Surrogate Antigen Processing Mediated by TAP-dependent Antigenic Peptide Secretion

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Abstract. MHC class I proteins assemble with peptides in the ER. The peptides are predominantly generated from cytoplasmic proteins, probably by the action of the proteasome, a multicatalytic proteinase complex. Peptides are translocated into the ER by the transporters associated with antigen processing (TAP), and bind to the MHC class I molecules before transport to the cell surface. Here, we use a new functional assay to demonstrate that peptides derived from vesicular stomatitis virus nucleoprotein (VSV-N) antigen are actively secreted from cells. This secretion pathway is dependent on the expression of TAP transporters, but is independent of the MHC genotype of the donor cells. Furthermore, the expression and transport of MHC class I molecules is not required. This novel pathway is sensitive to the protein secretion inhibitors brefeldin A (BFA) and a temperature block at 21°C, and is also inhibited by the metabolic poison, azide, and the protein synthesis inhibitor, emetine. These data support the existence of a novel form of peptide secretion that uses the TAP transporters, as opposed to the ER translocon, to gain access to the secretion pathway. Finally, we suggest that this release of peptides in the vicinity of uninfected cells, which we term surrogate antigen processing, could contribute to various immune and secretory phenomena.

Protein secretion has traditionally been thought to involve the action of the translocon located in the membrane of the ER of eukaryotic cells. Proteins are recognized cotranslationally when a signal sequence or a signal–anchor sequence emerges from the ribosome (Walter and Johnson, 1994). These sequences are recognized and bound by the signal recognition particle, and the resulting ribosomal complex then interacts with the signal recognition particle receptor on the ER membrane at the translocon (Andrews and Johnson, 1996). This results in the inclusion of proteins within the secretory pathway. This pathway is by far the best described route of protein secretion in eukaryotic cells. Recently, it has been proposed that some proteins are recognized by a component of the translocon, sec 61, exit the ER, and are transported into the cytoplasm where they are degraded (Wiertz et al., 1996).

The translocation into the ER of antigenic peptides for presentation by major histocompatibility complex (MHC) class I molecules is largely independent of the translocon. This form of translocation involves the action of two gene products that are members of the ATP binding cassette family. These genes encode transporters associated with antigen processing 1 and 2 (TAP-1 and -2), and have been implicated in the translocation of peptides from the cytoplasm to the lumen of the ER (Deverson et al., 1990; Bahram et al., 1991; Spies and DeMars, 1991; Spies et al., 1992; Gabathuler et al., 1994). After translocation into the ER, antigenic peptides bind to MHC class I molecules composed of a heavy chain (46-kD) and a light chain (12-kD) called β2m (Nuchtern et al., 1989; Yewdell and Bennink, 1989; van Bleek and Nathenson, 1990; Matsumura et al., 1992), before transport to the cell surface. The assembly and transport of MHC class I molecules appears to be regulated by a series of chaperones that includes calnexin (Degen and Williams, 1991), calreticulin, and tapasin (Sadasivan et al., 1996).

High performance liquid chromatography analysis of peptides eluted from acid-treated whole cells or MHC class I molecules has allowed the identification and characterization of the peptides associated with MHC class I molecules (Falk et al., 1990; Rötzschke et al., 1990; van Bleek and Nathenson, 1990). It is proposed that MHC

1. Abbreviations used in this paper: BFA, brefeldin A; HSP, heat shock protein; MHC, major histocompatibility complex; TAP, transporter associated with antigen processing; VSV, vesicular stomatitis virus; VSV-N, VSV nucleoprotein; VV, vaccinia virus.
class I molecules determine the final identity of MHC-restricted peptides and have an instructive role, in addition to a selective role, in peptide selection (Walnly et al., 1992). MHC binding to larger peptides followed by protected proteolytic trimming is a possible mechanism that could account for the observed MHC dependency of cellular peptides (Falk et al., 1990). Peptides unable to bind MHC class I because they are in excess or lack the correct MHC binding motif for the MHC haplotype are thus far undetectable by the techniques commonly used in the field, and are presumed to be short lived and degraded (Falk et al., 1990; Rötzsche et al., 1990). Recent results, however, suggest that peptides not able to bind to a MHC class I molecule intracellularly may be found bound to heat shock proteins (HSPs) such as gp96 (gp94; Arnold et al., 1995). These authors show that cellular antigens are represented by peptides associated with gp96 molecules independently of the MHC class I expressed, confirming earlier results (Udono and Srivastava, 1993, 1994). Gp96 extracted from a specific cell is able to induce cross-priming (Udono and Srivastava, 1993, 1994). Finally, two studies have demonstrated that peptides transported into the lumen of the ER, and do not bind to MHC class I molecules, can be transported out of the ER into the cytoplasm again by a process called “eflux” (Momburg et al., 1994; Schumacher et al., 1994), which may involve the action of the TAP molecules or the sec 61 protein associated with the translocon (Wiertz et al., 1996).

