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Exogenous Peptides Delivered by Ricin Require Processing by Signal Peptidase for Transporter Associated with Antigen Processing-Independent MHC Class I-Restricted Presentation

Daniel C. Smith,* Awen Gallimore,† Emma Jones,† Brenda Roberts,* J. Michael Lord,* Emma Deeks,* Vincenzo Cerundolo,† and Lynne M. Roberts2*

In this study we demonstrate that a disarmed version of the cytotoxic ricin can deliver exogenous CD8+ T cell epitopes into the MHC class I-restricted pathway by a TAP-independent, signal peptidase-dependent pathway. Defined viral peptide epitopes genetically fused to the N terminus of an attenuated ricin A subunit (RTA) that was reassOCIated with its partner B subunit were able to reach the early secretory pathway of sensitive cells, including TAP-deficient cells. Successful processing and presentation by MHC class I proteins was not dependent on proteasome activity or on recycling of MHC class I proteins, but rather on a functional secretory pathway. Our results demonstrated a role for signal peptidase in the generation of peptide epitopes associated at the amino terminus of RTA. We showed, first, that potential signal peptide cleavage sites located toward the N terminus of RTA can be posttranslationally cleaved by signal peptidase and, second, that mutation of one of these sites led to a loss of peptide presentation. These results identify a novel MHC class I presentation pathway that exploits the ability of toxins to reach the lumen of the endoplasmic reticulum by retrograde transport, and suggest a role for endoplasmic reticulum signal peptidase in the processing and presentation of MHC class I peptides. Because TAP-negative cells can be sensitized for CTL killing following retrograde transport of toxin-linked peptides, application of these results has direct implications for the development of novel vaccination strategies. The Journal of Immunology, 2002, 169: 99–107.

P roductive CD8+ CTL that recognize viral or tumor peptides in association with MHC class I are deemed valuable in controlling pathogen spread and, potentially, in reducing tumor progression. In recent years, several avenues have been investigated to pursue the challenge of artificially inducing protective CTL. (1–3). These methods have used both live and nonlive vectors to deliver Ags to the cytosol for subsequent presentation of peptides that correspond to epitopes recognized by CTL. Indeed, several protein toxins constitute a class of non-live vectors whose intracellular trafficking and/or membrane translocation abilities can be exploited to allow delivery of peptides for the induction of a CTL response (4–9).

In a previous study we showed that the A subunit of a catalytically defective Shiga-like toxin 1 (SLT1)3 could be engineered so that the holotoxin could successfully deliver a viral peptide into the conventional MHC class I processing and presentation pathway (7). However, due to the restricted cellular expression pattern of CD77 (10), the receptor for SLT1, the use of this protein as a generalized delivery vehicle may be limited. By contrast, the plant cytotoxin ricin has the capacity to bind to and be endocytosed into a vast number of different cell types (11). Ricin, like SLT1, has an unusual endocytic routing that takes it from the cell surface via the Golgi complex to the endoplasmic reticulum (ER) lumen (12–14). In a productive intoxication of the cell, the fraction of toxin that reaches the ER is somehow perceived as a substrate for ER-associated protein degradation (ERAD). This arm of the ER quality control process normally apprehends newly synthesized but aberrant proteins or orphan subunits of oligomers, and triggers their passage through Sec61 channels to the cytosol. In a tightly coupled sequence of events, such dislocated proteins are normally deglycosylated, certain lysyl residues become ubiquitinated, and the protein is then degraded by proteasomes (reviewed in Ref. 15). In the ER lumen, the catalytic subunit of ricin, though structurally native and endocytosed from the exterior of the cell, appears able to exploit this process to enter the cytosol (16).

From our understanding of these events, we reasoned that genetic fusions of immunodominant epitopes with toxins such as SLT1 and ricin could be introduced directly into the MHC class I processing pathway after their retrotranslocation to the cytosol from the ER. In the present study we have investigated the potential of using a disarmed version of ricin to deliver exogenous epitopes and found that, in contrast to SLT1 and most other toxin carriers, delivery and presentation of viral peptides is not dependent on proteasome activity or TAP. We present evidence for release of viral peptides from the toxin carrier by ER signal peptidase due to the presence of a potential signal peptidase cleavage site in the surface-exposed N-terminal segment of mature ricin A chain (RTA). Therefore, use of engineered ricin may be of particular value in delivering...
peptides to MHC class I molecules in TAP-deficient cells, e.g., in tumors where there is a down-regulation of TAP (17).

