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Factors Controlling the Trafficking and Processing of a Leader-Derived Peptide Presented by Qa-1

Ailin Bai,* † Carla J. Aldrich,‡ and James Forman* 2

Many leader-derived peptides require TAP for presentation by class I molecules. This TAP dependence can either be ascribed to the inability of proteases resident in the endoplasmic reticulum (ER) to trim leader peptide precursors into the appropriate epitope or the failure of a portion of the leader segment to gain access to the lumen of the ER. Using the Qa-1 binding epitope, Qdm derived from a class Ia leader as a model, we show that many cell types lack ER protease activity to trim this peptide at its C terminus. However, both T1 and T2 cells contain appropriate protease activity to process the full length Dd leader (DL) when introduced into the ER lummen. Nevertheless, both T1 cells treated with the TAP inhibitor ICP47 and TAP* T2 cells fail to present this epitope from either the intact Dd molecule or a minigene encoding the DL. This indicates that the portion of the leader containing Qdm does not gain access to the ER. However, changing the Arg at P7 of the DL to a Cys can alter its trafficking and allows for TAP-independent presentation of the Qdm epitope. The Journal of Immunology, 2000, 165: 7025–7034.

The conventional route for Ag processing in the class I pathway originates in the cytosol where nascent proteins are enzymatically degraded into peptides. The peptides are then transported into the endoplasmic reticulum (ER) through TAP, where they bind into the groove of class I molecules. The principal enzymatic activity involved in Ag processing in the cytosol is the proteasome, which has multicatalytic activity (1, 2). Evidence in support of the requirement for proteasome activity comes from studies using proteasome inhibitors that block the generation of specific class I epitopes as well as retard class I egress from the ER (3–7). Further, animals with a deletion of proteasome subunits or the proteasome regulator, PA28 show a decrease in class I expression and/or the generation of specific class I epitopes (8–10). In addition to the proteasome, other proteases have also been implicated in the processing of protein precursors (11–16). The relative contribution of different proteases to class I Ag processing has not been established.

Proteins in the secretary pathway can theoretically be processed in the ER without a requirement for cytosolic localization or TAP transport. However, the fact that very few full-length ER-targeted proteins are processed efficiently in a TAP-independent (TAP-I) fashion argues against the significance of such a pathway (17). Thus, many epitopes derived from membrane/secretory proteins appear to have a cytosolic origin (18). There must be a mechanism to allow these proteins to gain access to the conventional Ag-processing machinery. Although defective translation products retained in the cytosol remains a possible source of the precursor (19), increasing evidence suggests that protein retro-transport from the ER to the cytosol is a major pathway (20–23).

In contrast to the scarcity of TAP-I epitopes from full-length proteins, peptide fragments do frequently get processed in the ER. It has been shown that peptide precursors experimentally linked to a N-terminal signal sequence can be properly trimmed in TAP-deficient cells (24, 25). Such processing activities are also expected to operate on naturally occurring ER peptide fragments. Indeed, HLA-A2 molecules expressed on the surface of a TAP mutant cell line are found predominantly in association with leader-derived peptides (26, 27). An influenza matrix protein epitope incorporated into a synthetic signal sequence is also presented in a TAP-I manner (28). These data suggest that at least some leader peptides can be routed to the ER lumen following their cleavage. Another process that may facilitate the generation of peptide precursors is post-translational proteolytic modification. The type II protein Jaw1 can deliver a C-terminally appended epitope efficiently into the ER due to the orientation of its luminal region, which is exposed to the ER and thus subjected to proteolytic processing (29). The maturation of the hepatitis B HBe protein involves clipping of its C region by subtilisin proteases, which enables TAP-I presentation of a chimerically inserted epitope (30). However, most of these studies were performed with the TAP* cell line 721.174 and it derivatives including T2 cells. It is unlikely that this alternative processing pathway contributes significantly to class I expression because very little class I is expressed on the surface of cells from TAP-I knockout mice (31). Further, even in studies of cell lines, ER processing of peptide fragments is not invariably observed. Although N-terminal trimming can occur, the ability to process the C-terminal end of peptides is still controversial (13, 24).

