Lateral Diffusion of GFP-Tagged H2Ld Molecules and of GFP-TAP1 Reports on the Assembly and Retention of These Molecules in the Endoplasmic Reticulum

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Summary

Lateral diffusion of GFP-tagged H2Ld molecules in the ER membrane reports on their interaction with the TAP complex during synthesis and peptide loading. Peptide-loaded H2Ld molecules diffuse rapidly, near the theoretical limit for proteins in a bilayer. However, these molecules are retained in the ER for some time after assembly. H2Ld molecules, associated with the TAP complex, diffuse slowly, as does GFP-tagged TAP1. This implies that the association of H2Ld molecules with the TAP complex is stable for at least several minutes. It also suggests that the TAP complex is very large, perhaps containing hundreds of proteins.

Introduction

MHC class I molecules are synthesized and assembled in the endoplasmic reticulum (ER) (Pamer and Cresswell, 1998). Their assembly and retention in the ER involve binding to a number of ER chaperones (Degen and Williams, 1991; Hammond et al., 1994; Kearse et al., 1994; van Leeuwen and Kearse, 1996; Krause and Michalak, 1997), including a complex of proteins, containing TAP (Howard, 1995), calnexin, calreticulin, Erp57, and tapasin (Ortmann et al., 1997; Solheim et al., 1997; Lindquist et al., 1998). This complex supplies peptides, which are generated in the cytosol by proteasomes (Rock et al., 1994), to the nascent MHC molecule. Once loaded with peptides, MHC class I molecules appear to dissociate from the TAP complex and exit the ER (Suh et al., 1994). However, after dissociating from the TAP complex, MHC class I molecules may be retained in the ER long enough to exchange their peptide for others of higher affinity (Lewis and Elliott, 1998).

The mechanisms by which MHC molecules are transiently retained in the ER are not understood. While biochemical analyses detect complexes of MHC class I molecules with ER-resident proteins, they yield little information about the size, organization, and milieu of these complexes in native ER membranes. Hence, they cannot be used to determine the mechanisms of ER retention of nascent MHC class I molecules or the dynamics of association of these molecules with chaperones and the TAP complex.

Measurements of lateral diffusion can directly probe mechanisms of retention of MHC molecules in the ER during assembly and peptide loading. If nascent MHC class I molecules associate with immobile or slowly diffusing ER proteins, their apparent lateral diffusion coefficients (D) will be lower than those D previously reported for recycling proteins localized to the ER (Elson and Reidler, 1979; Cole et al., 1996). If they associate irreversibly with immobile ER proteins, the mobile fraction (R) of the MHC class I molecules would be low. Segregation of nascent molecules into membrane subregions, domains (Yechiel and Edidin, 1987), or their aggregation would also result in low mobile fractions.

We show here that D measured for GFP-tagged H2Ld molecules in the ER is lower compared to that of Golgi-resident proteins, while R is comparable to that of Golgi-resident proteins. The measured D is an average of D for two different populations of H2Ld molecules. One population is associated with the TAP complex; its D reflected the low D of the TAP complex itself. A second population of H2Ld molecules, loaded with peptide and apparently not associated with TAP, has a 5-fold higher D. Since TAP itself is retained in the ER, association with TAP is sufficient to retain some H2Ld molecules. However, completely assembled, peptide-loaded H2Ld molecules are also transiently retained in the ER, even though they are diffusing freely and are not associated with TAP. These H2Ld molecules cannot be retained by anchoring or aggregation, since R is high. Their retention may be due to their interaction with molecular machinery involved in optimization of peptide loading or to the limited availability of sites for trafficking molecules out of the ER.

Results

Cell Surface Expression of GFP-Tagged H2Ld Class I Molecules

We constructed two different GFP-tagged forms of the mouse MHC class I allele H2Ld as our probes for traffic of MHC class I molecules through the ER. These are shown in Figure 1A. One form, H2Ld-GFP, was tagged at the end of the cytoplasmic tail, the C terminus. The second form, H2Ld-GFPout, was tagged with GFP between the third exodomain of the heavy chain and the transmembrane domain. L cells were transfected with genes for the H2Ld GFP chimeras or for native H2Ld. Expression of the transfectants was monitored by flow cytometry using three different criteria: GFP fluorescence, labeling by H2Ld-specific mAb, and labeling by soluble 2CtK2lG (O’Herrin et al., 1997; Lebowitz et al., 1999), which detected specific peptide/H2Ld complexes (Figure 2). Cells transfected with genes for either native or GFP-tagged H2Ld bound 30-5-7S and 28-14-8S mAbs to approximately the same extent, while no specific staining was detectable on the surface of the parental
Using the same exposure time for image acquisition, no green fluorescence was observed in L cells or from cells expressing native H2Ld (data not shown).