Materials and Methods

Reagents

Synthetic peptides ASNENMDAM (peptide 366–374 of the influenza nucleoprotein (NP)) and KAVYNFATC (LCMV glycoprotein gp33), encompassing the 366–374 residues of influenza nucleoprotein and the 33–41 residues of lymphocytic choriomeningitis virus (LCMV), respectively, were obtained from Alta Bioscience (Birmingham, U.K.) and stored at –20°C in PBS. Lac-tactyosin (Calbiochem, La Jolla, CA), brefeldin A (BD Biosciences, San Diego, CA), and chloroquine (Sigma) were used to study the effect of various inhibitors and reagents on the processing. Cells were treated at the concentrations and durations stated in the text. Target cells (2 × 10^6) were treated with toxin (500 ng/ml) or mock-treated for 30 min on ice in lysis buffer (50 mM Tris-HCl (pH 7.5) containing 1% (v/v) Triton X-100 and 150 mM NaCl), and their nuclei were removed by centrifugation for 15 min at 15,000 × g. For aliquots to be lyzed in the presence of stabilizing peptide, 10 μM NP_pM_374 was added at this stage. Cell lysates were precleared overnight with 100 μl fixed Staphylococcus aureus (Sigma) in PBS, then incubated with the B22 H-2D^d conformational-specific Ab (23) for 90 min before adding a small volume of protein A-Sepharose for 45 min. All steps were performed at 4°C. Immunoprecipitates were washed three times in lysis buffer and subjected to Endo-β-N-glicosaminidase H (EndoH) digestion overnight (see EndoH treatment) and resolved by SDS-PAGE. Fluorographs were analyzed using a densitometer and ImageQuant software (Molecular Dynamics, Sunnyvale, CA).

EndoH treatment

EndoH (Boehringer Mannheim, Indianapolis, IN) digestions were performed by resuspending the pellets of protein A-Sepharose-bound immunoprecipitates in 30 μl of 0.5 M sodium citrate buffer (pH 5.5) containing 0.2% SDS and heating to 95°C for 5 min before addition of 3 U (1 μl) of EndoH and incubation overnight at 37°C.

ER import assays

In vitro transcription of mRNAs and translation in rabbit reticulocyte lysate (Promega, Madison, WI), in the presence or absence of canine pancreatic microsomes, was performed as described (24). Cotranslational import into microsomes and cleavage by signal peptidase was determined after a 40-min posttranslational treatment with 0.5 mg/ml proteinase K on ice (+1% Nonidet P-40) and visualizing bands by SDS-PAGE and fluorography.

Results

Ricin is a heterodimeric protein composed of two subunits, RTA and RTB, that are normally disulfide bonded together. RTA has a rRNA-specific N-glicosidase activity and is therefore capable of inactivating cytosolic ribosomes should it encounter them, while RTB is a galactose-specific lectin able to bind the holotoxin to the target cell surface before endocytic uptake. We created genetic fusions of DNA for the H-2D^d-restricted peptides, influenza nucleoprotein NP 366–374 peptide (ASNENMDAM) (25), or LCMV glycoprotein 1 (gp33) peptide, residues 33–41 (KAVYNFATC) (25), to either the 5' end of the cDNA encoding mature RTA (20) or spliced into an internal Cla site that permits exposure on the toxin surface (26) or onto the 3' end (data not shown). These proteins were expressed in and purified from E. coli as described previously (18) before being reassociated with glycosylated RTB purified from Ricinus communis (22). For most experiments, a catalytic site mutant (R180H) was used (18), and in one case a variant NP-ricin fusion was made using a lysine-enriched RTA_pM_180 (that is known to be more effectively degraded by proteases (19). The NP fusion proteins are schematically depicted in Fig. 1.

Expression and purification of RTA fusions and reassociation into ricin

Recombinant RTA fusions were expressed in a 1-L culture of E. coli JM101 cells and purified by cation exchange chromatography as previously described (18). The purified fusions were reassociated to plant-derived ricin B chain (RTB; Vector Laboratories, Burlingame, CA) by dialysis against PBS, following mixing 100 μg of each chain in the presence of the reducing agent 2-ME (2% in PBS containing 100 mM lactose), as described previously (22).

Ag presentation assays

Cells were pulsed with ricin fusions at the concentrations and durations stated in the text. Target cells (2 × 10^6) were radiolabeled with 100 μCi of ^51Cr for 2 h. Target cells were infected with 5 PFU/cell of recombinant vaccinia virus expressing the relevant viral protein for 90 min before labeling, or pulsed with 500 nM free peptide (NP or gp33) for 2 h during the ^51Cr labeling. Labeled cells were plated at 10^6 cells/well and CTL were mixed with the targets at the E:T ratios shown in the figures. Supernatants were harvested after 4 h. The percentage of specific lysis was calculated as described previously (7). To study the effect of various inhibitors and reagents on the processing, cells were treated at the concentrations and durations stated in the figures before performing the Ag presentation assay.