Although TAP-deficient T2 cells can present some leader-derived peptides, many recently defined leader-derived peptides require TAP to be expressed. These include the gp33 epitope from the lymphocytic choriomeningitis virus glycoprotein (32), the peptide from the leader of murine or human class I molecules that binds to Qa-1 (33) or HLA-E (34, 35), respectively, and an epitope from influenza nucleoprotein, which has been inserted in the H-2Dd leader (36). It is possible that the TAP dependency of leader peptide generation is a result of limited protease activity in the ER.
Consistent with this, Gallimore et al. (37) showed that the generation of the gp33 peptide is inhibited by the proteasome inhibitor lactacystin suggesting that this cytosolic enzymatic complex is required for correct processing. However, this result does not directly prove that there is a defect in processing of this leader peptide in the ER. Further, we showed that the presentation of another TAP-1 leader-derived peptide is not affected by lactacystin (3). An alternative possibility is that the trafficking of leader peptides may be altered so that their fate after cleavage from the nascent poly peptide is directly back into the cytosol rather than release into the lumen of the ER. This possibility has been demonstrated with a synchronized in vitro translation system (38). To test these alternatives, we have examined the processing of a leader-derived epitope present in class Ia molecules and presented by the class Ib molecule, Qa-1.

Materials and Methods

Cells and cell lines

HeLa-Qa-1b was generated by transfecting HeLa cells with Qa-1b cDNA (39). C1R-Qa-1b and L-Qa-1b have been described previously (33, 40). T1 and T2 cells were kindly provided by Dr. P. Cresswell (Yale University, New Haven, CT). Macrophages were prepared from thioglycollate-stimulated TAP-1-/- mice by peritoneal lavage and used after overnight culture. HeLa-Qa-1b was maintained in SMEM. Other cell lines were grown in RPMI 1640. All media were obtained from Life Technologies (Gainsberg, MD) and supplemented with 10% FCS. Qa-1-specific CTL clones 3C9 and 185.26E were generated by limiting dilution from B6.Tiaa anti-B6 secondary mixed lymphocyte cultures and maintained by weekly stimulation with irradiated B6 splenocytes in IL-2 containing MEM (33).

Recombinant vaccinia virus (rVV)

The minigene encoding MQdm was generated by ligating the annealed synthetic oligonucleotides into the vaccinia vector pSC11. Other minigenes were generated by PCR from D1, D1L13R, or D1R7C templates (41). The sequence encoding the signal sequence of the adenovirus E3/19K glycoprotein was fused to the N terminus by three rounds of PCR with overlapping 5' primers. In ESDL and ESMGQdm, an extra Ala residue was inserted immediately after the E3/19K leader to preserve the P1 residue of the signal peptide cleavage site. All minigenes were inserted into the vaccinia vector pSC11 and subjected to DNA sequencing for verification. Recombinant vaccinia virus was generated as described previously (41). rVVs expressing ICP47, furin, PC2, and PC3 were provided by Dr. J. W. Yewdell (National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD).

Pulse-chase and immunoprecipitation

L-Qa-1b cells were infected with rVVs at a multiplicity of infection of 20 for 2.5 h. Cells were then starved in methionine-free medium for 30 min and labeled with 0.2 μCi/ml Trasylol-S label (ICN Pharmaceuticals, Irvine, CA) for 15-30 min. Labeled cells were chased with complete medium containing excess methionine for various times. A total of 4 × 10^6 cells were lysed on ice with 0.5 ml lysis buffer (1% Nonidet P-40, 10 mM Tris-HCl (pH 7.5), 150 mM NaCl, 5 mM EDTA, 5 mM iodoacetamide, 1 mM PMSF, 0.1 trypsin inhibitor U/ml apropin) for 30 min. The post-nuclear supernatants were immunoprecipitated by the sequential incubation with an anti-CM against the cytoplasmic tail of Qa-1 and protein A-Sepharose beads (Pierce, Rockford, IL). Endo-H (Boehringer Mannheim, Indianapolis, IN) digestion was performed as described in the manufacturer’s instructions. Samples were separated by SDS-PAGE and the gels were visualized with a PhosphorImager (Molecular Dynamics, Seal Beach, CA).