We have developed a new bioassay to test the hypothesis that peptides translocated into the ER by the action of the TAP molecules become secreted. Using this assay, we present evidence of an alternative secretion pathway that exists in various mammalian cell types. These observations revise the model of peptide catabolism, and may provide an explanation for various immune and secretion phenomena.

Materials and Methods

Cell Lines and Cell Culture

RMA and RMA-S (H-2b) cell lines (Kärre et al., 1986) are maintained in culture in RPMI-1640 medium (GIBCO BRL, Gaithersburg, MD), supplemented with 10% heat-inactivated FBS, glutamine, penicillin, streptomycin, and Hepes. CMT.64 (H-2k), a small cell lung carcinoma cell line (Jeffieres et al., 1993; Gabathuler et al., 1994), is maintained in DME (GIBCO BRL) supplemented with 10% heat-inactivated FBS, glutamine, penicillin/streptomycin, and Hepes. CMT.12-12 are CMT.64 cells transduced with the rat TAP-1 and -2 cDNA (provided by G. Butcher, Agriculture and Food Research Council, Institute of Animal Physiology, Cambridge, UK). CMT.12-12 and L cells (American Type Culture Collection, Rockville, MD) are grown in DME (GIBCO BRL) with supplements. RIE (H-2k) cells, RIE expressing K0 and βm were provided by D. Williams (Williams et al., 1989).

Tissue Culture Infections Dose Affecting 50% of the Cultures (TCID50) Determination

Vero cells in 96-well plates were treated with vesicular stomatitis virus (VSV) preparations collected from the indicated cell lines 72 h after infection with 103 TCID50 units of VSV virus (multiplicity of infection [MOI] of 0.01). The plates were monitored for 5 d for cytopathic effect, and then the final titers were calculated as per TCID50.

Vaccinia Virus (VV) Constructs

The recombinant VV containing the VSV-N 52–59 peptide gene (VV–NP) was constructed by first ligating a double-stranded synthetic oligonucleotide encoding the VSV-N 52–59 peptide into a VV shuttle vector pJS5 (provided by B. Moss, National Institutes of Health [NIH], Bethesda, MD). This contained SacI and NheI restriction sites. Hydrolysis of the oligonucleotide, which contained SacI and NheI overhangs for ligation into pJS5, is as follows: 3′-tgag-tac-tct-cct-cag-att-gcg-gag-agt-gat-5′, with the complementary strand as 5′-c-agt-agga-tat-gtc-tac-c-aag-ggc-ctc-tga-g-3′. The VV–NP was then provided by J. Yewdell (NIH, Bethesda, MD) (Eisenlohr et al., 1992; Bacik et al., 1994).