Immunoprecipitations

RMA-S cells (2 × 10^6) were treated with toxin (500 ng/ml) or mock-treated for 90 min at 37°C. Cells were washed and starved for 60 min in 1 ml methionine-free RPMI 1640 medium supplemented with 10% (v/v) FCS, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37°C. They were then metabolically labeled in 500 μl fresh methionine-free RPMI 1640 medium with 1μCi [35S]methionine (Amersham Pharmacia Biotech, Little Chalfont, U.K.) at a concentration of 10 μCi/μl for 30 min at 37°C. Cells were lysed for 30 min on ice in lysis buffer (50 mM Tris-HCl (pH 7.5) containing 1% (v/v) Triton X-100 and 150 mM NaCl), and their nuclei were removed by centrifugation for 15 min at 15,000 × g. For aliquots to be lyzed in the presence of stabilizing peptide, 10 μM NP_pM_374 was added at this stage. Cell lysates were precleared overnight with 100 μl fixed Staphylococcus aureus (Sigma) in PBS, then incubated with the B22 H-2D^d conformational-specific Ab (23) for 90 min before adding a small volume of protein A-Sepharose for 45 min. All steps were performed at 4°C. Immunoprecipitates were washed three times in lysis buffer and subjected to Endo-β-N-glicosaminidase H (EndoH) digestion overnight (see EndoH treatment) and resolved by SDS-PAGE. Fluorographs were analyzed using a densitometer and ImageQuant software (Molecular Dynamics, Sunnyvale, CA).

FIGURE 1. Schematic representation of the ricin fusion proteins. A MHC class I-restricted antigenic peptide (NP) corresponding to residues 366–374 (ASNENMDAM) from the influenza nucleoprotein was made as a set of recombinant fusions with RTA. Three different fusion proteins are shown, in all of which RTA contained an active site mutation (R180H). The purified recombinant fusions were associated to plant-derived RTB.
Ricin was able to deliver NP peptide to MC57 cells for processing and presentation by MHC class I molecules, because cells treated with nNP-ricin or nNP6K-ricin were sensitized for specific lysis by NP-restricted CTL, but not by the irrelevant gp33-restricted CTL (Fig. 2). Controls show that these cells were able to process influenza nucleoprotein expressed in the cytosol from a vaccinia virus construct (VacNP). However, for both nNP-ricin or nNP6K-ricin, significant lysis was observed only in toxin-pulsed MC57 cells following pretreatment for 48 h with IFN-γ. Such treatment is known to up-regulate gene expression of MHC class I proteins, several proteasomal subunits, and the proteasome regulator PA28 (27). The fact that IFN-γ is not known to up-regulate extracellular proteases and that presentation of the NP peptide was only seen after IFN-γ treatment of MC57 cells suggests that extracellular processing of toxin is not occurring. Supporting this conclusion, peptide presentation was blocked when the nNP-ricin was prevented from binding and subsequently entering cells by pretreating the toxin with 100 mM lactose (data not shown). Interestingly, both the claNP-ricin fusion (Fig. 2) and a C-terminally located NP-ricin fusion (data not shown) failed to promote specific lysis. Therefore, it was decided to focus on the N-terminally located NP-ricin fusions.

Surprisingly, however, peptide presentation was not dependent on active proteasomes (Fig. 3). When MC57 cells were treated for 24 h with clasto-lactacystin-β-lactone, such that processing and presentation of NP peptides from vaccinia-expressed nucleoprotein was significantly reduced (Fig. 3), surface presentation still occurred under these conditions when cells were challenged with free NP peptide or the fusions nNP-ricin or nNP-6K-ricin. We also observed that the extent of specific lysis between nNP-ricin and the normally more degradable nNP-6K-ricin, in either the presence or absence of proteasome inhibition, was consistently similar. Most strikingly of all, processing and presentation of ricin-delivered NP was independent of functional TAP (Fig. 4). The TAP-deficient RMA-S cells were significantly lysed when ricin delivered NP peptide via endocytosis. Furthermore, this presentation was not dependent on IFN-γ (Fig. 4). Taken together, these data suggest that the processing of NP366–374 from ricin was occurring within the endosome of the cell rather than at the cell surface or within the cytosol.