To identify the intracellular structures containing the H2Ld, fixed permeabilized cells were labeled with antibodies against markers for the ER (calnexin or BiP) or for the Golgi complex (giantin or mannosidase II). The reticular pattern of both H2LdGFPin and H2LdGFPout fluorescence colocalized with fluorescence from labeled ER proteins (Figures 3B and 3C show the pattern for H2LdGFPin). Some H2LdGFP fluorescence colocalized with fluorescence from the labeled Golgi complex proteins (Figures 3E and 3F). The ER colocalization of H2LdGFP, while extensive, was not complete. H2LdGFP was evident in calnexin-poor subregions of the ER. The distribution of native H2Ld, detected with mAb, was similar to that of the GFP chimeras, though judging from fluorescence intensities, there was a higher ratio of ER-localized to Golgi-localized molecules for native H2Ld than for the GFP chimeras (data not shown).

The levels of expression and the strength of association of MHC class I molecules with β2m could influence their intracellular distribution and kinetics of maturation. Measuring the amount of H2Ld molecules immunoprecipitated from extracts of cells labeled to steady-state, we found that the transfects were expressed in amounts 5- to 10-fold higher than those of the endogenous MHC class I molecule of L cells, H2Kk. All three forms were associated with β2-microglobulin; however, the ratio of heavy chain to βm was much lower for H2LdGFPout (35:1) than for native H2Ld and H2LdGFPin (5:1) (Figure 4).

Overall, the intracellular distributions observed were consistent with earlier observations on the intracellular distribution of H2 by immunofluorescence (Yewdell and Bennink, 1992) and with the slow progress of H2Ld from ER to medial Golgi complex reported by Beck et al. (1986). We confirmed this slow progress in pulse-chase experiments (data not shown). Measured half-times for maturation of both native H2Ld and H2LdGFPin to endoH-resistant forms were about 4 hr, close to the value reported earlier (Beck et al., 1986). H2LdGFPout appeared to remain in the ER for much longer times; less than 40% of newly synthesized H2LdGFPout was endoH-resistant after 8 hr. This is consistent with its weak binding of β2m and its failure to associate with TAP (see below).

### Associations between H2LdGFP Chimeras and ER-Resident Proteins

Significant amounts of calnexin, calreticulin, TAP1, and TAP2 were detected by Western blotting after immunoprecipitation of native or GFP-tagged H2Ld with two different antibodies to the H2Ld heavy chain, mAb 28-14-8 (Ozato et al., 1980) and mAb 64-3-7 (Lie et al., 1991). As shown previously (Carreno et al., 1995), TAP1 and TAP2 were associated with native H2Ld or H2LdGFPin precipitated by 64-3-7 (Figure 4). In contrast, immunoprecipitates of H2LdGFPout were positive for calnexin but not for calreticulin or TAP molecules (Figure 4). We also probed quantitatively for complexes of native H2Ld with ER proteins by precipitating the complexes from extracts of pulse-labeled cells using specific antisera against TAP, calnexin, and calreticulin. After normalizing for the total amount of labeled extract...
Expression of native and GFP-tagged H2Ld molecules was measured by flow cytometry. The abscissa of each panel is logarithmic, covering 4 decades of fluorescence intensity.

Left panel: total GFP fluorescence. Incubation of cells with the peptide, QL9, slightly increased the total GFP fluorescence from L cells transfected with either form of GFP-tagged H2Ld gene.

Right panel: cell surface H2Ld measured using mAb 28-14-8, which binds both native H2Ld and free heavy chains, with mAb 30-5-7, which binds only native H2Ld, or with soluble divalent 2C TCR2IgG, which in this experiment binds only native H2Ld molecules loaded with peptide QL9. Cell surface H2Kk, an endogenous class I MHC molecule of L cells, was measured with mAb 11-4-1. Cells were first labeled with the mAbs or soluble TcR and then with Cy3 F(ab')2-conjugated goat anti-mouse IgG. For peptide loading, cells were incubated overnight in the absence (light line) or presence (bold line) of QL9 peptide. The dotted line represents background (the secondary antibody alone).

loaded in each lane, it appeared that all three forms of H2Ld associated to the same extent (with an uncertainty of 10%) with calnexin. However, though a cocktail of anti-TAP antibodies precipitated native H2Ld and H2LdGFPIn, no H2LdGFPOut could be detected in the precipitates, confirming the results of Western blotting. It appears that the GFP moiety on the cytoplasmic tail of H-2LdGFPIn does not interfere with its association with the TAP complex, while the GFP moiety near the α3 domain of H-2LdGFPOut does interfere. The α3 domain is important in β2m binding and in the interaction of class I molecules with calreticulin (Harris et al., 1998) and with the TAP complex (Carreno et al., 1995; Suh et al., 1996; Kulig et al., 1998). The GFP moiety could affect any or all of these interactions.