CTL assay

A total of 1.5 × 10^6 target cells were incubated with rVVs at a multiplicity of infection of 10 in 150 μl medium for 1 h. Fresh medium (1 ml) was then added to the culture and the infection was allowed to proceed for another 2 h. Targets were labeled with 100 μCi 35Cr at 37°C for 1 h and the standard 4-h 51Cr release assay was performed at E:T ratios from 4 to 40 (33).

Results

Ability of ER proteases to process the full-length D1 leader (DL) to generate the Qdm epitope

A general rule for ER processing is not available. Conflicting results have been reported when different model systems are used. Both the observation of the rat cin effect and results of some ER-targeting experiments indicate a lack of C-terminal trimming in the ER (13, 24, 43). Other ER targeting studies and the identification of TAP-I leader-derived peptides suggest that significant processing occurs at both ends of ER peptides (25–27, 30). We analyzed ER peptide trafficking and processing by determining the requirements for the generation of the Qdm epitope, a 9-mer peptide derived from aa 3–11 of the 24 amino acid leader of class Ia α-region molecules. This peptide, and a similar leader-derived peptide from class Ia HLA molecules in humans, are presented by Qa-1b and HLA-E, respectively, to CD94/NKG2 receptors on NK cells (44–47). Our previous studies have shown that the Qa-1b/Qdm complex can be recognized by αβ CD8+ T cells and induce an alloreactive response. A series of alloreactive CTL clones has been developed that are specific for Qa-1b either dependent or independent of the Qdm peptide (33). To test whether the activity of ER resident proteases restricts leader peptide processing, we generated a series of minigene constructs to direct the DL fragment into the desired cellular compartments (Fig. 1). We expected the minigene DL to be synthesized in the cytosol. Whether or not it would be inserted into the ER membrane by itself is uncertain. However, by appending this leader to the C terminus of the E3/19K signal sequence (ESDL), we could direct the DL into the ER. As an ER-targeting control, we engineered the precise nonamer Qdm peptide preceded by the E3/19K leader (ESQdm). All minigenes were inserted into vaccinia virus for expression in

![Figure 1](https://example.com/figure1.png)

**FIGURE 1.** Minigene constructs. DL is the 24 amino acids DC (containing the Qdm epitope) at position 3–11. MQdm comprises the minimum epitope with an initiating Met residue. The ER signal sequence (ES), of the E3/19K glycoprotein was fused to the N terminus of DL or Qdm to produce ESDL or ESQdm. The ER-targeted Qdm precursor containing the two N-terminal flanking residues, Met and Gly, is designated as ESMGQdm. ES fusion fragments containing the Qdm epitope extended at the C terminus by four or eight residues are designated as ESQdmC4 or ESQdmC8. In ESDL and ESMGQdm, an additional Ala residue was introduced at the junction site to preserve the P1 residue of the signal peptide cleavage site. The amino acid sequences of DL and ES are illustrated at the bottom. The Qdm epitope is underlined.
the target cells. Processing and presentation of the Qdm peptide was analyzed with a Qdm specific anti-Qa-1<sup>b</sup> CTL clone 3C9 (3).

L-Qa-1<sup>b</sup> cells infected with each rVV were recognized efficiently by clone 3C9 (data not shown), confirming the successful expression and processing of these minigenes. To examine leader processing by ER proteases, we used either TAP<sup>-</sup> targets or TAP<sup>+</sup> targets expressing the herpes virus protein encoded by ICP47 to block TAP function (48, 49). As a positive control, cells infected with rVV-ESQdm resulted in presentation of Qdm in all cases indicating efficient ER targeting by the E3/19K leader (Fig. 2A-D). However, no sensitization of macrophages from TAP<sup>1<sup>−/−</sup></sup> mice by rVV-ESQdm was observed (Fig. 2A), and its presentation in HeLa-Qa-1<sup>b</sup> and C1R-Qa-1<sup>b</sup> was markedly inhibited by ICP47 (Fig. 2, B and C). These results suggest there is a lack of sufficient ER processing activity to trim the full-length DL in these cells. In contrast, when ESDL was expressed in T2 cells, efficient presentation of Qdm did occur (Fig. 2D). This indicates that ER processing ability differs among different cell types.