It was of interest to locate the site of peptide processing and MHC class I loading within the endosome system. Ricin is a promiscuous toxin that, on its journey, can harmlessly enter all compartments of the endosomal lysosomal/trans-Golgi network system of the cell. Therefore, it is conceivable that ricin entering the endosomal system becomes processed by endopeptidase(s) to release the NP peptide such that it is able to interact with either surface recycling MHC class I proteins (28) or with MHC class I complexes that are being secreted. To investigate the possibility of ricin fusions being processed in the early endocytic pathway, we first studied the effect of inhibiting the ubiquitous furin-related serine proteases of the subtilisin family using the competitor inhibitor decanoyl-Arg-Val-Lys-Arg-chloromethylketone-CMK. Members of the furin family are frequently involved in activating proproteins in

**FIGURE 2.** Ricin-mediated delivery of the NP366–374 results in specific lysis by NP-restricted CTL. NP or gp33-specific CTL were tested for their ability to lyse target cells treated as indicated along the x-axis. MC57 target cells, with or without IFN-γ, were pulsed with control peptides, infected with vaccinia virus-expressing full-length Ags, or treated with the NP-ricin fusions. The percentage of specific lysis at three E:T ratios is shown. The data are representative of at least two similar experiments.

**FIGURE 3.** Presentation of ricin-delivered peptide is not dependent on functional proteasomes. NP-specific CTL were tested for their ability to lyse MC57 target cells treated as indicated along the x-axis. MC57 cells were incubated with either nNP-ricin or nNP-6K for 16 h. Where indicated, cells were pretreated with IFN-γ and subsequently with 20 μM clasto-lactacystin-β-lactone (βL) in 0.2% DMSO for 24 h before the addition of toxin. MC57 cells were used as target cells. Controls included MC57 cells infected with a vaccinia virus-encoding influenza nucleoprotein (VacNP), or pulsed with either influenza nucleoprotein (NP pep) or LCMV glycoprotein (gp33 pep) peptides. The percentage of specific lysis at three E:T ratios is shown. The data are representative of at least two similar experiments.
the late secretory pathway, and they have been shown able to generate class I peptides (29–31). However, the furin inhibitor did not block the processing of the N-terminally placed NP peptide from ricin (Fig. 5A). Likewise, presentation was not sensitive to the disruption of endosome/lysosome pH by the lysomotropic amine, chloroquine, which affects furin activity (32) and which would be predicted to indirectly reduce the proteolysis normally occurring within acidic compartments (Fig. 5B). To further investigate any processing in the early endocytic pathway, and to specifically rule out presentation by surface recycled MHC class I proteins, we studied the effect of disrupting both anterograde and retrograde traffic in the Golgi stack with BFA. Such treatment would prevent the toxin moving along its productive entry pathway to the ER and concomitantly block exocytosis of newly assembled MHC class I complexes from the ER. Fig. 5C shows that surface presentation of the NP peptide delivered into cells by ricin is extremely sensitive to BFA. These data support the idea that processing and loading were occurring in the early secretory pathway.

To more precisely investigate whether peptide processed from ricin interacted with MHC proteins as they were being transported through the early secretory pathway, or whether it interacted to stabilize MHC complexes in the ER, we looked at the loading of newly synthesized, empty MHC class I H chains (Fig. 6). This was achieved by radiolabeling RMA-S cells with a short pulse of [35S]methionine after treating cells with or without ricin or the ricin-peptide fusion, followed by detergent lysis, immunoprecipitation of any peptide-loaded H-2D\(^b\) complexes using the conformation-specific mAb B22, and EndoH treatment. Samples were compared with non-EndoH-treated controls. One hundred percent relative loading was seen when cell lysates were incubated with free NP peptide, while the background level of correctly assembled complexes was \(\sim 10\%\). The pulse-labeled MHC H chains were EndoH sensitive as judged by the faster migration of bands compared with a non-EndoH-treated sample (Fig. 6A). When cells were pretreated with nNP-6K ricin, the amount of immunoprecipitable peptide-loaded MHC complexes increased by \(\sim 180\%\) relative to untreated or ricin-only controls (Fig. 6, B and C). A structural-based algorithm (33) used to predict any H-2D\(^b\) MHC class I epitopes present in the nNP-6K ricin primary sequence identified a total of 23 putative epitopes. Of these only 12 had an energy score to indicate good binding interactions, with the NP epitope ranked first. Indeed, as shown in Fig. 6, B and C, there was no...