Virus Infections and Cytotoxic T Lymphocyte (CTL) Assay

CTL effectors were generated by injecting C57Bl/6 mice with VSV (5 × 102 TCID50 per site) behind the ears. 5 d after the draining lymph nodes were removed, and then lymphocytes were cultured at 4 × 106 cells/ml for 3 d in the presence of stimulation in RPMI-1640 with 10% heat-inactivated HyClone FBS (GIBCO BRL), glutamine, penicillin/streptomycin, Hepes, 0.1 mM essential amino acids, 1 mM Na-pyruvate, and 50 μM 2-ME. In the experiment described in Fig. 6, CTL effectors were generated from splenocytes. Briefly, spleens from C57Bl/6 VSV-infected mice were homogenized with a Dounce, and then the splenocytes were pelleted and washed three times before counting. Splenocytes were kept at 30 × 106 cells/ml per 1 ml medium as described previously. These splenocytes were kept for 10 d in culture with 1 μM VSV-N 52–59 peptides before the CTL assay. Cold targets were either treated with VSV (MOI of 2) for 12 h and subsequently washed, or treated with exogenous peptides (VSV-N 52–59) and subsequently washed. VV infection of cold targets was done as follows: VV–NP, VV–pJS5, and VV–ESNP were tetrasyprized in 0.1 vol 25 mg/ml tetrasyprin (C250; Difco Laboratories, Inc., Detroit, MI) for 30 min at 37°C, with vortexing every 10 min. After washing CMT.64, CMT.12-12, and L cells with PBS, the virus was added to the cells (MOI of 10), along with 0.5 ml RPMI supplemented with 2% heat-inactivated FBS, glutamine, penicillin, streptomycin, and Hepes. They were incubated for 1.5 h at 37°C for 3 d before adding 3 ml RPMI supplemented with 10% heat-inactivated FBS, glutamine, penicillin, streptomycin, and Hepes. They were incubated for 1.5 h at 37°C for 3 d. After infection the temperature block at 21°C was done from 3 h after infection until the beginning of the CTL assay. The cells were removed with versene and 0.05% tetrasyprin, and then washed two times with medium before addition to the CTL assay. The total infection time was 6 h until the start of the assay, or longer as indicated. Hot targets, which were RMA cells or as indicated, were treated with [51Cr] for 1 h at 37°C and subsequently washed. [51Cr] was used at 100 μCi per 106 cells. Cold targets were diluted two- or threefold when indicated, starting with 2 × 105 cells per well at a 20:1 ratio cold/hot targets. Each well received 106 effector CTL and 106 hot targets (100:1 ratio effector/hot targets). BFA at a concentration of 10 μg/ml was added to the assay when indicated. Effectors plus cold and hot targets were incubated together in V-bottom 96-well plates for 4 h for radioactivity release. In some assays, cold targets are replaced by medium containing peptides or viruses where indicated. The supernatant of VSV-infected cells was previously desalted through a G-25 column (Pharmacia Diagnostics, AB, Uppsala, Sweden), and then lyophilized before reconstitution in 2 ml of medium to achieve a 5× concentration. The [51Cr] release was measured by an LKB1282CS Compugamma counter (model 1282CS, LKB Pharmacia, Turku, Finland), and the specific [51Cr] release calculated as (experimental – medium control) / (total medium control) × 100%. The spontaneous release never exceeded 20% of the maximum release (treated with 1.5% Triton X-100).

Peptide Translocation Assay

Streptolysin-O–mediated transport assays were performed as described previously (Momburg et al., 1994) with minor modifications. Briefly, a peptide library was provided by J. Yewdell (NIH, Bethesda, MD) (Eisenlohr et al., 1992; Bacik et al., 1994).
permeabilized with 2 IU/ml of streptolysin-O (bioMerieux, Marcy-l’Etoile, France) for 10 min at 37°C. The iodinated peptide library (~66 ng) was added, and then the incubation was continued for 10 min in the presence or absence of 10 mM ATP (Sigma Chemical Co., St. Louis, MO). The cells were then transferred to ice, lysed in a buffer containing 1% Nonidet P-40, 150 mM NaCl, 5 mM MgCl₂, 50 mM Tris-HCl, pH 7.5, and then the nuclei were removed by centrifugation at 14,000 rpm for 10 min. Translocated peptides that had been glycosylated in the ER were recovered by adsorption to concavalin A-Sepharose beads (Pharmacia Diagnostics, AB). The beads were washed five times in lysis buffer, and then the associated radioactivity was measured in a LKB1282CS Compugamma counter (model 1282CS; LKB Pharmacia).

**BFA, Azide, and Emetine Treatment and Temperature Block**

BFA was used at a concentration of 10 μg/ml. BFA treatment of cold targets was started at the same time as the VSV infection at a MOI of 2, or for the last 4 h of infection before incubation with hot targets and effector CTL. BFA was kept in the CTL assay at a diluted concentration of 2 μg/ml that did not affect CTL recognition. BFA was added ~2 h after the start of the VV-NP or VV-ESNP infection, and then kept at 10 μg/ml throughout the infection and the assay or as indicated. The peptide translocation assay was done after overnight treatment of the cells with 10 μg/ml of BFA.

Azide, a metabolic poison, was added to VV-NP-infected L cells from the start or 3 h after the start of the infection at a concentration of 50 mM, and then removed by washing before the CTL assay. Emetine (Sigma Chemical Co.), a protein synthesis inhibitor, was added to VV-NP-infected L cells 3 h after the start of the infection at a concentration of 10 or 50 mM, and then maintained or washed before the CTL assay. The temperature block at 21°C was done for 3 h after a 3-h VV-NP infection of L cells until the beginning of the CTL assay. Exposure of the cells to 21°C would otherwise inhibit the infection of the cells or the CTL assay.

