

Lateral Diffusion of GFP-Tagged H2L^d Molecules and of GFP-TAP1 Reports on the Assembly and Retention of These Molecules in the Endoplasmic Reticulum

Didier Marguet,*§ Elias T. Spiliotis,*
Tsvetelina Pentcheva,* Michael Lebowitz,†
Jonathan Schneck,† and Michael Edidin*‡

*Department of Biology
The Johns Hopkins University
Baltimore, Maryland 21218

†Department of Pathology
The Johns Hopkins University School of Medicine
Baltimore, Maryland 21205

Summary

Lateral diffusion of GFP-tagged H2L^d molecules in the ER membrane reports on their interaction with the TAP complex during synthesis and peptide loading. Peptide-loaded H2L^d 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. H2L^d molecules, associated with the TAP complex, diffuse slowly, as does GFP-tagged TAP1. This implies that the association of H2L^d 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; Kearsse et al., 1994; van Leeuwen and Kearsse, 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 (Yeichiel and Edidin, 1987), or their aggregation would also result in low mobile fractions.

We show here that D measured for GFP-tagged H2L^d molecules in the ER is low 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 H2L^d molecules. One population is associated with the TAP complex; its D reflected the low D of the TAP complex itself. A second population of H2L^d 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 H2L^d molecules. However, completely assembled, peptide-loaded H2L^d molecules are also transiently retained in the ER, even though they are diffusing freely and are not associated with TAP. These H2L^d 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 H2L^d Class I Molecules

We constructed two different GFP-tagged forms of the mouse MHC class I allele H2L^d as our probes for traffic of MHC class I molecules through the ER. These are shown in Figure 1A. One form, H2L^dGFP_{in}, was tagged at the end of the cytoplasmic tail, the C terminus. The second form, H2L^dGFP_{out}, was tagged with GFP between the third exodomain of the heavy chain and the transmembrane domain. L cells were transfected with genes for the H2L^d GFP chimeras or for native H2L^d. Expression of the transfects was monitored by flow cytometry using three different criteria: GFP fluorescence, labeling by H2L^d-specific mAb, and labeling by soluble 2CTcR2IgG (O'Herrin et al., 1997; Lebowitz et al., 1999), which detected specific peptide/H2L^d complexes (Figure 2). Cells transfected with genes for either native or GFP-tagged H2L^d 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

‡ To whom correspondence should be addressed (e-mail: edidin@jhu.edu).

§ Present address: Centre d'Immunologie, INSERM CNRS, de Marseille Luminy, 13288 Marseille Cedex 09, France.

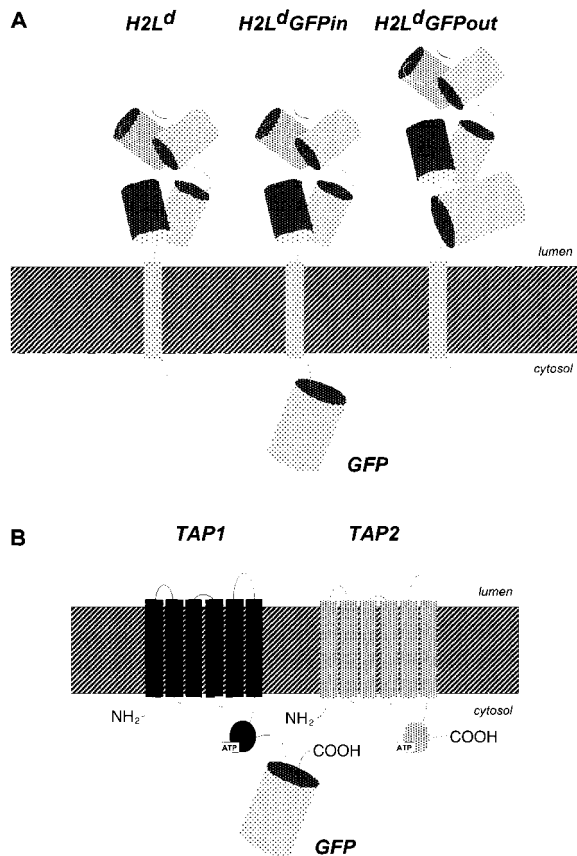


Figure 1. Native and GFP-Tagged H2L^d and TAP1 Molecules
(A) H2L^dGFP_{in} was constructed by linking the GFP gene to the C-terminal sequence of the native H2L^d gene. H2L^dGFP_{out} was constructed by inserting the GFP gene between the α3-domain sequence and the transmembrane domain sequence of the native H2L^d gene.
(B) TAP1 GFP was constructed by linking the GFP gene to the C-terminal sequence of the native gene. For details see Experimental Procedures.

L cells. It appears that GFP-tagged H2L^d molecules traffic the cell and reach the surface in native conformation.

GFP-tagged H2L^d loaded with a cognate peptide, QL9, specifically bound an engineered soluble divalent T cell receptor analog, 2C TCR2IgG. Binding of 2C TCR2IgG was specific; soluble receptor did not bind significantly to cells that had been incubated in the absence of peptide (compare the light line in Figure 2, right panel, to the dotted line, binding of secondary antibody only). In addition, 2CTCR2IgG also bound to H2L^dGFP_{in} loaded with other 2C-reactive peptides (p2Ca and SL9) but did not bind to H2L^dGFP_{in} loaded with MCMV peptides (data not shown). Thus, GFP-tagged H2L^d are specifically recognized by their functional receptor.

Distribution of GFP-Tagged H2L^d in Living Cells
In cells expressing GFP-tagged H2L^d, green fluorescence was observed at the plasma membrane and in intracellular structures. Much of the intracellular H2L^dGFP fluorescence was patchy and reticular in a pattern suggestive of ER; some was concentrated in a pattern suggestive of the Golgi complex (Figures 3A and

3D). Using the same exposure time for image acquisition, no green fluorescence was observed in L cells or from cells expressing native H2L^d (data not shown).

