995. Mining genomes: correlating tandem mass spectra of modified and unmodified peptides to sequences in nucleotide databases. Anal. Chem. 67, 3202–3210.

Yewdell, J.W., Bennink, J.R., 1992. Cell biology of antigen processing and presentation to major histocompatibility complex class I molecule-restricted T lymphocytes. Adv. Immunol. 52, 1–123.

Zarling, A.L., Ficarro, S.B., White, F.M., Shabanowitz, J., Hunt, D.F., Engelhard, V.H., 2000. Phosphorylated peptides are naturally processed and presented by MHC class I molecules in vivo. J. Exp. Med. 192, 1755–1762.