Anti-Peptide Antibody Blocks Peptide Binding to MHC Class I Molecules in the Endoplasmic Reticulum

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Anti-Peptide Antibody Blocks Peptide Binding to MHC Class I Molecules in the Endoplasmic Reticulum

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The finding that MHC class I molecules are physically associated with the TAP transporter has suggested that peptides may be directly transported into the binding groove of the class I molecules rather than into the lumen of the endoplasmic reticulum (ER) where they subsequently would encounter class I molecules by diffusion. Such a mechanism would protect peptides from peptides in the ER and/or escaping back into the cytoplasm. However, we find that an anti-peptide Ab that is cotranslationally transported into the ER prevents TAP-transported peptides from being presented on class I molecules. The Ab only blocks the binding of its cognate peptide (SIINFEKL) but not other peptides (KVVRFKDL, ASNENMETM, and FAPGNYPAL). Therefore, most TAP-transported peptides must diffuse through the lumen of the ER before binding stably to MHC class I molecules. The Journal of Immunology, 2001, 166: 3952–3956.

Major histocompatibility complex class I molecules are highly polymorphic cell surface glycoproteins that present peptide Ags of 8–10 aa to CTL (1, 2). The majority of class I-presented peptides are derived from proteins that are degraded in the cytoplasm (3) and are then transported into the endoplasmic reticulum (ER) by TAP (4, 5). In the lumen of the ER (3), peptides bind to newly synthesized MHC class I heterodimers, and these complexes are then transported to the plasma membrane for display (2). This process allows the immune system to detect cells that are synthesizing viral or abnormal (e.g., mutant) proteins.

When cells are lysed and solubilized in weak detergents, MHC class I molecules coimmunoprecipitate with TAP molecules, indicating that they are not covalently associated in the ER (5, 6). These molecules are brought together by tapasin, a 48-kDa protein that binds to both MHC class I and TAP (7). These findings suggested a model where peptides are translocated by TAP directly into the binding groove of MHC class I molecules (5, 7). Through this mechanism, transported peptides could be efficiently loaded onto class I molecules, escaping further trimming or destruction in the ER lumen. In support of this concept, it was found that mutant cells that lack tapasin (and the association between MHC class I and TAP) presented Ags inefficiently (8). However, the importance of the interaction between TAP and class I to the loading of peptides on class I molecules is still unclear. Several allelic forms of class I molecules and a mutant class I molecule do not coimmunoprecipitate with TAP yet do present peptides (9) (10–12). Similarly, transfection of a truncated soluble form of tapasin-deficient mutant cells restores Ag presentation, but TAP and class I molecules do not coimmunoprecipitate in these cells (13). These findings may indicate that peptides are not transported directly into TAP-associated class I molecules. Alternatively, it is possible that the association between TAP and class I molecules still occurs in these situations but is weaker and lost on detergent solubilization of the ER membrane. In fact, the association of TAP and class I molecules in normal cells is not detected in some detergents, e.g., Nonidet P-40 (7). Moreover, these findings would not rule out the possibility that peptides normally load primarily into TAP-associated class I molecules, but if cells lack the TAP-class I association, then peptides can diffuse through the ER lumen and bind to non-TAP-associated class I molecules. In addition, alternative roles for MHC class I association with TAP are possible. For instance, this association may function in regulating TAP levels (13).

In summary, there are no data that address whether the TAP-associated MHC class I molecules usually capture peptide directly after transport or from peptides that are diffusing through the ER lumen. To address this issue, we examined whether an anti-peptide Ab would compete for peptide loading on MHC class I molecules in the ER.

Materials and Methods

Cell lines

Cells were maintained in DMEM or RPMI 1640 (Irving Scientific, Santa Ana, CA) supplemented with 10% heat-inactivated FCS (Life Technologies, Grand Island, NY), 1000 U/ml penicillin, 1 mg/ml streptomycin, 25 μg/ml fungizone, and 2 mM glutamine. Cultures were incubated at 37°C in a humidified atmosphere of 10% or 5% CO2.