To identify the intracellular structures containing the H2L^d, 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 H2L^dGFP_{in} and H2L^dGFP_{out} fluorescence colocalized with fluorescence from labeled ER proteins (Figures 3B and 3C show the pattern for H2L^dGFP_{in}). Some H2L^dGFP fluorescence colocalized with fluorescence from the labeled Golgi complex proteins (Figures 3E and 3F). The ER colocalization of H2L^dGFP, while extensive, was not complete. H2L^dGFP was evident in calnexin-poor subregions of the ER. The distribution of native H2L^d, 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 H2L^d 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 H2L^d 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, H2K^k. All three forms were associated with β2-microglobulin; however, the ratio of heavy chain to β2m was much lower for H2L^dGFP_{out} (35:1) than for native H2L^d and H2L^dGFP_{in} (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 H2L^d 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 H2L^d and H2L^dGFP_{in} to endoH-resistant forms were about 4 hr, close to the value reported earlier (Beck et al., 1986). H2L^dGFP_{out} appeared to remain in the ER for much longer times; less than 40% of newly synthesized H2L^dGFP_{out} 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 H2L^dGFP 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 H2L^d with two different antibodies to the H2L^d 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 H2L^d or H2L^dGFP_{in} precipitated by 64-3-7 (Figure 4). In contrast, immunoprecipitates of H2L^dGFP_{out} were positive for calnexin but not for calreticulin or TAP molecules (Figure 4). We also probed quantitatively for complexes of native and GFP-H2L^d 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

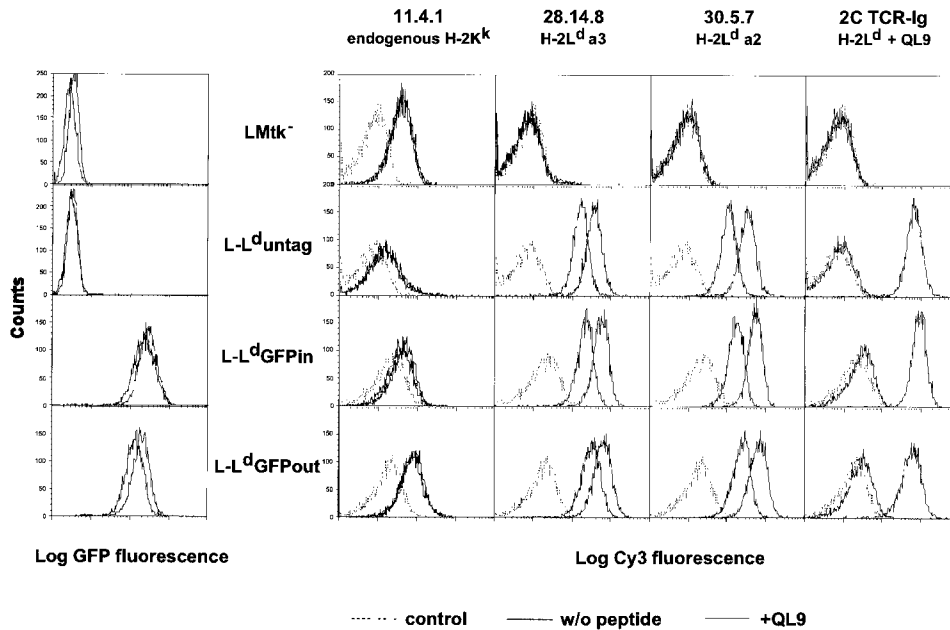


Figure 2. Expression of GFP-Tagged H2L^d Molecules by Transfected L Cells

Expression of native and GFP-tagged H2L^d 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 H2L^d gene.

Right panel, cell surface H2L^d measured using mAb 28-14-8, which binds both native H2L^d and free heavy chains, with mAb 30-5-7, which binds only native H2L^d, or with soluble divalent 2C TCR2IgG, which in this experiment binds only native H2L^d molecules loaded with peptide QL9. Cell surface H2K^k, 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 H2L^d associated to the same extent (with an uncertainty of 10%) with calnexin. However, though a cocktail of anti-TAP antibodies precipitated native H2L^d and H2L^dGFPin, no H2L^dGFPout could be detected in the precipitates, confirming the results of Western blotting. It appears that the GFP moiety on the cytoplasmic tail of H-2L^dGFPin does not interfere with its association with the TAP complex, while the GFP moiety near the α 3 domain of H-2L^dGFPout does interfere. The α 3 domain is important in β 2m 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 H2L^dGFP Chimeras in the Endoplasmic Reticulum

As shown above, a substantial fraction of GFP-tagged H2L^d molecules colocalized with a marker for ER membranes. We measured the lateral diffusion of these GFP-tagged H2L^d 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 H2L^dGFPout 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 H2L^dGFPin was about half that of H2L^dGFPout, $D = 2.0 \pm 0.1 \times 10^{-9} \text{ cm}^2\text{s}^{-1}$, and lower than found for Golgi proteins, though R was about the same (61%). This result suggested that H2L^dGFPin 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 H2L^dGFPin by comparing D in the plasma membrane of each GFP-tagged form of H2L^d with D of the native molecule. All D and R were similar, $D \sim 5 \times 10^{-9} \text{ cm}^2\text{s}^{-1}$ and $R \sim 85\%$, 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 H2L^d in the plasma membrane and the biochemical results reported above, it seemed likely that the relatively low D of H2L^dGFPin was due to its interaction with proteins of the TAP complex.