**FIGURE 4.** Ricin-dependent peptide presentation is more efficient in TAP-deficient RMA-S cells. MC57, RMA, and RMA-S cells were incubated with nNP-ricin or nNP-6K (50 ng/ml) for 16 h. NP-specific CTL were tested for their ability to lyse target cells treated as indicated along the x-axis. Controls include cells pulsed with free peptide from either influenza nucleoprotein (NP pep) or LCMV glycoprotein (gp33 pep). The percentage of lysis at three E:T ratios is shown. The data are representative of at least two similar experiments.

**FIGURE 5.** Presentation of toxin-derived NP peptide is both furin and chloroquine independent but is reliant on a functional secretory pathway. RMA-S cells (6 \(\times\) 10⁶) were incubated with nNP-6K ricin (nNP-6K) at 500 ng/ml for 2 h, following a 2-h pretreatment with increasing concentrations of the specific furin inhibitor decanoyl-Arg-Val-Lys-Arg-chloromethylketone (A), chloroquine (B), or BFA (C). Controls include RMA-S cells pretreated with the various inhibitors for 2 h, then pulsed with free peptide from either influenza nucleoprotein (NP pep) or LCMV glycoprotein (gp33 pep). The percentage of lysis is shown for three E:T ratios. The data are representative of at least two similar experiments.
increase above the background level in the loading of newly synthesized MHC proteins when the 6K-ricin lacking the NP epitope was tested. Collectively, the experimental and theoretical data indicated that the increased loading seen in TAP-negative RMA-S cells of newly synthesized, EndoH-sensitive MHC proteins could be attributed solely to the presence of the NP epitope within the ricin construct, rather than to processing and loading of internal RTA peptides. The significant increase in loading of newly synthesized, EndoH-sensitive MHC proteins when TAP-deficient cells were treated with nNP-ricin supported the idea that the ricin fusions were being processed in the ER or cis-Golgi.

Interestingly, examination of the N terminus of the nNP-RTA revealed the existence of two potential signal peptidase cleavage sites that fit the consensus defined by von Heijne et al. (34) (Fig. 7A) but that are never used in the biosynthesis of the toxin in the producing plant (35). Theoretical prediction analysis indicated that the second site (AGA ↓ TV) would be a more effective cleavage site than the first (IFP ↓ KQ). When these sequences were engineered into the secretory protein preprolactin to replace the natural preprolactin signal peptide cleavage site, they were indeed able to serve as cleavage sites during in vitro translation in the presence of canine microsomal membranes. After cotranslational import, a posttranslational incubation with protease showed that the lower, signal peptide-cleaved band was protected within the vesicles, unless the protease was added together with a membrane-disrupting detergent (Fig. 7B). As predicted, the first site, where incomplete cleavage was seen, was not as effective a substrate as the second site (Fig. 7B, *). To exclude the possibility that simply any sequence from the N terminus of RTA can serve to provide a signal peptidase cleavage site, a stretch of residues between the two putative cleavage sites (PIINF) was also engineered into preprolactin. Because the translation product in this case was resistant to proteinase K (i.e., protected within the membrane vesicles) but remained a full-length protein, we conclude that the sequence was not able to provide a cleavage site for signal peptidase (Fig. 7B).

Signal peptidase is the best-characterized endoprotease within the ER. To investigate any Ag processing function of this protease, the two potential cleavage sites within the nNP-6K ricin were separately rendered noncleavable by replacing the −3 and −1 positions with arginine. As described previously (36), such changes would preclude processing by signal peptidase. Indeed, replacement of the preprolactin cleavage site with either RFRKQ or RGRTV, followed by in vitro translation in the presence of canine microsomal membrane, confirmed that such substitutions prevented cleavage (data not shown). Therefore, these mutant proteins carrying the NP peptide were purified from E. coli. They were shown to be structurally sound because the modified RTAs readily reassociated with RTB, and when the catalytically active RTA equivalent was used the reassembled holotoxins exhibited native
potency (IC\textsubscript{50} 0.015 nM) (19). This indicated that the primary structural changes at the N terminus of RTA did not perturb toxin trafficking to the ER.