The RF.33.70 (SIINFEKL-Kb-specific) T-T hybridoma has been described previously (14). The T-T hybridoma 1G8, which recognizes the OVA peptide KVVRKFDL on H-2Kb, was kindly provided by Dr. James McCluskey (Flinders Medical Center, University of Melbourne, Parkville, Australia; see Ref. 15). The T-T hybridomas 12.33.03 and B3.4D8, which recognize influenza virus A nucleoprotein peptide (NP126–132) ASNENMETM on H-2Db and Sendai virus nucleoprotein peptide (NP324–332) FAPGNYPAL on H-2Kb, respectively, were kindly provided by Dr. David Woodland (St. Jude Children’s Research Hospital, Memphis, TN). A retroviral-transduced B16 melanoma cell line, which constitutively secretes murine IFN-γ, was a gift from Dr. Glen Dranoff (Dana-Farber Cancer Institute, Boston, MA).

Six-week-old female C57BL/6 mice (The Jackson Laboratory, Bar Harbor, ME) were immunized multiple times with keyhole limpet hemocyanin conjugated with SIINFEKL-biotin, and their spleen cells were fused with
the myeloma MOMS by established techniques (16). This fusion was plated into 96-well plates, and, after selection, individual wells were screened for the production of mAbs reactive with SIINFEKL. Two independent SIINFEKL-specific B cell hybrids were identified (IF10.2.2 and IF10.12.3). During the subcloning of these hybridomas by limiting dilution, we isolated spontaneous variants of the hybridomas IF10.2.15 and IF10.12.13 that no longer produced anti-SIINFEKL mAb. The hybridomas were maintained in DMEM supplemented with 20% FCS at 37°C and 10% CO₂ atmosphere in a humidified incubator.

**Antibodies**

The conformation-specific Ab to H-2Kb (Y-3) was obtained from the American Type Culture Collection (Manassas, VA). Anti-human MHC class I, HC10 mAb was kindly provided by Dr. Hidde Ploegh (Harvard Medical School, Boston MA). Rabbit antiserum against chicken OVA was purchased from ICN Immunochemicals (Costa Mesa, CA).

**Vaccinia virus constructs**

Recombinant vaccinia viruses containing the full-length OVA gene, a minigenic encoding the OVA257–264 peptide, a minigenic encoding the Sendai virus nucleoprotein NP324–332 peptide (FAPGNYPAL), a minigenic encoding the influenza virus nucleoprotein NP366–374 peptide (ASNENMETM), or the above minigenes with signal sequences were kindly provided by Drs. Jack Bennink and Jon Yewdell (National Institutes of Health, Bethesda, MD).

**Reagents**

The peptides SIINFEKL and KVVRFDKL were synthesized in a peptide synthesizer and purified to at least 90% purity by HPLC (Macromolecular Resources, Fort Collins, CO).

**ELISA**

Direct binding of Abs to peptides absorbed to plastic wells (after blocking with 1% FCS) was measured by the addition of 1:4000 alkaline phosphatase-conjugated goat anti-mouse IgG (Cappel, West Chester, PA) and detection by an ELISA amplification system (Life Technologies).

**Western blotting and fluorescent flow cytometric analysis**

For quantitation of OVA expression, equivalent numbers of cells (10⁶ cells/ml) were washed three times with PBS and lysed in ice-cold lysis buffer (1% Nonidet P-40, 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, and 1 mM EDTA) containing a cocktail of protease inhibitors (500 µM PMFS, 1 µg/ml leupeptin, 10 µg/ml aprotinin, and 1 µg/ml pepstatin) for 30 min on ice followed by centrifugation at 10,000 × g for 10 min. Proteins from cell lysates were resolved on SDS-polyacrylamide gels. After blocking with 1% milk, the membranes were incubated with 1:5000 dilution of rabbit anti-serum against chicken OVA (ICN Immunochemicals). Immunoreactive proteins were visualized by incubation with 1:10,000 peroxidase-conjugated antibody-purified goat anti-rabbit IgG serum (Sigma, St. Louis, MO), followed by the addition of a chemiluminescent substrate (NEN, Boston, MA).

Flow cytometric analysis was performed by using a Becton Dickinson (Mountain View, CA) FACScan flow cytometer. Cells were prepared by washing with PBS/1% FCS and resuspended in PBS/1% FCS at a concentration of 1 × 10⁸ cells/ml. A total of 2 × 10⁵ cells were stained with primary mouse Ab (30 min on ice) followed by two washes in PBS/1% FCS and staining with 1:100 FITC-conjugated rabbit anti-mouse Ab (30 min on ice in the dark) followed by two more washes and then resuspended in 400 µl of PBS/1% FCS/1% paraformaldehyde.