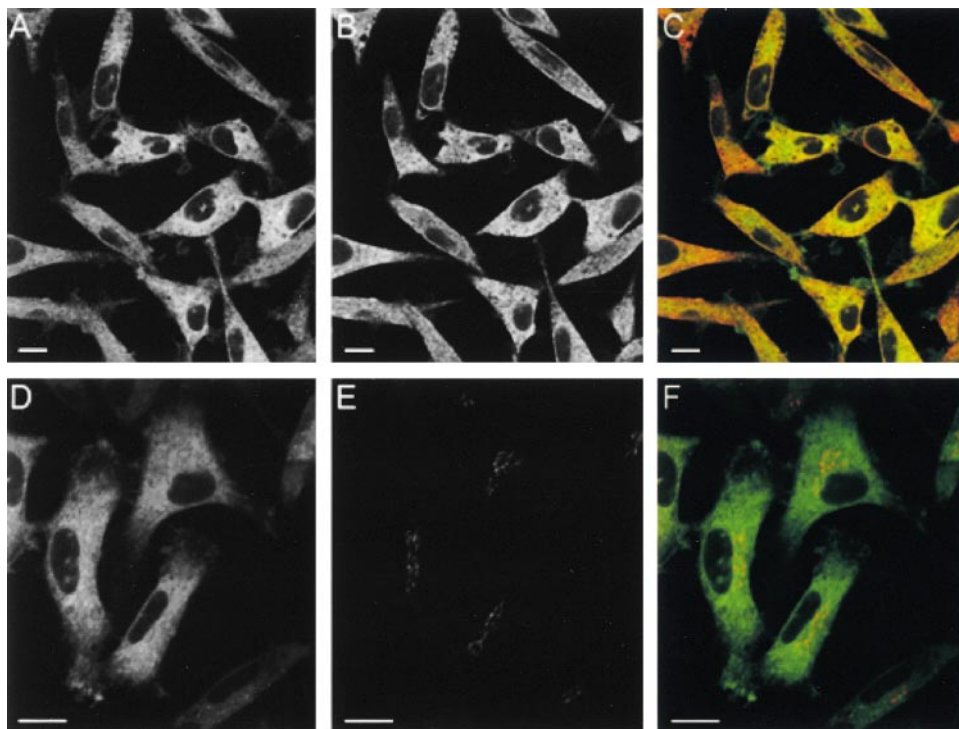


Figure 3. Colocalization of H2L^dGFPin with Markers for ER or Golgi Complex

L cells transfected with GFP-tagged H2L^d 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 μ m).

Lateral Diffusion of H2L^dGFP 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^d. 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 $\alpha\beta$ interferon, which induces the expression of TAP1 (Bikoff et al., 1991). H2L^dGFPin 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 $\alpha\beta$ 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^dGFPin in FT1⁻ fibroblasts derived from TAP1 knockout mice (Van Kaer et al., 1992) with D in FT1⁺ 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^dGFPin 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^dGFPin molecules loaded with peptide should dissociate from TAP (Suh et al., 1994) and so the average D for the population of H2L^d molecules should increase. Without a supply of peptides, effectively all H2L^dGFPin molecules should remain associated with 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^dGFPout, 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^dGFP loaded with peptide QL9, while it did not bind to H2L^dGFP 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^dGFP in the ER and at the plasma membrane after loading cells with MCMV, QL9, SL9, or p2Ca peptides. The affinity, K_A , of H2L^d for these peptides ranges from $\sim 10^9$ to $\sim 10^6 \text{ M}^{-1}$. After incubating cells with peptides, D of H2L^dGFPin 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^d for the peptide (Figure 6). The lateral diffusion of H2L^dGFPout in the ER increased slightly though significantly after cells were incubated with the high-affinity MCMV peptide, $D = 4.7 \pm 0.6 \times 10^{-9} \text{ cm}^2\text{s}^{-1}$

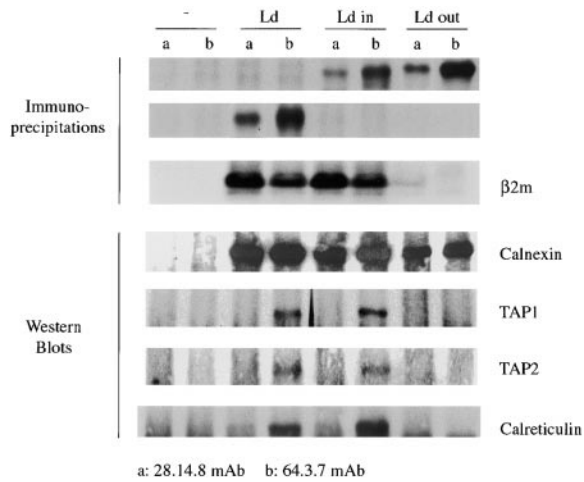


Figure 4. Association of Native and GFP-Tagged H2L^d with β 2m, Chaperones, and TAP in the ER

H2L^d was precipitated from cell lysates using mAb 28-14-8 or 64-3-7. 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 coimmunoprecipitation of β 2m is overexposed relative to the panels showing immunoprecipitation of heavy chains.

versus $3.7 \pm 0.3 \times 10^{-9} \text{ cm}^2\text{s}^{-1}$ for H2L^dGFPout in the absence of added peptide. In contrast, lateral diffusion of H2L^dGFPin at the plasma membrane was not changed by peptide loading.

D of H2L^dGFPin 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^dGFPin loaded by a peptide of a given affinity and, hence, the ratio of free and TAP-associated H2L^dGFPin. The largest D measured for H2L^dGFPin, $3.6 \times 10^{-9} \text{ cm}^2\text{s}^{-1}$, is significantly smaller than the maximum for H2L^dGFPout. Therefore, we infer

that a fraction of H2L^dGFPin is still associated with TAP even after incubation of cells with the high-affinity MCMV peptide. This is consistent with the report by Suh et al. (1994) that $\sim 10\%$ of H2 molecules are resistant to dissociation from TAP after peptide is added to cells.

The association of MHC class I with the TAP complex is prolonged when the supply of peptides is reduced. The main source of peptides presented by class I molecules is protein degraded by the 20S proteasome (Rock et al., 1994). We inhibited proteasome catalytic activity with lactacystin, a specific and irreversible inhibitor (Fenteany et al., 1995). Lactacystin treatment had no effect on the lateral diffusion of either H2L^dGFP chimera in the plasma membrane (Table 1). However, D of H2L^dGFPin in the ER was significantly reduced to $1.3 \pm 0.1 \times 10^{-9} \text{ cm}^2\text{s}^{-1}$ (versus $2.0 \pm 0.1 \times 10^{-9} \text{ cm}^2\text{s}^{-1}$ in untreated cells) (Figure 6). The effect of lactacystin treatment on D was reversed by added peptide. When treated cells were incubated with 100 μM MCMV or QL9 peptides for 1 hr, D of H2L^dGFPin increased to $3.9 \pm 0.3 \times 10^{-9} \text{ cm}^2\text{s}^{-1}$ (MCMV) or $3.0 \pm 0.2 \times 10^{-9} \text{ cm}^2\text{s}^{-1}$ (QL9), values similar to those for cells incubated in presence of peptide alone ($3.6 \pm 0.2 \times 10^{-9} \text{ cm}^2\text{s}^{-1}$ and $2.8 \pm 0.1 \times 10^{-9} \text{ cm}^2\text{s}^{-1}$, respectively).