To test that signal peptidase could act posttranslationally to process peptides appended to the N terminus of RTA, we added the purified mutant proteins described above to TAP-deficient cells and looked for peptide presentation. We observed that surface presentation of the NP peptide did not occur when the first of the predicted signal peptidase sites (IFP↓KQ) was deliberately disrupted by substitution with arginine (Fig. 8). By contrast, when the second predicted cleavage site (AGA↓TV) was mutated to prevent signal peptidase cleavage, presentation was reproducibly higher than that seen with the control (nonmutated) nNP-ricin (Fig. 8; for a possible explanation, see Discussion). These findings support the idea that CD8\textsuperscript{+} peptide epitopes positioned at the N terminus of RTA may be cleaved from the endocytosed toxin posttranslationally by signal peptidase (or an undetermined protease with signal peptidase-like activity), loaded onto MHC class I proteins and subsequently presented, only when the sequence IFP↓KQ is present at the start of mature RTA. Signal peptidase processing at this site would release the nine-residue N-terminal IFP-C-terminal extension of three residues of mature RTA.

To test whether such processing is confined to one particular peptide at the N terminus of RTA, we tested an alternative peptide. When the H-2D\textsuperscript{a}-restricted gp33 peptide from LCMV was positioned at the N terminus of RTA to generate gp33-ricin, specific lysis by gp33-specific CTL was observed using the standard assay. We reasoned that, because the productive intoxication pathway involves toxin dislocation from the ER as a pseudo-substrate of the ERAD pathway and an encounter with the ubiquitin/proteasome machinery, it may be possible to use ricin to deliver Ags directly into the MHC class I processing pathway. Our data now show that nanomolar amounts of a catalytically crippled form of ricin can indeed be used as a delivery vehicle in vitro. However, only peptides genetically fused to the N terminus of RTA were successfully processed and presented (Fig. 2), and processing was not dependent on active proteasomes (Fig. 3). Indeed, CTL-induced lysis was most efficient when ricin fusions were internalized by cells of a TAP-deficient line in the absence of IFN-γ (Fig. 4). So, although ricin can retrotranslocate from the ER of TAP-deficient cells (known because the native version can kill RMA-S cells (data not shown)) processing of the fused peptide from nNP-ricin is clearly not dependent on this step or other downstream events within the cytosol.

It is now recognized that the classical pathway for the presentation of endogenous antigenic peptides is not the only route by which peptides can reach MHC class I proteins. Indeed, processing of Ag and loading onto MHC class I can be quite flexible. For example, cross-presentation of exogenous Ag may occur. This may happen in some cell lines when exogenous Ags are internalized to intersect the classical processing pathway in the cytosol after release from endosomes or other intracellular compartments, or following endosomal degradation and recycling of peptide to bind cell surface class I molecules (38). However, raising endosomal pH to reduce proteolytic activity or blocking furin-related endoproteases did not prevent peptide presentation (Fig. 5, A and B). Furthermore, because protein traffic through recycling endosomes is known not to be affected by BFA, the effect of this drug in blocking the presentation of peptide delivered via the ricin carrier (Fig. 5C) would again preclude cross-presentation events in this particular case.

Class I Ag processing in the cytosol is normally mediated by proteasomes (for an example, see Ref. 39), although this may not

**FIGURE 8.** Signal peptidase is involved in Ag processing from ricin-mediated delivery. RMA-S cells (6 × 10\textsuperscript{4}) were incubated with 500 ng/ml nNP-6K ricin (nNP-6K), nNP(RFRKQ)-6K ricin (nNP-RFRKQ-6K), or nNP(RGRTV)-6K ricin (nNP-RGRTV-6K) for 16 h. NP-specific CTL were tested for their ability to lyse target cells treated as indicated along the x-axis. Control RMA-S cells were pulsed with free peptide from either influenza nucleoprotein (NP pep) or LCMV glycoprotein (gp33 pep). The percentage of lysis at three E:T ratios is shown. The data are representative of at least two similar experiments.

**FIGURE 9.** Ricin-mediated delivery and TAP-independent surface presentation is not epitope specific. RMA-S cells (6 × 10\textsuperscript{4}) were incubated with either 500 ng/ml nNP-6K ricin (nNP-6K) or 500 ng/ml gp33-6K ricin (gp33-6K) for 16 h. NP- and gp33-specific CTL were tested for their ability to lyse target cells treated as indicated along the x-axis. Control RMA-S cells were pulsed with free peptide from either influenza nucleoprotein (NP pep) or LCMV glycoprotein (gp33 pep). The percentage of lysis is shown for three E:T ratios. The data are representative of at least two similar experiments.
be an absolute requirement (40, 41). In this work we have shown that peptide processing from ricin and surface presentation of peptide can occur in cells where proteasomes are inhibited. Because presentation can also occur when TAP is absent, these data suggest that processing is occurring outside the cytosol. Although peptide transport through TAP is the optimal route by which peptides are loaded onto empty MHC proteins in the ER, it is now recognized that a physical interaction with TAP is not required for loading (42). Indeed, intracellular peptides can be presented on the surface of TAP-negative cells after delivery to the ER through Sec61 translocons via signal peptides (43, 44) or following direct presentation of exogenous peptides into cell organelles (45). Taken together, our data are consistent with processing and loading within the secretory pathway.