**Results**

The binding of peptides with MHC class I heavy chain and β₂m within the lumen of the ER has been well documented. However, alternative fates for peptides transported into the ER may exist. One such possibility is the expulsion of the peptide from the cell via the secretory pathway. To determine whether peptides transported into the ER are subsequently secreted from the cell, we have developed a novel assay in which the CTL-mediated lysis of uninfected cells provides a readout of the ability of virally infected cells to transfer sensitizing peptide. This was achieved by incubating virally infected cells with chromium (Cr)-loaded uninfected cells. Virus-specific CTL are then added to the culture, and any resulting Cr release is measured. In the absence of peptide transfer, the levels of release would be minimal. Fig. 1 (A) clearly shows that significant levels of lysis of the uninfected RMA cells (Kärre et al., 1986) are achieved after incubation with VSV-infected RMA cells, strongly suggesting that specific viral peptides had been transferred from the infected cells. We have used uninfected Cr-loaded RMA cell killing as a readout system because RMA cells are more sensitive to exogenous peptides than other cell lines under the conditions of our experiment. However, we are able to observe the same phenomenon when using Cr-loaded RMA-S and CMT.64 cells as recipients (data not shown).

To determine whether peptides shed from cell surface MHC class I complexes contributed to this transfer, we made use of the fact that in the context of an H-2Kb response, >90% of CTL recognize a single immunodominant epitope of the VSV-N protein (VSV-N 52–59; van Bleek and Nathenson, 1990; Zhang et al., 1992). Our results using cold target competition assays (Kulkarni et al., 1993), indicate that the peptide transferred is indeed VSV-N 52–59 (data not shown). Uninfected RMA cells were pulsed with a high concentration of VSV-N 52–59 peptide, and then the ability of these cells to sensitize Cr-loaded uninfected cells was measured (Fig. 1 A). We find that cells pulsed with saturating concentrations of antigenic peptide are not able to efficiently transfer peptides to recipient cells. Although this may happen over time, as suggested by Huang et al. (1994), we find no evidence for this in our assays. The inefficiency of cells pulsed with exogenous peptides to sensitize target cells for CTL killing suggests that transferred peptides do not originate from peptide–protein complexes present on the cell surface (Srivastava et al., 1994). Importantly, this assay indicates that the occurrence of classical bystander killing in this system is negligible.

We further used the existence of the single immunodominant epitope to calculate approximate concentrations of peptide transferred to recipient cells. Fig. 1 (B) shows curve allows approximation of the amount of peptide transferred from virally infected cells. RMA cells were incubated with different concentrations of the VSV-N peptide in presence of cold and hot RMA targets during 4 h. Striped bars represent RMA.

**Figure 1.** Specific CTL recognition after peptide transfer to uninfected RMA cells from VSV-infected RMA cells. (A) Peptide transfer occurs after viral infection but not after pulsing with synthetic viral peptide. Hot targets were RMA cells and cold targets were diluted twofold, starting with 2 × 10⁶ cells per well at a 20:1 ratio to cold/hot targets. [⁶⁷Cr] release was calculated as indicated in Materials and Methods. Black, striped, and white bars represent RMA+VSV, RMA+p, and RMA+-p, respectively. (B) A Peptide-RMA cell dose-response
the dose-response curve obtained when a mixture of cold and Cr-loaded RMA cells, mimicking the conditions of the transfer assay, are pulsed with different concentrations of peptide. When VSV-infected RMA cells (Kärre et al., 1986) are used as the peptide donors, we obtain 25% lysis of the uninfected cells at an infected/uninfected ratio of 20:1 (Fig. 1 A). Comparison with the dose-response curve (Fig. 1 B) suggests that this level of lysis represents exposure of the uninfected cells to \( \sim 5-50 \) nM of peptides. It must be stressed that this can only be an approximation because of the kinetics of the viral infection that contrast with the static concentrations achieved by adding exogenous peptide.

It was important to determine if the peptide transfer we observed could be explained by viral killing or CTL-mediated release of peptides from infected cells. By altering the parameters of VSV infection, we clearly demonstrate that the time course of the assay (4 h) does not allow Cr-loaded cells to be infected with VSV, process and degrade the proteins, and present the VSV-N peptides to specific CTL (Fig. 2 A). To further address this possibility, we studied cells that are not recognized by VSV-specific K\(^b\)-restricted CTL, but retain their ability to be infected by virus. CMT.64, T2, Ltk\(^-\), and RMA cell lines are infected to a similar level by VSV (Table I). We show that human T1 (Salter and Cresswell, 1986), and murine Ltk\(^-\) (H-2\(^k\)) transfer peptides to recipient uninfected RMA (H-2\(^b\)) cells (Fig. 2 B). However, none of these cell lines is recognized and lysed by VSV-specific CTL at different effector to target ratios (E:T). Mock infected Ltk\(^-\) cells (\( L^{+---} \)) were used as negative controls. Black and striped bars represent T1 VSV and L VSV, respectively.