**Viral infection and Ag presentation assays**

To assay for the presentation of endogenously synthesized Ag, B cell hybridomas were first grown in 20% FCS supplemented with 10 U/ml murine IFN-γ for 2 days to 3–5 × 10⁵ cells/ml to enhance Ag presentation. The cells were collected by centrifugation for 5 min at 200 × g at 4°C, washed once in RPMI 1640, and resuspended in serum-free OptiMEM (Life Technologies; supplemented with 1000 U/ml penicillin, 1 mg/ml streptomycin, 25 µg/ml fungizone, 2 mM glutamine, 50 µM 2-ME (Sigma), and 1% nutridoma (Boehringer Manheim, Indianapolis, IN)) at 5 × 10⁵ cells/ml (Life Technologies). Recombinant vaccinia virus was added at a multiplicity of infection of 10 and incubated for at least 2 h at 37°C with mixing every 15 min. They then were washed twice in PBS, incubated at 37°C with 1% paraformaldehyde for 10 min, washed again, and resuspended in HCM (RPMI 1640 supplemented with 10% FCS, 1000 U/ml penicillin, 1 mg/ml streptomycin, 25 µg/ml fungizone, 2 mM glutamine, 50 µM 2-ME (Sigma), 10 mM HEPES, and nonessential amino acids supplement (Invitrogen Scientific, Santa Ana, CA). The infected cells were serially diluted in a 96-well plate. The presence of peptide-Kb complexes were measured by quantifying the amount of IL-2 produced by the peptide-specific T-T hybridoma after stimulation with APCs in duplicate cultures, as described previously (17).

**Affinity determinations with BIACOREs**

Surface plasmon resonance analysis was used to measure the association and dissociation affinity constants for the binding of mAb IF10.2.2 to immobilized SIINFEKL by using BIACORE X (Biacore, Uppsala, Sweden) as described previously (18).

**Results**

**Characterization of B cell hybridomas producing anti-SIINFEKL Ab and their nonproducing pairs**

To investigate the fate of antigenic peptides in the ER lumen, we generated two B cell hybridomas that produce mAbs against the H-2Kb-restricted SIINFEKL epitope derived from chicken OVA (residues 257–264). The mAbs from the IF10.2.2 and IF10.12.3 cell lines were characterized by ELISA (Fig. 1). Both of these Abs bind SIINFEKL and SIINFEKL with an N-terminal extension (LESINFEKL), and also bind SIINFEKL with a C-terminal extension (SIINFEKLTE), although more weakly (Fig. 1). BIACORE analysis measured the affinity of the mAb for SIINFEKL as Kₐ = 5.8 × 10⁻⁸ M with a Kₐ of 4.4 × 10⁻⁷ M⁻¹s⁻¹ and a Kₐ of 2 × 10⁻³ s⁻¹. Their binding to SIINFEKL can be blocked by the presence of excess N-terminally extended SIINFEKL, but not by C-terminally extended SIINFEKL (data not shown). Presumably, the C-terminal flanking residues reduce the affinity of binding of the peptides to the mAbs. In contrast, these Abs do not stain SIINFEKL-Kₐ complexes on APCs and do not block the presentation of SIINFEKL-Kₐ when added exogenously to cultures with T cells (data not shown). Therefore, they bind free SIINFEKL-containing sequences but fail to recognize SIINFEKL when it is bound to Kₐ.

We isolated spontaneous variants of the hybridomas that no longer produced anti-SIINFEKL mAb. We verified that the Ab producer and nonproducer clones were closely matched by a number of criteria that were important for Ag presentation. As shown in Fig. 2, the Ab-producing and nonproducing cell lines (IF10.2.2 and IF10.2.15, respectively) express Kₐ on the surface equally both before and after incubation in IFN-γ. Similar results were obtained for the IF10.12.3/IF10.12.13 pair (data not shown). Moreover, the Ab-producing/nonproducing pairs (IF10.2.2/IF10.2.15 and IF10.12.3/IF10.12.13) present exogenous SIINFEKL and KVVRFDKL peptides equally well to the appropriate T cell hybridomas (Fig. 3). These results indicate that these producer/nonproducer pairs are matched for their expression of Kₐ and their ability to present peptide-MHC complexes to T cells. Finally, when infected with a vaccinia recombinant encoding OVA, the Ab-producing cell lines (IF10.2.2 and IF10.12.3) show...
cytometry. followed by FITC-conjugated anti-Ig. Fluorescence was quantified by flow cytometry.

higher expression of OVA protein than their nonproducing variants (IF10.2.15 and IF10.12.13, respectively; Fig. 4), possibly because of adaptations for the synthesis of high levels of Ab. If anything this difference should mitigate against the effects that we describe below.