Lateral Diffusion of TAP1 GFP in the ER

The lateral diffusion of H2L^dGFPin appeared to be constrained by its association with the TAP complex. This meant that either H2L^dGFPin 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 1B). TAP1 GFP appeared to be integrated into a functional TAP complex, since the cell surface level of endogenous class I molecules increased in cells transfected with TAP1 GFP (data not shown). GFP fluorescence largely colocalized with the marker for the ER,

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

Cell	Treatment	H2L ^d GFPin				H2L ^d GFPout			
		ER		PM		ER		PM	
		D	R	D	R	D	R	D	R
L	-	-	-	6 ± 0.8	78 ± 3	-	-	5.6 ± 0.3	81 ± 2
	20°C	-	-	-	-	-	-	-	-
	Cy3 Fab	-	-	5 ± 1.8	85 ± 6	-	-	4.7 ± 0.4	79 ± 4
	20°C	-	-	-	-	-	-	-	-
	-	20 ± 1	61 ± 2	6.6 ± 0.4	68 ± 2	37 ± 3	68 ± 2	7.5 ± 0.5	75 ± 2
	p2Ca	22 ± 2	77 ± 2	8.6 ± 0.7	86 ± 2	-	-	-	-
	SL9	28 ± 2	63 ± 2	8.1 ± 0.7	74 ± 2	-	-	-	-
	QL9	28 ± 1	66 ± 2	7.5 ± 0.4	73 ± 2	-	-	-	-
	MCMV	36 ± 2	68 ± 2	9.1 ± 0.6	81 ± 2	47 ± 6	75 ± 4	7.7 ± 0.7	81 ± 3
	lactacystin	13 ± 1	71 ± 3	10.0 ± 1	72 ± 2	14 ± 1	61 ± 4	9.5 ± 0.7	78 ± 3
	lactacystin + MCMV	39 ± 3	69 ± 2	8.0 ± 0.8	82 ± 3	44 ± 7	38 ± 5	13 ± 2	77 ± 3
lactacystin + QL9	30 ± 2	59 ± 2	8.0 ± 0.5	72 ± 3	-	-	-	-	
EE2H3	-	46 ± 4	81 ± 1	7.8 ± 0.6	88 ± 2	-	-	-	-
	$\alpha\beta$ IFN	18 ± 1	84 ± 2	7.8 ± 0.7	85 ± 2	-	-	-	-
	$\alpha\beta$ IFN + MCMV	39 ± 2	72 ± 2	6.8 ± 0.5	80 ± 1	-	-	-	-
FT1 ⁺	-	19 ± 1	71 ± 3	-	-	-	-	-	
FT1 ⁻	-	40 ± 3	59 ± 4	-	-	-	-	-	

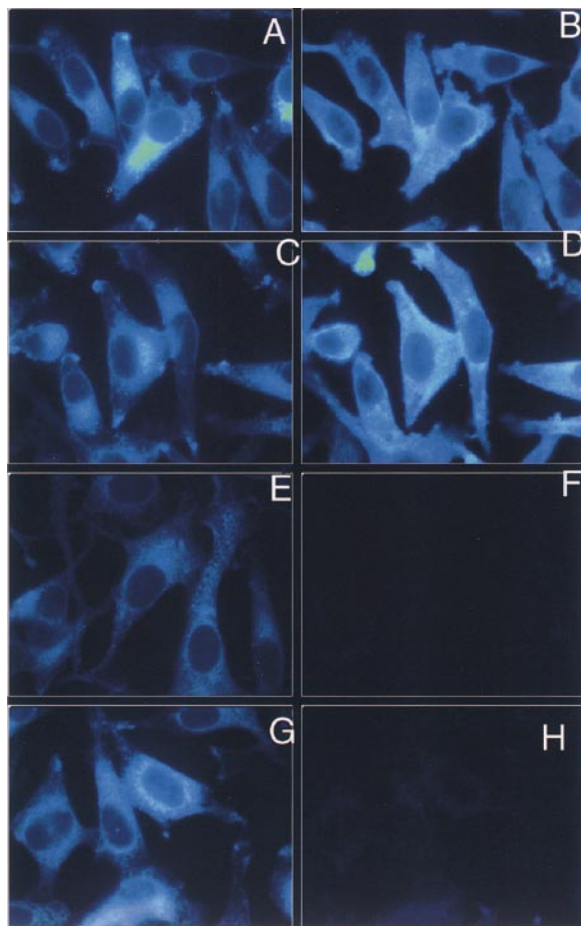


Figure 5. Peptide-Loaded H2L^d GFPIn

Cells cultured on coverslips for 24–36 hr were fixed in 4% paraformaldehyde in PBS. The fixed cells were permeabilized with 0.05% saponin and 1% BSA in PBS, then labeled with 2CTCR2IgG, followed by labeled anti-Ig. Left panel, GFP fluorescence; right panel, 2CTCR2IgG plus Cy3-anti-Ig. (A), (B) + peptide QL9; (C), (D) + peptide SL9; (E), (F) + peptide MCMV; (G and H), no peptide added.

calnexin; however, some regions of cytoplasm were rich in TAP1 GFP but calnexin poor, a pattern similar to that observed for H2L^dGFPIn. TAP1 GFP fluorescence did not colocalize with the Golgi complex marker giantin.

D measured for TAP1 GFP, $\sim 1.2 \pm 0.1 \times 10^{-9} \text{ cm}^2\text{s}^{-1}$, was, within error, identical to that of H2L^dGFP in the ER membranes of cells treated with lactacystin, $1.3\text{--}1.4 \times 10^{-9} \text{ cm}^2\text{s}^{-1}$. D of TAP1 GFP was not changed when cells were treated with lactacystin. This result strongly supported the idea that the constraints to lateral diffusion of MHC class I molecules are due to the stable association of otherwise mobile molecules with the TAP complex.