Lateral Diffusion of H2LdGFP Chimeras in the Endoplasmic Reticulum

As shown above, a substantial fraction of GFP-tagged H2Ld molecules colocalized with a marker for ER membranes. We measured the lateral diffusion of these GFP-tagged H2Ld by bleaching a spot in the ER rather than a stripe, as we had in our earlier work on diffusion in the Golgi complex (Cole et al., 1996). Given the size of the spot and the tubular geometry of the ER, we expected recovery of fluorescence after bleaching to be due to one-dimensional diffusion down the long axis of a tubule (see Cole et al., 1996). This assumption was validated by measuring the lateral diffusion of GFP-tagged galactosyl transferase, a Golgi complex marker. Assuming one-dimensional diffusion, we measured $D = 7.1 \times 10^{-9} \text{cm}^2\text{s}^{-1}$, close to $D = 5.4 \times 10^{-9} \text{cm}^2\text{s}^{-1}$ that we had measured for this protein in the Golgi complex of HeLa cells (Cole et al., 1996).

The diffusion of H2LdGFPOut in the ER, $D = 3.7 \pm 0.3 \times 10^{-9} \text{cm}^2\text{s}^{-1}$ and $R = 68\%$ (see Table 1 for a summary of all $D$ and $R$), was within the range of values that we found earlier for Golgi proteins diverted to the ER (Cole et al., 1996). Lateral diffusion of H2LdGFPIn was about half that of H2LdGFPOut, $D = 2.0 \pm 0.1 \times 10^{-9} \text{cm}^2\text{s}^{-1}$, and lower than found for Golgi proteins, though $R$ was about the same (61%). This result suggested that H2LdGFPIn molecules were free to diffuse in the ER membrane but that their lateral diffusion was hindered, either by the GFP tag on the cytoplasmic tail or by interactions with other proteins.

We ruled out an effect of the size of the GFP tag on diffusion of H2LdGFPIn by comparing $D$ in the plasma membrane of each GFP-tagged form of H2Ld with $D$ of the native molecule. All $D$ and $R$ were similar, $D \approx 5 \times 10^{-9} \text{cm}^2\text{s}^{-1}$ and $R \approx 85\%$, whether measured by bleaching GFP fluorescence or after labeling a Cy3-conjugated Fab fragment. Individual values are tabulated in Table 1. Given the results on lateral diffusion of GFP-tagged H2Ld in the plasma membrane and the biochemical results reported above, it seemed likely that the relatively low $D$ of H2LdGFPIn was due to its interaction with proteins of the TAP complex.
Figure 3. Colocalization of H2L<sup>4</sup>GFP<sub>i</sub>n with Markers for ER or Golgi Complex

L cells transfected with GFP-tagged H2L<sup>4</sup> were fixed in ice-cold methanol, stained with antibodies against the indicated organelle marker proteins and Cy5-conjugated secondary antibodies, and imaged by confocal microscopy. (A and D) GFP fluorescence, (B) calnexin (ER marker), (C) colocalization image for GFP fluorescence (green) and calnexin (red), (E) giantin (Golgi complex marker), and (F) colocalization image for GFP fluorescence and giantin. Colocalization is seen as yellow to orange/red due to the combination of the green and the red signal (Scale bar, 10 μm).

Lateral Diffusion of H2L<sup>4</sup>GFP and the TAP Complex

To test more directly for the role of a functional TAP complex in constraining lateral diffusion, we transfected EE2H3 embryonic cells (Silverman et al., 1988) with the vectors for GFP-tagged H2L<sup>4</sup>. The EE2H3 cell line is defective in assembly and expression of MHC class I, but the defect is remedied by treatment of the cells with αβ interferon, which induces the expression of TAP1 (Bikoff et al., 1991). H2L<sup>4</sup>GFP<sub>i</sub>n was successfully expressed in EE2H3. D in the ER was high, \(4.6 \pm 0.2 \times 10^{-9} \text{ cm}^2\text{s}^{-1}\). After inducing functional TAP expression by a 48 hr incubation with αβ interferon, D was almost identical to that in L cells, \(1.8 \pm 0.1 \times 10^{-9} \text{ cm}^2\text{s}^{-1}\).