Kb association with ER-targeted SIINFEKL is impaired by the presence of anti-SIINFEKL in the ER

In initial experiments we tested whether anti-SIINFEKL Ab affected the presentation of SIINFEKL peptide that was targeted into the ER via a signal sequence. In this situation, the peptide enters the ER via the sec61 translocon and presumably must diffuse through the ER lumen to reach MHC class I molecules. The Ab-producing (IF10.2.2) and -nonproducing (1F10.2.15) clones were infected with recombinant vaccinia encoding a SIINFEKL minigene with a signal sequence, fixed, and the presence of infected with recombinant vaccinia encoding full-length OVA (see Materials and Methods).

Kb binding of TAP-transported SIINFEKL is impaired by the presence of anti-SIINFEKL in the ER

Given the finding that the MHC class I presentation of ER-targeted SIINFEKL was inhibited by the presence of anti-SIINFEKL in the ER of the APC, we next analyzed how this Ab would affect the presentation of TAP-transported peptides. In one set of experiments, we expressed peptides in the cytoplasm from minigenes in a vaccinia-expressed minigene construct encoding the Sendai virus nucleoprotein epitope (Fig. 5C) with a signal sequence was presented on Kb equally well by the producer and nonproducer clones (1F10.2.2 and 1F10.2.15). The kinetics of presentation of the Sendai virus epitope are slower than SIINFEKL and therefore Ag presentation is only detected at the 5-h time point. Again, similar results were obtained with the second producer-nonproducer pair (data not shown). These results are important because they indicate that the producer and nonproducer are equally competent to present an Ag unrelated to SIINFEKL on Kb. Therefore, the anti-SIINFEKL mAb is selectively blocking the presentation of a SIINFEKL construct that enters the ER lumen and therein binds Kb.

FIGURE 3. Presentation of exogenous peptides on surface Kb of producer and nonproducer cells. Ab-producing B cell hybridoma IF10.2.2 (■) and its nonproducing variant IF10.2.15 (○) were fixed and incubated with the indicated peptide. These peptide-pulsed cells were then assayed for their ability to present Ag to either RF.33.70 (specific for Kb/SIINFEKL) or 1G8 (specific for Kb/KVVRFKDL; see Materials and Methods).

FIGURE 4. OVA is expressed in producer and nonproducer cells. Western blot analysis of OVA expression after infection of IF10.2.2, IF10.2.15, IF10.12.3, and IF10.12.13 with recombinant vaccinia encoding full-length OVA (see Materials and Methods).

FIGURE 5. Ag presentation of ER-targeted SIINFEKL is impaired in Ab-producing cells. The anti-SIINFEKL-producing IF10.2.2 (squares) and its nonproducing variant IF10.2.15 (circles) were infected with recombinant vaccinia encoding ER leader sequence SIINFEKL (from OVA; A and B), or FAPGNYPAL (from Sendai virus nucleoprotein; C). These cells were then fixed after 2 h (A and C) or 5 h (B and C) and assayed for their ability to stimulate SIINFEKL-specific (A and B) or FAPGNYPAL-specific (C) T-T hybridomas.

FIGURE 2. Kb expression on the surface of a B cell hybridoma producer and nonproducer pair. Producer (IF10.2.2) and nonproducer (IF10.2.15) cells were incubated with or without 10 U/ml IFN-γ for 2 days and then stained with the mAb Y-3 (specific for Kb) or control Ab (HC10) followed by FITC-conjugated anti-Ig. Fluorescence was quantified by flow cytometry.
Ag presentation of TAP-transported SIINFEKL is impaired in Ab-producing cells. The indicated peptides were expressed from minigenes in vaccinia in Ab producer (●) and nonproducer cells (■) (IF10.2.2.4 and IF10.2.15, respectively, in upper panels and IF10.2.3 and IF10.2.13, respectively, in lower panels). Ag presentation was assayed as described in Fig. 5.