The Fraction of H2L^d Molecules Associated with TAP

The lateral diffusion coefficients that we measured are 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

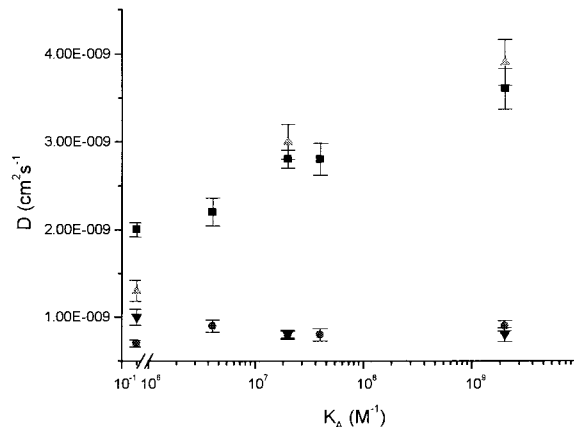


Figure 6. The Effects of Modulating Peptide Supply to the ER on the Lateral Diffusion of H2L^dGFPIn in ER and Plasma Membranes

Lateral diffusion coefficients, D, are plotted as a function of the affinity of H2L^d for the peptide added, p2Ca, 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.

for rapidly diffusing molecules as $4.7 \times 10^{-9} \text{ cm}^2\text{s}^{-1}$, the value measured for H2L^dGFPout after adding peptide to L cells and the value for H2L^dGFPIn in cells that lack a functional TAP complex. We took D for slowly diffusing molecules as $1.2 \times 10^{-9} \text{ cm}^2\text{s}^{-1}$, the value measured for GFP-TAP1. These values were used to resolve the measured D, D_{avg} , as $D_{\text{avg}} = f1(D_{\text{fast}}) + f2(D_{\text{slow}})$, where f1 equals the fraction of rapidly diffusing H2L^d molecules, f2 equals the fraction of slowly diffusing H2L^d molecules, and $f1 + f2 = 1$. Rather than determine f1 and f2 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_{avg} for known mixtures of free fluorescein and fluorescein-IgM (Edidin, 1994). These data fit the relationship $1/D_{\text{avg}} = f1(1/D_{\text{fast}}) + f2(1/D_{\text{slow}})$. We used this relationship and the estimates of D_{fast} and D_{slow} to evaluate f1, the fraction of H2L^d not associated with TAP, and f2, the fraction of H2L^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^dGFPIn molecules in the ER are associated with TAP. The dissociation of H2L^dGFPIn from TAP depended upon the affinity of H2L^d for the peptides. Only 10% of H2L^dGFPIn was still associated with TAP after adding the high-affinity peptide MCMV, while about 25% of H2L^dGFPIn remained TAP associated after adding peptides QL9 or p2Ca. These fractions approximate those reported earlier for H2K^b and H2Db associated with TAP in immunoprecipitates (Suh et al., 1994; Suh et al., 1996).

Even though H2L^dGFPout was not coprecipitated with TAP (Figure 5), analysis of D_{avg} indicated that at steady-state a small fraction, $\sim 10\%$, of these molecules was TAP associated. This result is consistent with the observation that D of H2L^dGFPout 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 H2L^d. Molecules tagged with GFP on either the C-terminal cytoplasmic tail, H2L^dGFPin, or in the exodomain, H2L^dGFPout, reached the cell surface. They reacted with mAb against native H2L^d molecules and, after loading with peptide, with a soluble T cell receptor specific for H2L^d and peptide. Hence, it appears that the GFP tag does not interfere with the proper folding, assembly, and peptide loading of H2L^d GFP chimeras.

Most of the intracellular GFP-tagged H2L^d was in the ER and the Golgi complex. This was evident both from the pattern of intracellular fluorescence and from the colocalization of GFP fluorescence with fluorescence from antibody-labeled proteins marking ER and Golgi complex. Native H2L^d, labeled with mAb, gave the same distribution of fluorescence as the GFP-tagged molecules. This ER and Golgi localization has also been observed in earlier work visualizing H2 using fluorescent antibodies (Yewdell and Bennink, 1992).

The diffusion coefficients measured for GFP-tagged H2L^d in ER membranes differed between H2L^dGFPin and H2L^dGFPout and also changed with the availability of peptides in the ER. D of H2L^dGFPout molecules, most of which were not associated with TAP, was $\sim 4 \times 10^{-9}$ cm²s⁻¹, near the limit set by viscosity of bilayer lipids. D for H2L^dGFPin molecules, many of which were associated with TAP, was significantly smaller, $\sim 2 \times 10^{-9}$ cm²s⁻¹. When either GFP-tagged H2L^d was loaded with peptides, D increased, reflecting an increased fraction of H2L^d molecules not associated with TAP. Evidence that D measured for H2L^d depends upon the fraction of molecules associated with TAP also came from the lateral diffusion of H2L^dGFPin in EE2H3 embryonic cells, which lack a functional TAP complex. In these cells, D is $\sim 4 \times 10^{-9}$ cm²s⁻¹. After inducing TAP1 expression with interferon, D decreased to the value measured in L cells, $\sim 2 \times 10^{-9}$ cm²s⁻¹. When we loaded molecules with exogenous peptide, dissociating them from TAP, D returned to $\sim 4 \times 10^{-9}$ cm²s⁻¹. The same effects were seen comparing D in FT1⁻, TAP1 knockout cells, with D in FT1⁺, TAP1⁺ cells.

The correlation of D and association with TAP suggested two possibilities for the interaction of H2L^d and TAP. If the complex of molecules including TAP is immobile, then stable association of H2L^d with the complex should immobilize the H2L^d molecules; this will lower R but should not change D. On the other hand, cycles of rapid association and dissociation of H2L^d and the TAP complex would lower D (Elson and Reidler, 1979). If the TAP complex is diffusing, then either stable or cyclic associations of H2L^d and TAP would lower D, but we do not expect R to change. Using TAP1 GFP, we found that TAP1 is indeed mobile, with $D \sim 1-2 \times 10^{-9}$ cm²s⁻¹, 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 H2L^dGFPin after lactacystin treatment, we conclude that the association of H2L^d 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 $\sim 10^6$ (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

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

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 $D_1 = 1 \times 10^{-9}$ cm²s⁻¹ and for $D_2 = 5 \times 10^{-9}$ cm²s⁻¹, we found that the ratio a_1/a_2 is $\sim 150-200$. That is, the radius of the slowly diffusing TAP complex is $\sim 150-$ to 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 $\sim 4-5$ Å. The radius of a well-studied multispan membrane protein, bacteriorhodopsin (molecular weight $\sim 24,000$, 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 $\sim 600-1,000$ 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 H2L^d 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 H2L^d 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 H2L^d 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 H2L^d 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 H2L^d 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 H2K^b (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^d molecules is also not likely due to the 5- to 10-fold overexpression of H2L^d. 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 with some machinery for optimizing peptide loading. Alternatively, retention of peptide-loaded MHC class I molecules 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^d genes *H2L^d*, *H2L^dGFPin*, and *H2L^dGFPout* were transferred to mouse fibroblasts, L-M(tk⁻) (H2^b) (ATCC CCL 1.3), or to EE2H3 (H2^b) 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 cells were maintained by 1:10 passage three times weekly in RPMI-1640 supplemented with 2 mM L-glutamine, 10 mM HEPES, and 10% heat-inactivated fetal calf serum. Expression of the TAP complex was induced in EE2H3 cells by incubating the cells for 48 hr in the presence of 500 U/mL interferon $\alpha\beta$ (Sigma). Other treatments prior to FPR measurements of lateral diffusion were performed as described in the following sections.