Since interferon treatment of EE2H3 cells may have multiple effects on cell function, we also compared D of H2L<sup>4</sup>GFP<sub>i</sub>n in FT1<sup>-</sup> fibroblasts derived from TAP1 knockout mice (Van Kaer et al., 1992) with D in FT1<sup>+</sup> fibroblasts derived from normal mice. D in TAP1 knockout cells was \(4 \pm 0.3 \times 10^{-9} \text{ cm}^2\text{s}^{-1}\), while D in normal controls was \(1.9 \pm 0.1 \times 10^{-9} \text{ cm}^2\text{s}^{-1}\).

The results with EE2H3 and FT1 cells implied that D of H2L<sup>4</sup>GFP<sub>i</sub>n in L cells was low because of interactions of this molecule with the TAP complex. These interactions can be modulated by the supply of peptide to the ER. H2L<sup>4</sup>GFP<sub>i</sub>n molecules loaded with peptide should dissociate from TAP (Suh et al., 1996; Bai, 1997), and so their average D should decrease. We expected modulation of the peptide supply to have little effect on D of H2L<sup>4</sup>GFP<sub>o</sub>ut, since this chimera appears not to be associated with TAP.

Day et al. (1997) reported that peptides added to culture medium are transported to the ER, where they are bound by nascent MHC class I molecules. We confirmed this using 2C TCR2IgG as a probe. As we had found when labeling cell surfaces with this reagent, 2C TCR2IgG bound specifically to H2L<sup>4</sup>GFP loaded with peptide QL9, while it did not bind to H2L<sup>4</sup>GFP loaded with peptide MCMV (Figure 5; Lebowitz et al., 1999). Cytoplasmic labeling by 2C TCR2IgG was not entirely coincident with GFP fluorescence. Some GFP fluorescence was concentrated in a perinuclear region that may represent both the Golgi complex and the region of exit sites in the ER (Chao et al., 1999).

We measured lateral diffusion of H2L<sup>4</sup>GFP in the ER and at the plasma membrane after loading cells with MCMV, QL9, SL9, or p2Ca peptides. The affinity, \(K_a\), of H2L<sup>4</sup> for these peptides ranges from \(\sim 10^4\) to \(\sim 10^6 \text{ M}^{-1}\) after incubating cells with peptides. D of H2L<sup>4</sup>GFP<sub>i</sub>n molecules in the ER ranged from slightly higher than controls, \(2 \times 10^{-9} \text{ cm}^2\text{s}^{-1}\), to significantly higher than controls, \(3.6 \times 10^{-9} \text{ cm}^2\text{s}^{-1}\); D was proportional to the affinity of H2L<sup>4</sup> for the peptide (Figure 6). The lateral diffusion of H2L<sup>4</sup>GFP<sub>o</sub>ut in the ER increased slightly though significantly after cells were incubated with the high-affinity MCMV peptide, D = \(4.7 \pm 0.6 \times 10^{-9} \text{ cm}^2\text{s}^{-1}\).
versus $3.7 \pm 0.3 \times 10^{-9}$ cm$^2$s$^{-1}$ for H2L$^d$GFPout in the absence of added peptide. In contrast, lateral diffusion of H2L$^d$GFPin at the plasma membrane was not changed by peptide loading.

D of H2L$^d$GFPin correlated well with the affinity of the peptides used to load it (Figure 6). Since we used a constant concentration of peptide, this correlation reflects the fraction of H2L$^d$GFPin loaded by a peptide of a given affinity and, hence, the ratio of free and TAP-associated H2L$^d$GFPin. The largest D measured for endogenous class I molecules increased in cells transfected with TAP1 GFP, 3.6 to 3.7. After electrophoresis on SDS-PAGE, samples were transferred to nylon membranes and the blots were probed with specific antibodies to calnexin, TAP1 and TAP2, and calreticulin. Note that the panel showing immunoprecipitation of β2m is overexposed relative to the panels showing immunoprecipitation of heavy chains.