Figure 2. CTL and viral lysis do not mediate peptide transfer. (A) Peptide sensitization is not because of VSV infection of Cr-loaded cells. RMA cells were infected for 12 h with VSV as previously described (RMA+VSV), or alternatively, RMA cells were treated with VSV immediately before adding the effectors (RMA.4+VSV). The effector to target (E:T) ratios ranged from 12.5:1 to 100:1. Black, striped, and white bars represent RMA+VSV, RMA+-, and RMA.4+VSV, respectively. (B) Peptide transfer from infected to uninfected cells is independent of the MHC haplotype of the cell infected by VSV and not dependent on CTL-mediated release. A human T1 cell line and the mouse Ltk\(^-\) (L) were treated with or without (\( L^{+---} \)) VSV (MOI of 2) for 12–18 h as described previously. RMA cells were used as hot targets as described previously. Black, striped, and white bars represent T1+VSV, L+VSV, and L++-, respectively. (C) VSV-infected T1 and Ltk\(^-\) were not recognized by CTL used in the assay. T1, Ltk\(^-\) (L) cells were added to effectors after VSV infection and \(^{[51]}\)Cr labeling to examine whether or not they are recognized as targets for VSV-specific CTL at different effector to target ratios (E:T). Mock infected Ltk\(^-\) cells (\( L^{+---} \)) were used as negative controls. Black and striped bars represent T1+VSV and L+VSV, respectively.
Table I. TCID_{50} Results: 10\(^{6}\) Cells Infected with VSV at a MOI of 0.1\(^{*}\)

| Cell line | Titer     | Exp. 1 | Exp. 2 |
|-----------|-----------|--------|--------|
| CMT 64    | 9.6 \times 10^6 | 5.4 \times 10^6 |
| RMA       | 1.2 \times 10^7  | 7.2 \times 10^6  |
| T2        | 9.3 \times 10^6  | 6.2 \times 10^6  |
| Ltk\(^{-}\) | 1.1 \times 10^8  | 8.1 \times 10^8  |

\(^{*}\) The cell lines used in this assay were approximately the same in their ability to reproduce VSV.

To establish the significance of individual components of the antigen processing pathway for peptide transfer, we investigated whether cell variants deficient in one or more elements of this pathway are able to transfer peptide. Human T2 (Salter and Cresswell, 1986), and murine CMT.64 (H-2\(^b\)), cells (Jefferies et al., 1993; Gabathuler et al., 1994) do not express either TAP-1 or -2. After infection with VSV, we find that these cell lines are neither recognized by VSV-specific K\(^b\)-restricted CTL (Jefferies et al., 1993; data not shown), nor are they able to efficiently transfer peptides to the recipient RMA cells (Fig. 3A). As shown above, the wild-type T1 cells, though not recognized by VSV-specific CTL (Fig. 2C), are able to transfer a VSV peptide (Fig. 2B). CMT.64 cells induced with IFN-\(\gamma\) to express TAP-1 and -2 are able to transfer peptides (Fig. 3A). Furthermore, CMT.64 transfected with the rat TAP-1 and -2 genes (CMT.12-12) are able to transfer a peptide to RMA cells (Fig. 3A). These data reveal a dependency on the expression of the TAP transporters for the exchange of peptide(s). This infers that the peptides must be translocated into the ER to be transferred.

To investigate whether interaction with and transport by MHC class I molecules is a prerequisite for peptide transfer, we used the murine R1.E (H-2\(^b\)) cells (Williams et al., 1989) that lack \(\beta_2m\), and therefore do not transport MHC class I heavy chain. R1.E cells are able to efficiently transfer peptides to RMA cells (Fig. 3B). The contribution of K\(^b\) molecules alone, and of K\(^b\) and \(\beta_2m\) to this peptide transfer was established by examining R1.E cell derivatives transfected with heavy or heavy and light chains. The expression of K\(^b\) alone and both K\(^b\) and \(\beta_2m\) does not affect the efficiency of peptide transfer of transfected R1.E cells (Fig. 3B). This indicates that peptide binding and transport of MHC class I molecules is not required for transfer of the peptide.