Where in the secretory pathway could the processing occur? In addition to the possibility of endosomal processing outlined above, Ag processing may take place within the secretory pathway by unknown exoproteases, or by endoproteases of the furin family, for example. Furin is a member of the subtilisin family of serine proteases that is found predominantly in the TGN from where it cycles via the cell surface. The TAP-independent generation of MHC class I peptides by furin has been observed when placed at the C terminus of the hepatitis B virus core protein (29–31). However, the specific furin inhibitor we used had no effect, and the lack of putative furin sites toward the N terminus of RTA would argue against a role for this protease in the release of epitopes joined to the N terminus of RTA. There are, of course, numerous other endoproteases within the late secretory pathway, most being cell type specific for the activation of proproteins such as hormones, growth factors, extracellular enzymes, etc. In our study, we saw a significant increase in peptide-stabilized MHC complexes that were still EndoH sensitive when TAP-deficient cells are treated with the toxin fusions (Fig. 6). This strongly suggested that loading was occurring within the ER lumen itself. If this were happening it would clearly make sense for the peptides to be proteolytically released within this compartment, in the vicinity of empty class I H chains. Indeed, this may help explain the IFN-γ-independent processing and presentation in TAP-deficient RMA-S cells (Fig. 4). Treatment of RMA-S cells with IFN-γ would lead to an increase in MHC class I proteins in the ER lumen (27). However, such an increase would not be predicted to enhance presentation of the toxin-delivered NP peptide because, in the absence of other peptides entering from the cytosol, there is always a high concentration of available class I proteins within the ER. Conversely, in RMA cells, an IFN-γ-mediated increase in class I molecules within the ER would permit increased loading of the processed NP peptide in the face of competition from other peptides entering via TAP. The ER lumen is a major site of protein folding in an environment not compatible with protein degradation events. Therefore, the consequent likely dealth of endoproteases within the ER would appear to make endoproteolytic release of peptides from ricin unlikely. Nevertheless, some endoproteases do exist within the ER membrane and lumen (e.g., signal peptide peptidase (46) and the chaperones ERP60 and ERP72 (47, 48)). The best-characterized of these is the signal peptidase complex that cleaves short ER-targeting peptide sequences from newly synthesized polypeptides. However, in addition to the removal of signal peptides, this enzyme may also be responsible for the processing the H-2d subunit of the mammalian asialoglycoprotein receptor (49) and in the degradation of aberrant membrane peptides in yeast (50).

Close examination of the mature N terminus of RTA revealed two potential signal peptidase cleavage sites, IFF<sup>T</sup> +<sup>3</sup> ↓ KQ and AGA<sup>T</sup> +<sup>16</sup> ↓ TV, although it should be noted that neither is used in the ricin-producing plant (35). According to theoretical predictions (34), the second of these sites would be the more effective substrate. Indeed, when the natural signal peptidase cleavage site of a preprolactin reporter was substituted with either of these sequences and tested in a translation system supplemented with ER-derived microsomes, it was clear that they could serve as substrates for signal peptidase with the predicted efficiencies (Fig. 7). Furthermore, when the first of these sites was replaced with the nonfunctional arginyl sequences in nNP-RTA, peptide presentation in TAP-deficient cells was completely abolished (Fig. 8). However, a similar mutation of the second cleavage site in nNP-RTA, predicted to be a more effective signal peptidase substrate, actually provoked an increase in peptide presentation.

In the absence of a more extensive investigation we can only speculate as to why this happens. We propose that, although N-terminal peptide fusions to ricin are normally accessible to a signal peptidase-like activity for the production of both peptides, it is somehow more favorable to load only the shorter peptide of the two for subsequent surface presentation. Cleavage at the first site would release a peptide predicted to contain the nine-residue NP peptide with an additional three residues at its C terminus (Fig. 7). By contrast, cleavage at the second site would generate a longer peptide (the nine-residue peptide epitope with a 16-residue C-terminal extension of the RTA sequence; Fig. 7). This hypothesis is consistent with a need for further processing to remove the C-terminal extensions, because we consider it unlikely that 12- and 25-mer peptides generated by signal peptidase would be presented at the cell surface. Although it is known that the NP<sub>366–374</sub> nonamer with a C-terminal extension of three residues can directly assemble with class I molecules, this species has much faster off rates than the optimal nonamer (51). Therefore, it is likely that another enzyme(s) is involved, one that is only able to cut or trim the shorter 12-mer peptide to optimal size, possibly because the longer peptide possesses a structure incompatible for further signal peptidase cleavage and/or subsequent processing. In this scenario, successful generation and loading of NP<sub>366–374</sub> would normally be a competitive process. Abrogating cleavage at the second site, as in nNP-(RGRTV)-6K (Fig. 9), would in effect ensure the production of only the shorter peptide, rather than a mixed population, and thereby increase the opportunities for successful loading and presentation.