Table 1. Diffusion Coefficients, D ($\times 10^{10}$ cm$^2$s$^{-1}$), and Percent Recovery, R, for H2L$^d$

<table>
<thead>
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<th>Treatment</th>
<th>H2L$^d$GFPin</th>
<th>H2L$^d$GFPout</th>
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<tr>
<td></td>
<td>ER</td>
<td>PM</td>
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<tr>
<td>L</td>
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<td>Cy3 Fab</td>
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<td>-</td>
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<tr>
<td>MCMV</td>
<td>36 ± 2</td>
<td>68 ± 2</td>
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<tr>
<td>lactacystin</td>
<td>13 ± 1</td>
<td>71 ± 3</td>
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<tr>
<td>lactacystin + MCMV</td>
<td>39 ± 3</td>
<td>69 ± 2</td>
</tr>
<tr>
<td>lactacystin</td>
<td>30 ± 2</td>
<td>59 ± 2</td>
</tr>
<tr>
<td>EE2H3</td>
<td>-</td>
<td>146 ± 4</td>
</tr>
<tr>
<td>αβ IFN</td>
<td>18 ± 1</td>
<td>78 ± 0.7</td>
</tr>
<tr>
<td>αβ IFN + MCMV</td>
<td>39 ± 2</td>
<td>72 ± 2</td>
</tr>
<tr>
<td>FT1</td>
<td>19 ± 3</td>
<td>71 ± 3</td>
</tr>
<tr>
<td>FT1</td>
<td>-</td>
<td>40 ± 3</td>
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Lateral Diffusion of TAP1 GFP in the ER

The lateral diffusion of H2L$^d$GFPin appeared to be constrained by its association with the TAP complex. This meant that either H2L$^d$GFPin undergoes cycles of rapid association with and dissociation from an immobile TAP complex, or that it bound to a slowly diffusing TAP complex. To distinguish between these possibilities, we tagged the TAP1 subunit (Monaco et al., 1990; Marusina et al., 1997) with GFP at its cytosolic C terminus (Figure 4). TAP1 GFP appeared to be integrated into a complex, or that it bound to a slowly diffusing TAP complex.
Figure 6. The Effects of Modulating Peptide Supply to the ER on the Lateral Diffusion of H2L\(^{\text{GFPin}}\) in ER and Plasma Membranes

Lateral diffusion coefficients, \(D\), are plotted as a function of the affinity of H2L\(^{\text{d}}\) for the peptide added, \(P_{2Ca}\), SL9, QL9, or MCMV. The left-most points are for cells that were not fed peptide: filled squares, \(D\) in the ER of untreated cells; filled triangles, \(D\) in the ER of cells treated with lactacystin; filled circles, \(D\) in the plasma membrane of untreated cells; filled inverted triangles, \(D\) in the plasma membrane of cells treated with lactacystin.

The lateral diffusion coefficients that we measured are state averages, since they are measured for a mixture of more rapidly diffusing molecules not associated with the TAP complex and less rapidly diffusing molecules associated with the complex. To resolve this mixture, we took \(D\)

for rapidly diffusing molecules as \(4.7 \times 10^{-9}\) cm\(^2\)s\(^{-1}\), the value measured for H2L\(^{\text{GFPout}}\) after adding peptide to L cells and the value for H2L\(^{\text{GFPin}}\) in cells that lack a functional TAP complex. We took \(D\) for slowly diffusing molecules as \(1.2 \times 10^{-9}\) cm\(^2\)s\(^{-1}\), the value measured for GFP-TAP1. These values were used to resolve the measured \(D\), \(D_{\text{avg}}\), as \(D_{\text{avg}} = f_1(D_{\text{fast}}) + f_2(D_{\text{slow}})\), where \(f_1\) equals the fraction of rapidly diffusing H2L\(^{\text{d}}\) molecules, \(f_2\) equals the fraction of slowly diffusing H2L\(^{\text{d}}\) molecules, and \(f_1 + f_2 = 1\). Rather than determine \(f_1\) and \(f_2\) from each experimental FPR curve, we generalized the way in which two diffusing species contribute to a single recovery curve, using data that we had obtained on \(D_{\text{avg}}\) for known mixtures of free fluorescein and fluorescein-IgM (Edidin, 1994). These data fit the relationship \(1/D_{\text{avg}} = f_1(1/D_{\text{fast}}) + f_2(1/D_{\text{slow}})\). We used this relationship and the estimates of \(D_{\text{fast}}\) and \(D_{\text{slow}}\) to evaluate \(f_1\), the fraction of H2L\(^{\text{d}}\) not associated with TAP, and \(f_2\), the fraction of H2L\(^{\text{d}}\) associated with TAP, in control cells and in cells that had been fed peptides. We estimate that in the absence of added peptide, approximately half of the H2L\(^{\text{GFPin}}\) in the ER are associated with TAP. The dissociation of H2L\(^{\text{GFPin}}\) from TAP depended upon the affinity of H2L\(^{\text{d}}\) for the peptides. Only 10% of H2L\(^{\text{GFPin}}\) was still associated with TAP after adding the high-affinity peptide MCMV, while about 25% of H2L\(^{\text{GFPin}}\) remained TAP associated after adding peptides QL9 or \(P_{2Ca}\). These fractions approximate those reported earlier for H2K\(^{\beta}\) and H2Db associated with TAP in immunoprecipitates (Suh et al., 1994; Suh et al., 1996).