Ear skin fibroblasts derived from B6 \times 129 TAP1 knockout mice, FT1⁻, and B6 \times 129 normal controls, FT1⁺ were transformed by passage every 3 days until the cells passed crisis and continuous lines were developed. The cells were the kind gift of Dr. Luc Van Kaer (Vanderbilt University School of Medicine). The cells were transiently transfected with the gene for H2L^dGFPin, and lateral diffusion of the fluorescent protein in the ER was measured 48 hr later.

Mouse mAbs 28-14-8S (ATCC HB-27), 30-5-7S (ATCC HB-31), and 11-4-1 (ATCC TIB-95) were isolated from supernatants of hybridoma cell cultures using GammaBindPlus Sepharose (Pharmacia Biotech). Monoclonal antibody 28-14-8S binds to native molecules and to free heavy chains, mAb 30-5-7S binds only to native H2L^d molecules, and mAb 11-4-1 binds to the endogenous H2K^k molecules of L cells. Monoclonal antibody 64-3-7 against the α 1 domain of H2L^d (Shiroishi et al., 1985) was the kind gift of Dr. T. H. Hansen (Washington University School of Medicine). Cy3-conjugated F(ab')₂ fragment goat anti-mouse IgG (Jackson ImmunoResearch Laboratories) was used at 10 μ g/mL.

Antibody to the Golgi complex marker mannosidase II was the kind gift of Dr. Marilyn Farquhar (University of California, San Diego) (Velasco et al., 1993). Antibody to the Golgi complex marker protein giantin (Linstedt and Hauri, 1993) was the kind gift of Dr. André Leblivic. Antibodies to calnexin, calreticulin, and BiP were from Stressgen Biotechnologies.

Construction of the Chimeras

The pUC18-L^d vector containing the *H2L^d* class I cDNA was kindly provided by Dr. M. Zuniga (University of California, Santa Cruz). The 3' EcoRI site was deleted by mung bean nuclease digestion to prepare the *H2L^dGFPin* fusion gene. The BamHI-blunted EcoRI fragment was subcloned into the pEGFP-N3 (Clontech Laboratories) vector at the BglIII and SmaI sites to give the *pH2L^dGFPin* vector. The BamHI-BglIII H2L^d cDNA sequence coding for amino acids 1–218 was cloned in the pEGFP-N3 vector at the BglIII site to give the *pH2L^dint1-EGFP* vector. The *H2L^d* cDNA sequence coding for the α 3 domain was amplified by PCR with the oligonucleotides 5'-ACGCG ACGCTGCTGCGCACAGATT-3' and 5'-TAGCCCGGGGACGGAGGA

GGCTCC-3'. The PCR product was digested by BglIII and SmaI and then cloned into the recombinant pH2L^dint1-EGFP at the BglIII and SmaI to give the *pH2L^dint2-EGFP* vector. The *H2L^d* cDNA sequence coding for the amino acids 308–363 (transmembrane and cytoplasmic domains) was amplified by PCR with the oligonucleotides 5'-AAGGGTGATCATGGTGATCGTTGCT-3' and 5'-GCTCTAGAATT CACGCTTACAATC-3'. The PCR product was digested by BsrGI and XbaI and then cloned into the recombinant *pH2L^dint2-EGFP* at the BsrGI and XbaI to give the *pH2L^dGFPout* vector. All PCR-amplified DNA and cloning junction fragments were sequenced to confirm fidelity. The *pH2L^duntag* vector was produced by the deletion of the GFP coding sequence from the pEGFP-N3 vector followed by the introduction of the full *H2L^d* cDNA.

TAP1 cDNA was kindly provided by Dr. John Monaco (University of Cincinnati). It was GFP tagged on the C-terminal cytoplasmic domain by deleting the stop-codon of the gene and ligating the GFP gene, following the procedures described above for *H2L^d*.

Flow Cytometry

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 F(ab')₂ 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 \times 10⁴ cells.

Peptides

p2Ca (LSPFPFDL) (Udaka et al., 1992), SL9 (SPFPFDLL) (Sykulev et al., 1994a), and QL9 (QLSPFPFDL) (Sykulev et al., 1994b) peptides from α -ketoglutarate dehydrogenase, and MCMV (YPHFMPTNL), a peptide from MCMV pp89 (Reddehase 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, cells were incubated for 30 min with labeled 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 Axiovert, fitted with a Photometrix slow-scan CCD camera) 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 μ Ci/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-[(3cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS, Sigma). After preclearing, lysates were incubated with mAb 28-14-8 and mAb 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' TAP1 (mouse) and rabbit anti-GST-3' TAP2 (mouse) (Kulig et al., 1998), kindly provided by Dr. John Monaco (University of Cincinnati). Immunoprecipitates and molecular weight standard were electrophoresed on 7%–15% SDS-PAGE gels.

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 s 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 H2L^dGFP 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 H2L^d in the ER were not changed if plasma membrane H2L^d molecules were cross-linked and immobilized by mAb followed by anti-mouse IgG before measuring lateral diffusion in the ER.

Acknowledgments

We thank Drs. Marilyn Farquhar, Ted Hansen, André Lebivic, John Monaco, Luc Van Kaer, and Martha Zuniga for providing cells and reagents, Mrs. Taiyin Wei for expert technical support, and Mr. Andrew Nechkin of the Integrated Imaging Center, Department of Biology. This work was supported by National Institutes of Health grants AI14584 to M. E. and AI29575 to J. S. and by a grant from the National Multiple Sclerosis Foundation to J. S. (RG 2637A2/1). D. M. was supported in part by the Centre National de la Recherche Scientifique of France and by a fellowship from the North Atlantic Treaty Organization.