Because T2 were the only cells of the four tested that could process Qdm in the ER from ESDL, additional experiments were performed to validate the data obtained with this cell line. T2 is derived from a TAP<sup>+</sup> T and B fusion cell hybrid cell line T1, which was originally derived from cell line 721.174 (50,51). To ensure the equivalence of ICP47 blocking and the deletion of TAP, both T1 and T2 cells were examined with minigene rVV in the presence of ICP47. Not surprisingly, ICP47 had no effect on the presentation of minigene products by T2 cells (Fig. 3A). Further, while the processing of DL was blocked by ICP47 in T1 cells, the presentation of ESDL remained unchanged (Fig. 3B). Therefore, in terms of minigene processing, the phenotype of T2 cells can be

FIGURE 2. TAP-I processing of the minigene products. TAP-I<sup>−/−</sup> (Qa-1<sup>b</sup>) macrophages (A), HeLa-Qa-1<sup>b</sup> (B), C1R-Qa-1<sup>b</sup> (C), and T2 (D) cells were infected with rVVs expressing minigenes for 3 h and used as targets in a <sup>51</sup>Cr release assay. The effector cell is a Qa-1<sup>b</sup>/Qdm-specific allogeneic CTL clone 3C9. D, T2 was coinfectected with rVV-Qa-1<sup>b</sup>. HeLa-Qa-1<sup>b</sup> and C1R-Qa-1<sup>b</sup> were coinfectected with either rVV-Ova or rVV-ICP47. Data are representative of multiple experiments.
reproduced by ICP47 in T1 cells. These results also suggest that the ER processing activity we observed is not a result of clonal variability, but an inherent property for both T1 and T2 cells.

The defect of ER processing lies in the trimming of the C terminus

The finding that many cell types are unable to process the full-length DL in the ER prompted us to further evaluate their ER processing potentials. Because the DL consists of 24 amino acids and the Qdm epitope spans residues 3–11, to generate the precise nonamer peptide 2 residues at the N terminus and 13 residues at the C terminus must be removed. To approach the restricting step of the processing, we generated ER-targeted constructs extended either at the N or the C terminus of the Qdm epitope (Fig. 1). The size limit was addressed with constructs bearing a C-terminal extension of four (ESQdmC4) or eight (ESQdmC8) residues. In T2 cells, as determined by CTL recognition, all constructs were properly processed in a manner similar to the ER-targeted full-length DL, although we consistently noted that the ESQdmC4 construct was processed less well than the ESQdmC8 construct (Fig. 4A). When tested in HeLa-Qa-1b cells in the presence of ICP47, only the construct with the N-terminal extension was presented (Fig.

**FIGURE 3.** Processing of the DL in the ER by T1 and T2 cells. T2 (A) and T1 (B) cells were coinfected with rVV encoding minigenes, and either rVV-Ova or rVV-ICP47 for 3 h and used as targets in a 51Cr release assay. Both targets were also infected with rVV-Qa-1b. The effector is the same as in Fig. 2. Data are representative of multiple experiments.

**FIGURE 4.** Processing of ER-targeted N- or C-terminally extended Qdm precursors. A, T2-Qa-1b cells were infected with ER-targeted Qdm precursors extended at the N terminus (ESMGQdm) or C terminus (ESQdmC4 and ESQdmC8). B, HeLa-Qa-1b cells were coinfected with rVV-ICP47 to block TAP function. The effector is the same as in Fig. 2. Data are representative of multiple experiments.
The trimming of the C-terminal end of the epitope is deficient, because even with an extension of four amino acids, the presentation is blocked by ICP47 (Fig. 4B).

So far, we have tested the processing of minigene products containing either full-length or truncated leader fragments in different cell types and the data are summarized in Table I. Although efficient processing is observed in all targets with normal TAP function, absence of TAP-I processing of ER-targeted precursors at the C terminus appears to be a property of most cell types. Many components of the Ag processing machinery are regulated by IFN-γ (52). It is possible that some ER proteases remain quiescent in these cells. However, we were unable to induce significant ER processing activity in HeLa-Qa-1b cells by IFN-γ treatment (data not shown).

The DL in the context of the whole molecule is not routed to the ER in T1/T2 cells

With the knowledge that T1/T2 cells are capable of processing the full-length DL in the ER, we tested whether the naturally cleaved leader from the whole Dd molecule has access to such ER proteases. The answer to this question allows for the determination of the trafficking pathway of class I leader peptides.