Discussion

We have used B cell hybridomas that produce an anti-SIINFEKL Ab as APCs to study MHC class I antigenic peptides in the ER. We find that for endogenously produced SIINFEKL peptide, APCs producing an anti-SIINFEKL mAb present this peptide poorly compared with non-Ab-producing cells. A key issue for interpreting this finding is whether these cell pairs differ solely in their production of the anti-peptide Ab and not in other aspects of MHC class I Ag presentation. Several lines of evidence argue that these pairs are identical for Ag presentation. The cells that are being compared are daughter clones of the original hybridomas and therefore are closely related. They express the same levels of MHC class I molecules. Moreover, they present exogenous peptides identically, indicating that in addition to class I, all of the other components that are needed to interact with T cells (e.g., adhesion molecules) are present and function in a comparable manner. Perhaps most importantly, these APC pairs present other peptides from endogenous Ags on K^b (the Sendai peptide FAPGNYPAL and OVA peptide KVVRFDKL) and D^b (the influenza peptide ASNENMETM) equally well. Therefore, all components of the MHC class I pathway (proteasomes, TAP, etc.) function identically in these APCs. In addition, the finding that the producer cells presented KVVRFDKL from OVA equally well to the nonproducer (while SIINFEKL was reduced) indicates that differences in expression of recombinant Ag cannot account for the reduced presentation of SIINFEKL. Western blots further verified that the producer cells were expressing OVA at least as well as the nonproducer.

The anti-SIINFEKL mAb does not inhibit the presentation of SIINFEKL-K^b complexes from APCs if the Ab is added exogenously to culture or when the APC is cocultured with Ab-producing cells. This is not surprising because the affinity of K^b for SIINFEKL (K_D = 1.5 × 10^{-9} M) is 40-fold higher than the mAb (K_D = 5.8 × 10^{-8} M), but more importantly, the K^b-bound SIINFEKL has a very slow off rate (K_off = 9.1 × 10^{-6} s^{-1}) (15). Therefore, the anti-SIINFEKL mAb is unlikely to be blocking Ag presentation after the peptide-MHC complexes are fully formed. Instead, the anti-peptide mAb must be inhibiting Ag presentation by binding peptides before they tightly bind to class I molecules. Peptides bind to newly synthesized class I molecules in the ER (2). The mAb is cotranslationally transported into the ER and then follows the secretory pathway. Therefore, the mAb is most likely interfering with peptide binding in the ER. It is formally possible that if peptide is transiently unloaded from class I molecules during exocytosis then its rebinding could be blocked by Ab in the secretory compartment; however, there is no evidence for peptides being unstably bound or unloaded after class I molecules leave the ER.

Because the anti-SIINFEKL mAb blocks the presentation of peptides that are generated in the cytosol, these peptides must be freely accessible to the Ab after transport into the ER. This finding strongly
argues against a model where peptides are transported directly into the binding groove of TAP-associated MHC class I molecules. Alternatively, if such binding occurs, it must be highly unstable, with the majority of peptides rapidly dissociating. Instead, our finding support a model where peptides that are transported by TAP must enter the ER lumen directly or after an initial reversible binding to class I molecules (11, 21–23) before becoming tightly bound by class I molecules. Most probably this process occurs through simple diffusion, although it is possible that other ER luminal molecules (such as chaperones) could facilitate this process. However, if other peptide-binding molecules are involved, the current study would argue that these molecules (as with MHC class I) do not interfere with the accessibility of the transported peptide to the Ab. SIINFEKL is presented in the mAb-producer cell lines after long incubations, and it is possible that this arises from peptides that are transported directly into TAP-associated class I molecules. If this occurs, our data would indicate that it is a minor pathway. However, similar presentation also is observed with SIINFEKL transported through the translocon sec61 and therefore this uninhibitable component may simply represent incomplete blocking by the mAb.

It has been known that peptides don’t need to be transported through TAP to be presented on MHC class I molecules. For example, peptides derived from signal sequences or minigenes fused to signal sequences can be presented in TAP-deficient cells and presumably reach class I molecules by diffusion through the ER (24–26). However, before our study, it has not been clear how efficient this process is relative to the presentation of peptides that are transported through TAP. Our findings suggest that peptides are unlikely to be preferentially loaded onto the class I molecule that is associated with the TAP that transported them. These findings strongly suggest that the major-ty of transported peptides will diffuse into the ER lumen and be accessible to competing processing events such as binding heat shock proteins (27) and transport out of the ER into the cytoplasm (28), which may limit Ag presentation. In addition, there is functional data that the ER contains an aminopeptidase that can trim peptides (20, 29). This could lead to the destruction of peptides that are already of the appropriate size for presentation. In contrast, peptides that are too long to be presented could be trimmed to eight or nine residues, and our data would suggest that these will be presented as efficiently as if they had been generated in the cytoplasm. Peptide diffusion in the ER lumen could also facilitate editing by peptide exchange (22, 30, 31), providing an environment of competing peptides.

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