Received April 30, 1999; revised June 28, 1999.

References

- Bai, A., and Forman, J. (1997). The effect of the proteasome inhibitor lactacystin on the presentation of transporter associated with antigen processing (TAP) dependent and TAP-independent peptide epitopes by class I molecules. *J. Immunol.* **159**, 2139–2146.
- Beck, J.C., Hansen, T.H., Cullen, S.E., and Lee, D.R. (1986). Slower processing and weaker β2-m association, and lower surface expression of H-2Ld are influenced by its amino terminus. *J. Immunol.* **137**, 916–923.
- Bikoff, E.K., Jaffe, L., Ribaldo, R.K., Otten, G.R., Germain, R.N., and Robertson, E.J. (1991). MHC class I surface expression in embryo-derived cell lines inducible with peptide or interferon. *Nature* **354**, 235–238.
- Carreno, B.M., Solheim J.C., Harris, M., Stroynowski, I., Connolly, J.M., and Hansen, T.H. (1995). TAP associates with a unique class I conformation, whereas calnexin associates with multiple class I forms in mouse and man. *J. Immunol.* **155**, 4726–4733.
- Chao, D.S., Hay, J.C., Winnick, S., Prekeris, R., Kulmperman, J., and Scheller, R.H. (1999). SNARE membrane trafficking dynamics in vivo. *J. Cell Biol.* **144**, 869–881.
- Cole, N.B., Smith, C.L., Sciaky, N., Terasaki, M., Edidin, M., and Lippincott-Schwartz, J. (1996). Diffusional mobility of Golgi proteins in membranes of living cells. *Science* **273**, 797–801.
- Day, P.M., Yewdell, J.W., Porgador, A., Germain, R.N., and Bennink, J.R. (1997). Direct delivery of exogenous MHC class I molecule-binding oligopeptides to the endoplasmic reticulum of viable cells. *Proc. Natl. Acad. Sci. USA* **94**, 8064–8069.
- Degen, E., and Williams D.B. (1991). Participation of a novel 88-kD protein in the biogenesis of murine class I histocompatibility molecules. *J. Cell Biol.* **112**, 1099–1115.
- Edidin, M. (1994). Lateral mobility of membrane proteins—a journey from heterokaryons to laser tweezers. In *The Legacy of Cell Fusion*, S. Gordon, ed. (Oxford: Oxford University Press), pp. 101–114.
- Elson, E.L., and Reidler, J.A. (1979). Analysis of cell surface interactions by measurements of lateral mobility. *J. Supramol. Struct.* **12**, 481–489.
- Fenteany, G., Standaert, R.F., Lane, W.S., Choi, S., Corey, E.J., and Schreiber, S.L. (1995). Inhibition of proteasome activities and subunit-specific amino-terminal threonine modification by lactacystin. *Science* **268**, 726–731.
- Hammond, C., Braakman, I., and Helenius, A. (1994). Role of N-linked oligosaccharide recognition, glucose trimming, and calnexin in glycoprotein folding and quality control. *Proc. Natl. Acad. Sci. USA* **91**, 913–917.
- Harris M.R., Yu Y.Y., Kindle C.S., Hansen T.H., and Solheim J.C. (1998). Calreticulin and calnexin interact with different protein and glycan determinants during the assembly of MHC class I. *J. Immunol.* **160**, 5404–5409.
- Howard, J.C. (1995). Supply and transport of peptides presented by class I MHC molecules. *Curr. Opin. Immunol.* **7**, 69–76.
- Hughes, B.D., Pailthorpe, B.A., White, L.R., and Sawyer, W.H. (1982). Extraction of membrane microviscosity from translational and rotational diffusion coefficients. *Biophys. J.* **37**, 673–676.
- Joyce, S. (1997). Traffic control of completely assembled MHC class I molecules beyond the endoplasmic reticulum. *J. Mol. Biol.* **266**, 993–1001.
- Kearse K.P., Williams, D.B., and Singer, A. (1994). Persistence of glucose residues on core oligosaccharides prevents association of TCR alpha and TCR beta proteins with calnexin and results specifically in accelerated degradation of nascent TCR alpha proteins within the endoplasmic reticulum. *EMBO J.* **13**, 3678–3686.
- Krause, K.H., and Michalak, M. (1997). Calreticulin. *Cell* **88**, 439–443.
- Kulig, K., Nandi, D., Bacik, I., Monaco, J.J., and Vukmanovic, S. (1998). Physical and functional association of the major histocompatibility complex class I heavy chain α3 domain with the transporter associated with antigen processing. *J. Exp. Med.* **187**, 865–874.
- Lebowitz, M.S., O'Herrin, S.M., Hamad, A.-R., Fahmy, T., Marguet, D., Barnes, N.C., Pardoll, D., Bieler, J.G., and Schneck, J.P. (1999). Soluble high-affinity dimers of T-cell receptors and class II major