Coexpression of Dd and Qa-1b readily sensitizes T cells for recognition by Qdm-specific CTLs, suggesting that this cell line can liberate the Qdm peptide from its naturally translated precursor (Fig. 5A). To distinguish the contributions of cytosolic vs ER proteases to the processing, ICP47 was coexpressed in these target cells. As shown in Fig. 5A, while the presentation from the ER-targeted DL is not affected by ICP47, the processing of the whole Dd molecule is completely blocked. The absence of ER processing of the DL in the context of the whole molecule was further confirmed with T2 cells (Fig. 5B). Here it is demonstrated that the presentation of Qdm from Dd and ESDL is strikingly different. Our Ag processing data support this leader peptide trafficking model. However, there are examples of TAP-I processing of epitopes derived from the N-terminal region of leader peptides. By comparing these two groups of signal peptides, Dobberstein and colleagues (38, 53) proposed that the presence of N-region charged residues might affect the trafficking of leader peptides. The Arg7 is the only charged residue in the N region of DL (Fig. 1). Our previous studies have shown that replacing Arg7 with a noncharged Cys residue (DdR7C) did not affect the ER targeting function of this leader (41). This Arg represents the P5 residue of the Qdm epitope. We have synthesized the corresponding Qdm5C peptide and shown that it binds to Qa-1b with an affinity similar to that of the wild-type Qdm peptide (54).

We first examined whether the mutant peptide can be generated intracellularly from the full DdR7C mutant. We have previously established an experimental system that allows for monitoring the availability of Qa-1b-binding peptides by following the kinetics of Qa-1b maturation (3). L-Qa-1b cells infected with RVVs encoding either the full-length DdR7C mutant or the ER-targeted Qdm5C epitope were labeled with 35S and the maturation of Qa-1b was analyzed by pulse-chase and immunoprecipitation. Although most Qa-1b molecules are retained in the ER in cells infected with irrelevant RV, infection with RVV-ESQdm5C greatly enhances Qa-1b maturation (Fig. 6A), confirming our in vitro peptide binding data. Additionally, infection with RVV-DdR7C also significantly promotes the exit of Qa-1b molecules from the ER, indicating Qa-1b-binding peptides can be generated from the full-length mutant (Fig. 6A).

To test whether this mutant leader can enter the ER and undergo TAP-I processing, we had to assess this in T1/T2 cells because of their unique ER processing capacity. Because the biochemical maturation of Qa-1b in these cells does not correlate with the peptide generation (data not shown), a CTL assay was employed in the following studies. The Arg residue at P5 of Qdm plays a direct role in T cell recognition. Our standard Qdm-specific CTL clones do not recognize the Qdm5C mutant peptide. Therefore, we used clone 185.2E6, which recognizes RAMS cells only when the exogenous Qdm5C peptide is present. This recognition can be enhanced by addition of the reducing reagent DTT, indicating 185.2E6 recognizes unmodified Cys in this peptide (data not shown). Furthermore, 185.2E6 does not cross-react with cells pulsed with wild type Qdm, although they do recognize another Qdm variant with a noncharged residue at P5 (data not shown). The recognition is restricted by Qa-1b, because T2 cells pulsed

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<sup>a</sup> Target cells were infected with RVVs containing minigene inserts for 3 h. Presentation of Qdm was measured with the CTL clone 3C9. Each target was tested at least twice with similar results.
<sup>b</sup> T1 and T2 cells were co-infected with rVV-Qa-1b.
<sup>c</sup> NT, Not tested.

Table I. Processing of minigene products in different cell lines

Effect of a N region charged residue in the leader segment on the trafficking of the Qdm peptide

It has been demonstrated in an in vitro system that the N-terminal fragment of a signal peptide can be released into the cytosol following a second cleavage in the hydrophobic core region (38). Our Ag processing data support this leader peptide trafficking model.