Consistent with the hypothesis that extensive Ag presentation can occur within the ER lumen, it has previously been shown that, in cells devoid of TAP, the embedded NP<sub>366–374</sub> epitope within a full-length, ER-targeted NP mutant that lacked N-linked glycans was released for assembly with class I proteins (52). However, a full-length glycosylated NP targeted to the ER in an identical fashion was not capable of sensitizing TAP-negative target cells for lysis (44). These results strongly suggest that the tertiary conformation of polypeptides within the ER lumen may influence the generation of MHC class I epitopes. It should be noted that the longer peptide predicted to arise from the ricin fusion carries a glycosylation sequon. Whether the longer peptide becomes posttranslationally glycosylated (14) to similarly preclude further processing, or whether its structure is simply inappropriate for processing, remains unknown.

Although proteases are known to exist in the ER lumen (Refs. 46–48 and reviewed in Ref. 53), the exact complement and role of resident carboxypeptidases, aminopeptidases, and endoproteases in Ag processing is unclear. Where Ags have been extensively processed in the ER (44), the enzyme(s) responsible have not usually been identified. One ER endoprotease that is known to be involved in the generation of HLA-E binding epitopes is signal...
peptide peptides (54). This enzyme cleaves transmembrane signal peptides within their hydrophobic domain, liberating the resulting N-terminal fragment to the cytosol (46). However, the peptides we predict to arise upon signal peptide proteolysis in the present study appear neither sufficiently hydrophobic nor membrane associated to serve as good signal peptide substrate. Therefore, for the moment, questions regarding the precise mechanism of posttranslational signal peptide cleavage of the N-terminal ricin fusions, any interplay between the peptides generated, and the exact nature and sequence of the processing steps remain unknown and are clearly beyond the scope of the present study.

Signal peptide has been previously speculated to posttranslational process the endogenous ER membrane protein JAW1, releasing fused peptides for surface presentation by class I MHC (55). However, functional evidence for the involvement of signal peptide was not presented in this study. By contrast, the data presented here show that a protein, physiologically able to reach the ER lumen from the exterior of the cell, can be processed there by signal peptide or a signal peptide-like activity. Such processing may be coupled with the protein disulfide isomerase-catalyzed unfolding that toxins appear to require pretranslocation (56) and/or the retrotranslocation process itself. By definition, the early stages of the ERAD of ricin would occur in proximity to the dynamic, bidirectional translocation machinery that contains, on its periphery, signal peptide. Further characterization of these events is now required.

To check that these events were not limited to presentation of the NP epitope, we created a similar N-terminal fusion of the T cell epitope gp33 with RTA. This was also processed and presented in a TAP-independent manner (Fig. 9), in marked contrast to the TAP-dependent presentation of gp33 from the LCMV glycoprotein (57, 58). This finding supports a model of signal peptide processing within the sequence of mature RTA and suggests that, provided the fused peptide did not cover the cleavage site, any added peptide would be similarly processed.

Administration of ex vivo toxin-treated APCs to promote protective CTL as part of a vaccination protocol remains an obvious long-term goal of toxin exploitation. Of the toxins tested for such an application, only pertussis toxin (8) and ricin (this study) appear an application, only pertussis toxin (8) and ricin (this study) appear able to deliver peptides in a BFA-sensitive, TAP-independent manner. Although the processing of an N-terminal peptide Ag borne by pertussis toxin clearly occurred within the secretory pathway, the involvement of signal peptide or other ER protease, and the site of subsequent peptide loading, were not demonstrated (8). The behavior of the ricin vector is particularly striking. The strong TAP-independent bias exhibited by this vehicle may pave the way for developing disarmed ricin vectors directed solely against certain TAP-negative tumors in vivo. Theoretically, it follows that preferential TAP-independent presentation of a common viral epitope, such as the influenza nucleoprotein NP peptide, by a TAP-deficient tumor cell might allow tumor clearance by preexisting viral-specific CTL.

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References