- histocompatibility complexes: biochemical probes for analysis and modulation of immune responses. *Cell. Immunol.* **192**, 175–184.
- Lewis, J.W., and Elliott, T. (1998). Evidence for successive peptide binding and quality control stages during MHC class I assembly. *Curr. Biol.* **8**, 717–720.
- Li, Y., Smith, T., Grabski, S., and DeWitt, D.L. (1998). The membrane association sequences of the prostaglandin endoperoxidase synthases-1 and -2 isozymes. *J. Biol. Chem.* **273**, 29830–29837.
- Lie, W.R., Myers, N.B., Connolly, J.M., Gorka, J., Lee, D.R., and Hansen, T.H. (1991). The specific binding of peptide ligand to Ld class I major histocompatibility complex molecules determines their antigenic structure. *J. Exp. Med.* **173**, 449–459.
- Lindquist, J.A., Jensen, O.N., Mann, M., and Hammerling, G.J. (1998). ER-60, a chaperone with thiol-dependent reductase activity involved in MHC class I assembly. *EMBO J.* **17**, 2186–2195.
- Linstedt, A.D., and Hauri, H.P. (1993). Giantin, a novel conserved Golgi membrane protein containing a cytoplasmic domain of at least 350 kDa. *Mol. Biol. Cell* **4**, 679–693.
- Marusina, K., Iyer, M., and Monaco, J.J. (1997). Allelic variation in the mouse Tap-1 and Tap-2 transporter genes. *J. Immunol.* **158**, 5251–5256.
- Monaco, J.J., Cho, S., and Attaya, M. (1990). Transport protein genes in the murine MHC: possible implications for antigen processing. *Science* **250**, 1723–1726.
- Müller, D.J., Sass, H.-J., Müller, S.A., Buldt, G., and Engel, A. (1999). Surface structures of native bacteriorhodopsin depend on the molecular packing arrangement in the membrane. *J. Mol. Biol.* **285**, 1903–1909.
- O'Herrin, S.M., Lebowitz, M.S., Bieler, J.G., al-Ramadi, B.K., Utz, U., Bothwell, A.L., and Schneck, J.P. (1997). Analysis of the expression of peptide-major histocompatibility complexes using high affinity soluble divalent T cell receptor. *J. Exp. Med.* **186**, 1333–1345.
- Ortmann, B., Copeman, J., Lehner, P.J., Sadasivan, B., Herberg, J.A., Grandea, A.G., Riddell, S.R., Tampe, R., Spies, T., Trowsdale, J., and Cresswell, P. (1997). A critical role for tapasin in the assembly and function of multimeric MHC class I-TAP complexes. *Science* **277**, 1306–1309.
- Ozato, K., Hansen, T.H., and Sachs, D.H. (1980). Monoclonal antibodies to mouse MHC antigens. II. Antibodies to the H-2Ld antigen, the product of a third polymorphic locus of the mouse major histocompatibility complex. *J. Immunol.* **125**, 2473–2477.
- Pamer, E., and Cresswell, P. (1998). Mechanisms of MHC class I restricted antigen processing. *Annu. Rev. Immunol.* **16**, 323–358.
- Reddehase, M.J., Rothbard, J.B., and Koszinowsky, U.H. (1989). A pentapeptide as minimal antigenic determinant for MHC class I-restricted T-lymphocytes. *Nature* **337**, 651–653.
- Rock, K.L., Gramm, C., Rothstein, L., Clark, K., Stein, R., Dick, L., Hwang, D., and Goldberg, A.L. (1994). Inhibitors of the proteasome block the degradation of most cell proteins and the generation of peptides presented on MHC class I molecules. *Cell* **78**, 761–771.
- Shiroishi, T., Evans, G., Appella, E., and Ozato, K. (1985). In vitro mutagenesis of a mouse MHC class I gene for the examination of structure-function relationships. *J. Immunol.* **134**, 623–629.
- Silverman, T., Rein, A., Orrison, B., Langloss, J., Bratthauer, G., Miyazaki, J., and Ozato, K. (1988). Establishment of cell lines from somite mouse embryos and expression of major histocompatibility class I genes in these cells. *J. Immunol.* **140**, 4378–4387.
- Solheim, J.C., Harris, M.R., Kindle, C.S., and Hansen, T.H. (1997). Prominence of beta 2-microglobulin, class I heavy chain conformation, and tapasin in the interactions of class I heavy chain with calreticulin and the transporter associated with antigen processing. *J. Immunol* **158**, 2236–2241.
- Suh, W.-K., Cohen-Doyle, M.F., Fruh, K., Wang, K., Peterson, P.A., and Williams, D.B. (1994). Interaction of MHC class I molecules with the transporter associated with antigen processing. *Science* **264**, 1322–1326.
- Suh, W.-K., Mitchell, E.K., Yang, Y., Peterson, P.A., Waneck, G.L., and Williams, D.B. (1996). MHC class I molecules form ternary complexes with calnexin and TAP and undergo peptide-regulated interaction with TAP via their extracellular domains. *J. Exp. Med.* **184**, 337–348.
- Sykulev, Y., Brunmark, A., Jackson, M., Cohen, R.J., Peterson, P.A., and Eisen, H.N. (1994a). Kinetics and affinity of reactions between antigen-specific T cell receptor and peptide-MHC complexes. *Immunity* **1**, 15–22.
- Sykulev, Y., Brunmark, A., Tsomides, T.J., Kageyama, S., Jackson, M., Peterson, P.A., and Eisen, H.N. (1994b). High-affinity reactions between antigen-specific T-cell receptors and peptides associated with allogeneic and syngeneic major histocompatibility complex class I proteins. *Proc. Natl. Acad. Sci. USA* **91**, 11487–11491.
- Szczesna-Skorupa, E., Chen, C.-D., Rogers, S., and Kemper, B. (1998). Mobility of cytochrome P450 in the endoplasmic reticulum membrane. *Proc. Natl. Acad. Sci. USA* **95**, 14793–14798.
- Tatu, U., and Helenius, A. (1997). Interactions between newly-synthesized glycoproteins, calnexin and a network of resident chaperones in the endoplasmic reticulum. *J. Cell Biol.* **136**, 555–565.
- Udaka, K., Tsomides, T.J., and Eisen, H.N. (1992). A naturally occurring peptide recognized by alloreactive CD8+ cytotoxic T lymphocytes in association with a class I MHC protein. *Cell* **69**, 989–998.
- Van Kaer, L., Ashton-Rickardt, P.G., Ploegh, H.L., and Tonegawa, S. (1992). TAP1 mutant mice are deficient in antigen presentation, surface class I molecules and CD4+ T cells. *Cell* **71**, 1205–1214.
- van Leeuwen, J.E., and Kears, K.P. (1996). Deglycosylation of N-linked glycans is an important step in the dissociation of calreticulin-class I-TAP complexes. *Proc. Natl. Acad. Sci. USA* **93**, 13997–134001.
- Velasco, A., Hendricks, L., Moremen, K.W., Tulsiani, D.R., Touster, O., and Farquhar, M.G. (1993). Cell type-dependent variations in the subcellular distribution of alpha-mannosidase I and II. *J. Cell Biol.* **122**, 39–51.
- Yechiel, E., and Edidin, M. (1987). Micrometer scale domains in fibroblast plasma membranes. *J. Cell Biol.* **105**, 755–760.
- Yewdell, J.W., and Bennink, J.R. (1992). Cell biology of antigen processing and presentation to major histocompatibility complex class I molecule-restricted T lymphocytes. *Adv. Immunol.* **52**, 1–123.