Even though H2L\(^{\text{GFPout}}\) was not coprecipitated with TAP (Figure 5), analysis of \(D_{\text{avg}}\) indicated that at steady-state a small fraction, ~10%, of these molecules was TAP associated. This result is consistent with the observation that \(D\) of H2L\(^{\text{GFPout}}\) increased in cells fed peptides and decreased in cells treated with lactacystin (Table 1).
Discussion

To better understand the way in which MHC class I molecules are retained in the ER as they are assembled and to probe the associations of class I molecules with resident ER proteins, we GFP tagged the mouse class I molecule H2L4. Molecules tagged with GFP on either the C-terminal cytoplasmic tail, H2L4GFPin, or in the exodomain, H2L4GFPout, reached the cell surface. They measurements imply a much larger size. Lateral diffusion of H2L4 and peptide. Hence, it appears that (Hughes et al., 1982). The equation given there can be after loading with peptide, with a soluble T cell receptor to the logarithm of the radius of the diffusing molecule of TAP complex is stable for association of H2L4 and TAP would lower D, but we can immobilize the H2L4 molecules; this will lower R should immobilize the H2L4 molecules; this will lower R but should not change D. On the other hand, cycles of rapid association and dissociation of H2L4 and the TAP complex would lower D (Elson and Reider, 1979). If the TAP complex is diffusing, then either stable or cyclic associations of H2L4 and TAP would lower D, but we do not expect R to change. Using TAP1 GFP, we found that TAP1 is indeed mobile, with D ~ 1-2 x 10^-9 cm^2/s, comparable to D measured for two other ER-resident proteins (Li et al., 1998; Szczesna-Skorupa et al., 1998). Comparing D for TAP1 with that for H2L4GFP in after lactacystin treatment, we conclude that the association of H2L4 molecules with the TAP complex is stable for minutes (the time of an FPR measurement).

Peptide-loaded class I molecules diffuse at the viscosity limit in ER membrane, while a resident ER protein, TAP1, like other resident proteins does not. Given this difference, we cannot ascribe the lower D of TAP to molecular crowding. Instead, it is likely to reflect the large size of the TAP complex. Biochemical analyses set a lower limit for the molecular weight of the complex of ~10^6 (Ortmann et al., 1997), but our lateral diffusion measurements imply a much larger size. Lateral diffusion of a protein embedded in a bilayer is proportional to the logarithm of the radius of the diffusing molecule (Hughes et al., 1982). The equation given there can be approximated as

\[ D = k \ln \left( \frac{c}{a} \right) - 0.577 \]

where D is the diffusion coefficient, k and c are constants that include the membrane bilayer thickness, 5 nm, and viscosity, 1P, and the viscosity of the surrounding medium, 2 cP, and a is the radius of the transmembrane portion of the diffusing protein.

Solving simultaneous equations for D = 1 x 10^-9 cm^2/s and for D = 5 x 10^-9 cm^2/s; we found that the ratio a/a1 is ~150-200. That is, the radius of the slowly diffusing TAP complex is ~150-200 fold larger than the radius of a single MHC class I molecule. To scale this, we note that the radius of a single transmembrane helix is ~4-5 Å. The radius of a well-studied multispan membrane protein, bacteriorhodopsin (molecular weight ~24,000, 7-transmembrane helices arranged in an ellipse) is 20 Å in the long axis, and the radius of the trimer in which it crystallizes (21-transmembrane helices) is 30 Å (Müller et al., 1999). From this we estimate that the TAP complex has a radius of ~600-1,000 Å. If the low D of TAP indeed reports the size of the complex of which it is a part, then this complex could consist of hundreds of proteins, perhaps including those of a matrix of ER chaperones (Tatu and Helenius, 1997). Size alone might be enough to keep such a large complex from exiting the ER.