The role of the secretory pathway in peptide transfer was studied using BFA, an inhibitor of protein secretion. BFA was used at concentrations reported previously (Nuchtern et al., 1989; Yewdell and Bennink, 1989; Jefferies et al., 1993), which effectively inhibit protein secretion in these cells, but do not harm cell viability (data not shown). BFA-treated RMA cells were infected with VSV, and then examined for their ability to transfer peptide to recipient cells. We find that BFA dramatically inhibits peptide transfer (Fig. 4A), suggesting that the peptide must be actively transported through the secretory pathway for efficient transfer to take place. To confirm that BFA did not simply inhibit CTL recognition, different concentrations of BFA from 4–10 \(\mu\)g/ml were added directly to a CTL assay, and then the effects were monitored (Fig. 4B). It is clear from these data that VSV-specific CTL recognition of peptide-pulsed targets (RMA cells) was not affected by BFA. There are no significant differences between CTL lysis and presence or absence of BFA in the effector/target ranges of 100:1 to 0.046:1 (Fig. 4B). To determine that BFA does not merely inhibit peptide translocation and that the observed effects are truly on the secretory pathway, BFA was used in the well-established in vitro peptide transport assay. The peptides were designed with an iodination site (Tyr) and an N-glycosylation site (Heemels et al., 1993), which permits ER translocation to be assessed by the ac-
Figure 4. BFA inhibition demonstrates the dependency of peptide transfer on the secretory pathway. (A) BFA inhibits peptide transfer in VSV-infected RMA cells. Hot targets were RMA cells treated as previously described. Cold RMA cells were treated with BFA at 10 μg/ml from the start of VSV infection (RMA + BFA + VSV), only for the last 4 h of the VSV infection (RMA + VSV + BFA), or without BFA at 10 μg/ml (RMA + VSV). VSV infection was done for 12 h at a MOI of 2. BFA was kept in the assay during incubation with effector CTL at a diluted concentration of 2 μg/ml.

Black, striped, and grey bars represent RMA + BFA + VSV, RMA + VSV + BFA, and RMA + VSV, respectively. (B) BFA treatment does not inhibit the CTL-dependent lysis of target cells. Hot targets were RMA cell-pulsed (RMA + p) or not (RMA), with VSV-N 52–59 peptide at a concentration of 1 μM. BFA was added in the assay during incubation with effector CTL at a concentration indicated (4, 5, or 10 μg/ml). (C) BFA does not affect the TAP-dependent peptide translocation in the ER. Cells as indicated treated or not with BFA (10 μg/ml) were permeabilized with streptolysin-O, and then used to monitor the ER translocation of radioiodinated peptides in presence (ATP) or absence (control) of ATP. The protocol is described in Materials and Methods. The radioactivity in counts per minute is represented.
peptide transfer was observed in BFA-treated or -untreated cells. This is consistent with the TAP dependency of these processes observed previously. Conversely, the peptide transfer process of the TAP-expressing cells (L and CMT.12-12 cells) was grossly inhibited by the presence of 10 μg/ml of BFA. In addition, emetine, which inhibits protein synthesis (Braciale et al., 1988), and azide, which inhibits respiratory metabolism, were used to demonstrate that this process was energy- and protein synthesis dependent, and was not a result of general cell deterioration because of virus-induced cell death. Emetine was shown (Fig. 6 D) to be an excellent inhibitor of the process when incubated with the donor cells demonstrating that protein synthesis is required and, moreover, peptide secretion is an active process. Similarly, azide (Fig. 6 E) is able to inhibit this process, and shows that energy metabolism is required for the secretion of the peptides that sensitize recipient target cells. Temperature reduction to 21°C has been shown to block protein secretion (Matlin and Simons, 1983; Kuismanen and Saraste, 1989), and its effects were examined on the peptide transfer process. Temperature block before incubation with CTL clearly affects the peptide transfer process (Fig. 6 D). The data demonstrates that the blockage is not complete, and this is likely because of the fact that the temperature block must be released to incubate the cells with the CTL in this assay. Thus, some sensitization is expected. These data demonstrate surrogate antigen processing is dependent on TAP translocation, secretion, and energy, and on de novo protein synthesis.