4B). The trimming of the C-terminal end of the epitope is deficient, because even with an extension of four amino acids, the presentation is blocked by ICP47 (Fig. 4B).
with Qdm5C are not lysed unless they are infected with rVV-Qa-1b (Fig. 6B). 185.2E6 was then used to analyze the intracellular processing of the DdR7C. T2 cells infected with rVV-Qa-1b can be sensitized for 185.2E6-mediated lysis efficiently by either adding the exogenous Qdm5C peptide or expressing the ER-targeted Qdm5C (Fig. 6B). Coexpression of the wild-type Dd molecule does not lead to 185.2E6 recognition, nor is the corresponding Qdm peptide produced. However, coinfection with rVV-DdR7C causes significant target cell recognition. Because DdR7C only differs from Dd by a single amino acid residue, the lysis is likely conferred by the generation of Qdm5C in the ER. This is further confirmed with T1 cells. Although both C1R and T1 can present the Qdm5C peptide from the DdR7C mutant, ICP47 completely blocks the recognition of C1R but only partially of T1 (Fig. 6C). This partial inhibition is not due to a lesser degree of sensitivity of T1 cells to ICP47, because the processing of Qdm from the wild-type Dd molecule in such cells is suppressed completely (Fig. 6D). This suggests that both ER and cytosol-derived peptides contribute to the recognition of T1 cells by 185.2E6. Thus, changing the N-region charged residue Arg to Cys renders the DL segment accessible to TAP-I ER processing.

Discussion

Leader-derived peptides presented by MHC class I molecules can be grouped into two types, TAP-I and TAP-D. The former group represents the major peptide species in some TAP-deficient cells, leading to the conclusion that leader peptides have full access to the ER and constitute an alternative source for class I binding peptides (26, 27). However, some newly identified epitopes fall into the second group (32–36). The pathway for processing the TAP-D leader-derived epitopes is poorly understood. One question raised by this observation is the general accessibility of leader peptides to the luminal side of the ER. Although biochemical studies have provided the first evidence that different segments of the leader peptide may travel differently (38), direct demonstration of the trafficking of a class I epitope-containing leader peptide has not been available. For functional studies using CTL recognition as the readout, additional factors, especially the processing capacity of the ER, has to be taken into consideration.

The first finding in this study is that different cell types vary in their ability to process ER-targeted minigene products. Several cell lines that we examined could not process ESDL in the absence of TAP function. In contrast, T1/T2 cells are fully competent to liberate the Qdm epitope from the 24 amino acid leader precursor. Based on the current understanding of class I assembly, we think this processing event most likely occurs in the ER. However, a retrograde transport pathway from golgi to the ER has been demonstrated (55). We cannot formally exclude the possibility that peptidases in the distal secretory pathway are involved. Theoretically, ER-targeted minigene products can even be secreted and presented through the exogenous pathway. It has been shown that this pathway operates more efficiently in phagocytes (56). In our system, the lack of ESDL processing in TAP-2 macrophages strongly opposes this possibility. Regardless of the exact processing site in the secretory pathway, the processing machinery involved seems to be cell-type specific. Although we are assuming that the proteolytic component accounts for this different processing pattern, until the processing activity of nonpermissive cells can be reconstituted by a certain protease, other possibilities cannot be excluded. For example, there could be differences in the level of factors required for selective retention of peptide precursors to prevent their rapid export from the processing compartment.

The observation that T2 is unique in processing the ER-targeted peptide precursor is potentially important because this cell line is a representative of TAP-2 cell lines used in ER processing studies (24–27, 29, 30). If these cells are not representative of most other cells’ proteolytic profile, the conclusion based on the studies with this cell line may overestimate the significance of this processing pathway. However, we should be cautious to generalize this observation to other epitopes because such comparisons have rarely been done.