The functional importance of TAP-class I interaction in the assembly of MHC class I molecules has been the subject of conflicting reports. Here, we show that TAP-class I interaction restricts free diffusion of MHC class I H2L4 molecules in the ER membrane, but this alone does not mediate temporal retention of MHC class I in the ER. Our results on the high D and ER localization of H2L4 molecules after loading with exogenous peptide suggest that in some cases a later step, export from the ER, or recycling to optimize peptide loading may be limiting. In control cells, approximately half of all GFP-tagged H2L4 molecules diffuse at the viscosity limit. While some of these may be newly synthesized molecules that have not yet been loaded with peptide, our biochemical results, together with those on peptide loading, indicate that most of this fraction of H2L4 molecules are peptide loaded. If there is no processing or export step after the TAP complex, then we would expect this population to have left the ER.

The retention of peptide-loaded H2L4 molecules in the ER probably is not an aspect of the slow maturation of these molecules (Beck et al., 1986). We have preliminary
results on GFP-tagged H2κb (an MHC class I molecule with a maturation time of about 45 min) which also show that peptide-loaded molecules are highly diffusible but remain in the ER for some time after loading. ER retention of peptide-loaded H2L molecules is also not likely due to the 5- to 10-fold overexpression of H2L. J. Joyce (1997) has shown that up to 50-fold overexpression of MHC class I molecules does not change the extent of their peptide loading or the rate of their biochemical maturation. ER retention may be due to association of peptide-loaded MHC class I molecules which may reflect the limited availability of ER export sites requiring fully assembled MHC class I molecules to queue at sites of vesicle formation and protein export.

**Experimental Procedures**

**Cells and Antibodies**

To produce stable transfected cell lines, native and GFP-tagged H2L* genes H2L, H2L*GFP, and H2L*GFPout were transfected to mouse fibroblasts, L-Mtk (H2)* (ATCC CCL 1.3), or to EE2H3 (H2)* embryonic cells (Silverman et al., 1988) in the vectors described below. Cells were transfected using LipofectAMINE reagent (Life Technologies), then selected for G418 resistance and cloned. All FT1 described in the following sections.

**Stressgen Biotechnologies.** Bated for 2 hr with anti-calnexin serum. To probe for TAP1, lysates of MCMV (YPHFMPTNL) a peptide from MCMV pp69 (Reddeshae et al., 1989), were made by F-MOC chemical synthesis and then purified by preparative HPLC.

**Construction of the Chimeras**

The BamHI±BglII H2Ld cDNA sequence coding for amino acids 1±218 were transferred to OPTITRAN membranes (Schleicher and Schuell).

**Western Blotting**

Cells were transfected using LipofectAMINE reagent (Life Technologies), then selected for G418 resistance and cloned. All FT1 described in the following sections.

**Immunoprecipitation**

Cells were harvested in PBS, 1.5 mM EDTA, washed once with PBS containing 1% BSA, and incubated with a saturating concentration of mAb or with PBS 1% BSA alone for 30 min at 4°C. The cells were washed three times with PBS 1% BSA and incubated with a saturating concentration of Cy3-conjugated Fab', goat anti-mouse IgG for 30 min at 4°C, washed with PBS 1% BSA three times, and resuspended in 1% paraformaldehyde in PBS. Labeled cells were analyzed on an EPICS 752 flow cytometer (Coulter Instruments). Each sample analyzed comprised a minimum of 5 × 10⁶ cells.

**Peptides**

p2Ca (SPPFPFDL) (Udaka et al., 1992), SL9 (SPPFPFDL) (Sykulev et al., 1994a), and QL9 (QLSPFPFDL) (Sykulev et al., 1994b) peptides from α-ketoglutarate dehydrogenase, and MCMV (YPHFMPTNL), a peptide from MCMV pp69 (Reddeshae et al., 1989), were made by F-MOC chemical synthesis and then purified by preparative HPLC.

**Conventional and Confocal Fluorescence Microscopy**

Cells for immunofluorescence were cultured on glass coverslips for 24–36 hr, then fixed for 30 min at room temperature with 4% paraformaldehyde in PBS. Cells to be labeled with antibody to endomembrane markers were permeabilized with PBS containing 0.05% saponin and 1% BSA, then incubated for 30 min at room temperature, with primary antibodies diluted in PBS containing 0.05% saponin and 1% BSA. Unbound antibodies were removed by washing in the same medium; after this, the cells were incubated for 30 min with secondary antibodies and washed in PBS containing 0.05% saponin and 1% BSA. Coverslips were mounted onto glass slides in anti-fading medium. Samples were imaged either on a conventional microscope (Zeiss Axiosvert, fitted with a Photometrix slow-scan CCD camera) or on a confocal laser scanning microscope, Leica TCS 4D (Leica Lasertechnik, Heidelberg, Germany).