As a final confirmation that a novel route of entry into the secretory pathway had truly been identified, it was reasoned that the TAP dependency of this pathway could be bypassed by the addition of a signal sequence to the NH₂-terminus of the VSV-N peptide. This peptide should enter the secretory pathway via the translocon, and thus bypass the TAP translocation process. A VV construct containing the E3/19k signal sequence (Eisenlohr et al., 1992; Bacik et al., 1994) was obtained (a gift of J. Yewdell), and peptide transfer was monitored in TAP-expressing (L cells) and -nonexpressing cells (CMT.64) infected with this recombinant VV (VV–ESNP) or with the recombinant VV containing the VSV-N peptide without the signal sequence (VV–NP; Fig. 7 A). In L cells that express TAP molecules, both the peptide with and without the signal sequence are able to be transferred. The VSV peptide without the signal sequence is slightly more efficient; this could be because of the greater activity of the TAP translocator versus the translocon, or simply because of the heterogeneity in expression levels between the two recombinant viruses. These data contrast with that obtained in cells lacking TAP molecules (CMT.64 cells). Expression of the peptide containing the signal sequence results in its transfer in the absence of the TAP molecules, whereas the peptide lacking a signal sequence is not able to be transferred from these cells. These data demonstrate that the secretory pathway, which has been identified, is distinct from that involving the translocon, and that the new secretory pathway involves translocation by the TAP molecules.

To demonstrate the BFA sensitivity of the peptide transfer process involving the translocon secretory pathway, BFA was added to the assays and its effects were observed (Fig. 7 B). Peptide transfer from the cells expressing the VSV-N peptide containing the signal sequence is clearly affected in TAP-expressing and -nonexpressing cells. These data demonstrate that peptide transfer can take place through the translocation of peptides via the translocon if the signal exists in the peptide to do so. These data also suggest that it is the initial translocation event involving the translocon or TAP complexes that controls the peptide transfer process, and the rest of the secretory pathway may be shared by the two translocators.

Discussion

Here we show that endogenously produced antigenic peptides have alternative fates within the cell. Not only can peptides bind to MHC class I molecules and be transported through the cell to the cell surface, they can also be secreted independently of MHC class I molecules from the cell. This secretion appears to be dependent on TAP-based translocation into the ER, and is BFA sensitive. Therefore, the normal secretory pathway is important for this process. Data from other laboratories (Falk et al., 1990; Rötzschke et al., 1990; Wallny et al., 1992) suggest that peptides, once generated, are able to bind to MHC class I molecules in the lumen of ER, and they speculate this protects them from total degradation. On this basis, an instructive role for MHC class I molecules in cellular peptide processing has been postulated (Wallny et al., 1992). Recently, new results (Arnold et al., 1995) have shown an alternative fate for antigenic peptides that do not bind self-MHC class I molecules. These peptides are found bound to HSPs, like gp96, and were previously undetectable in these cells. Furthermore, other work has demonstrated that peptides transported into the lumen of the ER can be transported out of the ER and back into the cytosol by a
process referred to as an efflux (Momburg et al., 1994; and Schumacher et al., 1994). It is possible that these peptides could bind to proteins like gp96 and then be protected from degradation. Our results show that peptides do not bind to MHC class I molecules, either because they are made in excess or because the cells lack the MHC class I molecules capable of binding the VSV peptide, are secreted from the cell. Thus, in a situation in which there are high concentrations of peptides produced, the equilibrium can be pushed towards either secretion of the unbound peptide or degradation by lysosomal enzymes.
peptide, or return of the peptide to the cytoplasm, which presumably results in further degradation. It is speculated that the return of the peptide to the cytoplasm involves the TAP transporters, but it could involve the recently described translocon function involving sec61 that exudes some fraction of MHC heavy chains to the cytoplasm (Andrews and Johnson, 1996; Wiertz et al., 1996).

Overall, the pathway we describe is a hitherto unknown pathway for peptide secretion that does not involve the translocon for translocation into the ER, but relies upon the TAP transporters for ER delivery. The limitations for this transport mechanism are likely to be the size and composition of the peptides that can be transported by the TAP molecules. Clearly, the specificities of the TAP molecules from different species and different TAP alleles within a species would alter the type of peptides delivered to the ER. Additionally, the affinity of the MHC molecule for a peptide could contribute to its secretion. The size of the peptides that could conceivably be secreted by this mechanism would be limited by the size of peptides transported by the TAP molecules. Furthermore, the broader specificity of action of the TAP transporters as compared to the binding of the MHC molecules may result in a pool of peptides that could also be secreted in this way, but do not have the ability to bind to MHC molecules. This could result in secretion of small peptides that could function in processes not involving MHC molecules.

The phenomenon we describe differs from the previous observations (Arnold et al., 1995), where gp96 and HSPs are ER resident or cytosolic proteins, and under normal circumstances not released from cells. Gp96 with bound peptides must be extracted from homogenized cells. It is even possible that peptides become bound to gp96 during the extraction process. In our case, secretion from live cells allows peptides to become associated with the MHC class I molecules expressed on the cell surface of surrounding, uninfected cells. We do not know in which form the peptide is secreted: it may be bound to a carrier protein, for example. A thorough characterization of the medium containing the sensitizing peptide is needed to identify such a protein. It is unlikely that these secreted peptides would have been detected in previous extraction protocols used in the field.