Cells that were unable to process the DL in the ER likely had a defect in their ability to trim the peptide at the C terminus. The deficiency does not seem to be quantitative because progressive shortening of the C-terminal flanking region does not improve processing. There is at least one additional example that indicates the absence of C-terminal processing in non-T2 cells. Craiu et al. (13) showed that a C-terminally extended Ova epitope fragment is not trimmed in the ER in TAP-1−/− bone marrow cells. Therefore, in these cells, ER peptides likely recycle back to the cytosol for further processing. Several studies suggest that the proteasome is the only enzyme that can liberate the correct C-termini in the cytosol.
However, we did not observe any inhibition on the processing of our minigene DLs by the proteasome-specific inhibitor lactacystin, suggesting cytosolic proteases other than the proteasome are capable of liberating the Qdm epitope from the leader peptide precursor (data not shown and Refs. 12 and 16). The identity of the proteases used in Ag processing in T2 cells is unknown. The same enzymes may be responsible for the trimming of other TAP-I leader-derived peptides and a large ER-targeted influenza peptide precursor (25–27). The subtilisin protease furin has been reported to participate in the processing of an epitope introduced in the secretory pathway in T2 cells (30). The DL minigene does not contain the dibasic motif recognized by this type of enzyme. Furthermore, we expressed several subtilisin proteases including furin, PC2, and PC3 (57) in HeLa cells using recombinant vaccinia viruses. No detectable enhancement of ER processing was observed (data not shown), indicating these enzymes are not responsible for the processing of the DL minigene. The ER protease(s) in T2 cells does not promiscuously act on any peptides, because several ER-targeted minigenes are not processed in the same cell line (13, 24). Snyder et al. (58) showed that an influenza epitope with C-terminal flanking residues is not presented unless exogenous angiotensin-converting enzyme is introduced into the secretory pathway. They also showed that the N-terminal determinant of a tandem epitope construct is not presented efficiently (24). This indicates the flanking residues can affect whether C-terminal trimming will occur.

By clarifying the restriction of the ER enzymatic activity, we were able to provide strong evidence concerning the effect of the leader peptide trafficking on epitope processing. We demonstrated that T2 cells can process the ER target DL but cannot present Qdm from a DL minigene or the entire Dd molecule. This clearly indicates that being a leader peptide does not guarantee the segment itself to be able to enter the ER lumen. Uger et al. (36) inserted an epitope derived from the influenza nucleoprotein in a similar position of the Dd leader as Qdm and found its processing to be TAP-D even in T2 cells. Our previous studies showed that disrupting leader function without altering the epitope itself directed Dd synthesis to the cytosol and abrogated epitope generation (41). This clearly rules out mistargeted and defective translation products as the main source of this TAP-D epitope. Our interpretation is that leader peptide trafficking is the determining factor for its processing pathway. Leader peptides are inserted in the ER membrane cotranslationally and remain there until its cleavage from the protein precursor. To maintain the integrity of the membrane, these

![Figure 6](http://www.jimmunol.org/)
peptides must be removed. Lyco et al. (38) showed that the pre-
lactacystin leader is further processed by a signal peptide peptidase.

During protein translocation, the leader peptide is cleaved by
signal peptidase (SP). In the presence of N-region charged res-


d 

d'

charged amino acid;

charged amino acid; Cys.

charged amino acid; Arg.

charged amino acid; HLA-E.

charged amino acid; TAP-

charged amino acid; Qdm.

charged amino acid; class I.

charged amino acid; leader

charged amino acid; leader peptide.

charged amino acid; leader peptide with N-region.

charged amino acid; leader peptide without N-region.

charged amino acid; N-terminal fragment of the leader.

charged amino acid; leader peptide without the N-region.

charged amino acid; N-terminal charged residue.

charged amino acid; leader peptide harbored in the ER.

charged amino acid; leader peptide processed in a TAP-D manner.

charged amino acid; leader peptide processed in a TAP-I manner.

charged amino acid; leader peptide processed independent of TAP.

charged amino acid; leader peptide processed in the same way.

charged amino acid; leader peptide with N-region.

charged amino acid; leader peptide without the N-region.

charged amino acid; N-terminal charged amino acid.

charged amino acid; leader without the N-region charged amino acid.

charged amino acid; leader with the N-region charged amino acid.

charged amino acid; leader peptide harbored in the ER.

charged amino acid; leader peptide processed in the same way.

charged amino acid; leader peptide harbored in the ER lumen.

charged amino acid; leader peptide or its N-terminal fragment.

charged amino acid; leader peptide without the N-region.

charged amino acid; leader peptide harbored in the ER membrane.

charged amino acid; leader peptide or its N-terminal fragment.

charged amino acid; leader peptide harbored in the ER.

charged amino acid; leader peptide processed in a TAP-D manner.

charged amino acid; leader peptide processed in a TAP-I manner.

charged amino acid; leader peptide processed independent of TAP.

charged amino acid; leader peptide processed in the same way.

charged amino acid; leader peptide harbored in the ER.

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