**Immunoprecipitation**

Cells were starved of methionine for 60 min, then labeled with 300 μCi/mL Tran35S-label for 30 min or for 12 hr. The labeled cells were washed three times in cold PBS and lysed in lysis buffer containing 1% 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonate (CHAPS, Sigma). After preclearing, lysates were incubated with μAb 28-14-8S and μAb 64-3-7. To probe for calnexin, lysates were incubated for 2 hr with anti-calnexin serum. To probe for TAP1, lysates were incubated for 12 hr with a cocktail of anti-TAP serum containing rabbit anti-GST-3' TAGP1 (mouse) and rabbit anti-GST-3' TAP2 (mouse) (Kulig et al., 1998), kindly provided by Dr. J. John Monaco (University of Cincinnati). Immunoprecipitates and molecular weight standard were electrophoresed on 7%–15% SDS-PAGE gels.

**Flow Cytometry**

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**Western Blotting**

Immunoprecipitates, resolved by SDS-PAGE as described above, were transferred to OPTITRAN membranes (Schleicher and Schuell). After incubation with specific anti-sera for 2 hr and washing, membranes were incubated for 1 hr at 4°C with horseradish peroxidase-conjugated donkey anti-rabbit Ig (Amersham Life Sciences), washed three times with PBS/0.3% Tween-20, and then incubated with...
Western blotting detection reagents (Amersham Life Sciences) for 1 min. Membranes were applied to BioMax MR film (Eastman Kodak) for 10 to 2 min.

Measurement of Lateral Diffusion by FPR

Cells were cultured on cover slips at 37°C for 2 days before fluorescence photobleaching recovery (FPR) experiments. Coverslips were washed twice in Hank's balanced salts buffer (Sigma) supplemented with 10 mM HEPES (pH 7.2) and 1% heat-inactivated fetal calf serum (HH 1%) prewarmed to 37°C. Coverslips were mounted on slides in HH 1% buffer and sealed with nail polish. For experiments in presence of peptides, cells on coverslips were incubated at 37°C in medium 100 μM peptide for 1 hr before FPR measurements. To inhibit proteasomes, cells on coverslips were incubated at 37°C in presence of 10 μM lactacystin (Kamiya Biomedical) for 30 min, then washed twice in HH 1% prewarmed to 37°C. When lactacystin treatment was followed by an incubation with a peptide, the lactacystin-treated cells were washed twice in culture medium and incubated as described for peptide loading.

Lateral diffusion of GFP-tagged or antibody-tagged class I molecules was measured on a Zeiss Axioplan fluorescence microscope using a 1.30 NA 100× Zeiss Plan-neofluor objective to focus the 488 nm line of an argon ion laser to a spot of ~0.6 μm radius. Cells were imaged using DIC optics to locate the region of interest (perinuclear, or plasma membrane). The attenuated laser beam was used to monitor the fluorescence signal in the region before and after partial bleaching of the fluorophore by a 2 ms pulse of laser light at full intensity. All measurements of D in the ER were made at 36°C–37°C.

Data were collected and analyzed using custom software. Two parameters were extracted from each curve: the half-time for recovery, from which the diffusion coefficient, D, was calculated, and the percent recovery of fluorescence, R D was calculated assuming recovery by one-dimensional diffusion in the ER. We assume this because the bleaching beam is wider than the tubules of the ER and hence bleaches the full widths of a number of tubular structures; recovery then occurs by diffusion of unbleached molecules in the long axes of the tubules. The differences in D, ~50%, measured for the same molecules some months apart are likely due to errors in practice, 25%–30%) in measuring the radius of the laser beam used for bleaching and measuring fluorescence recovery.

When making measurements of lateral diffusion of H2Ld-GFP in the ER, we took care to focus the spot to a region of the nuclear envelope, which was sampled in several places before making a measurement. This avoided fluorescence from GFP chimeras in the Golgi complex or in vesicles, since the fluorescence of the GFP-tagged proteins in the latter regions was much brighter than in the ER. In one series of experiments, we also measured D in ER far from the nuclear membrane; there was no difference between D measured in the perinuclear ER and that measured in more distal ER. Diffusion of plasma membrane molecules made little or no contribution to the measured recoveries. D and R for GFP-tagged H2Ld in the ER were not changed if plasma membrane H2Ld molecules were cross-linked and immobilized by mAb followed by anti-mouse IgG before measuring lateral diffusion in the ER.

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