To support the model of peptide secretion, it was crucial to exclude a role for virally induced or CTL-mediated lysis as a source of priming peptide. To achieve this, many of our assays utilized CTL that do not recognize the virally infected cell type used. Furthermore, one would not expect a dependency on TAP expression if peptides were transferred by viral lysis per se. Additionally, the virus titre produced by these different cell lines was similar, also demonstrating no TAP dependency for VSV infection. Although the phenomenon we describe is dependent on TAP-mediated translocation into the ER and general protein secretion, it is not specifically reliant on MHC transport. The possibility that the VSV-N peptide is somehow unique because of its potentially high affinity for Kb is unlikely to provide an explanation: a high affinity for Kb would drive the equilibrium towards assembly with MHC heavy chain rather than secretion; yet, we easily detect it. It is interesting to note that Huang et al. (1994) have elucidated mechanisms in which proteins or peptides may either be exchanged from donor to recipient after release from the cell surface or released after uptake of antigen by the antigen processing donor. Though these phenomena may operate under specific circumstances, they relate to the processing of exogenous antigens rather than the endogenous antigens we examine here. Furthermore, notwithstanding complete protease digestion of small peptides to amino acids, peptide secretion seems a reasonable fate for peptides that enter the secretory pathway. It will be of great interest to examine this phenomenon in other systems and to determine whether other proteins, such as carrier proteins, are required for peptide secretion.

Our results using a VV construct expressing the VSV-N peptide epitope demonstrate clearly that the peptide trans-
fer is not the result of the VSV replication pathway. The VV-encoded peptide (VV-N 52–59) also requires a functional TAP transporter, strongly supporting the view that the normal antigen peptide processing machinery is required. In addition, these data demonstrate that the 8-amino acid peptide itself can be transferred, and transfer is thus not dependent on the proteolytic cleavage of a larger fragment extra- or intracellularly. Furthermore, peptide transfer of the VV-expressed VSV-N peptide is also BFA sensitive and has the same characteristics as the VSV-expressed peptide. To address the temperature, energy, and secretory dependency of this process, inhibitory methods were used. Emetine inhibits protein synthesis (Braciale et al., 1989), and clearly inhibits surrogate antigen processing (Fig. 6 D). Likewise, azide also inhibits energy metabolism and ablates peptide transfer (Fig. 6 E). Furthermore, a cold temperature (21°C) block (Matlin and Simons, 1983; Kuismanen and Saraste, 1989) that inhibits protein secretion also inhibits peptide transfer (Fig. 6 D). The data presented here, and confirmed by all of the controls described above, demonstrate the dependency of this process on energy metabolism, de novo synthesis, and protein secretion.

Thus, these data and controls conclusively show that this phenomenon is not because of a trivial release of peptide by dead cells. Overall, peptide transfer is not dependent on VSV infection nor the viral lytic cycle. Finally, we demonstrated that peptides containing a signal sequence can bypass the TAP-dependent translocation event and use the classic translocon for entrance into the lumen of the ER. Once there, the pathway for peptide transfer is BFA sensitive. These data suggest the TAP molecules regulate access to the peptide secretory pathway of peptides lacking signal sequences, but that the translocon regulates the access of signal sequence-containing peptide to the secretory pathway. Therefore, the downstream components of the secretory pathway may be shared by both translocators. The interpretation that this is a new secretory pathway is based on our assertion that protein translocation across the ER membrane is an initial event in secretion, and our evidence demonstrating that peptides transported by the TAP complex can be secreted illustrates a hitherto unknown mechanism for gaining access to the downstream components of the secretory pathway.

The peptide transfer phenomenon we have described in vitro represents a hitherto undescribed fate for antigenic peptides. It will be of great interest to determine if this is indeed a general phenomenon relevant to many peptides. If this is the case, it is intriguing to speculate that peptide secretion may play a role in some currently poorly understood phenomena, such as pathogen-induced autoimmunity, where viral infection in one anatomical location results in tissue damage in another (Sinha et al., 1990). Additionally, it may account for mechanisms of rejection of organs on the basis of minor histocompatibility type and cross-priming phenomena (Bevan et al., 1976; Gooding and Edwards, 1980; Huang et al., 1994; Huang et al., 1996). Lastly, this mechanism could account for T cell selection in the thymus in the absence of thymic cells expressing the selecting antigen.

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