Peptide-receptive Major Histocompatibility Complex Class I Molecules Cycle between Endoplasmic Reticulum and cis-Golgi in Wild-type Lymphocytes

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Prior to binding to a high affinity peptide and transporting it to the cell surface, major histocompatibility complex class I molecules are retained inside the cell by retention in the endoplasmic reticulum (ER), recycling through the ER-Golgi intermediate compartment and possibly the cis-Golgi, or both. Using fluorescence microscopy and a novel in vitro COPII (ER-to-cis-Golgi intermediate compartment) vesicle formation assay, we find that in both lymphocytes and fibroblasts that lack the functional transporter associated with antigen presentation, class I molecules exit the ER and reach the cis-Golgi. Intriguingly, in wild-type T1 lymphoma cells, peptide-occupied and peptide-receptive class I molecules are simultaneously exported from ER membranes with similar efficiencies. Our results suggest that binding of high affinity peptide and exit from the ER are not coupled, that the major histocompatibility complex class I quality control compartment extends into the Golgi apparatus under standard conditions, and that peptide loading onto class I molecules may occur in post-ER compartments.

MHC class I molecules enable the immune system to survey the proteome of all nucleated cells and thus to detect viruses and intracellular parasites. Their assembly with antigenic peptides and transport to the cell surface are regulated by a quality control mechanism that is insufficiently understood. The peptides, which are mostly generated by the cytosolic proteasome, are transported into the ER lumen by the ABC transporter, TAP (1), and bind there to class I molecules, which are dimers of a transmembrane heavy chain and the soluble light chain, β2-microglobulin (2). Initially, newly synthesized class I molecules bind mainly the abundant low affinity peptides that do not optimally match the length (8–10 amino acids) and sequence requirements for tight binding to a given heavy chain allele. During this time, these peptide-receptive (immature) class I molecules do not acquire endoglycosidase H (EndoH) resistance, i.e. they are retained in a compartment proximal to the medial Golgi (3). In an optimization process termed “peptide editing,” the low affinity peptides are exchanged for peptides of gradually increasing affinity until, with the binding of a sufficiently high affinity peptide, class I-peptide complexes are transported to the cell surface (4). There, a specific peptide, for example of viral origin, can be detected by the cognate T cell receptor on a cytotoxic T lymphocyte, which then induces the apoptosis of the presenting cell.

Peptide editing and regulated transport together ensure that every class I molecule will travel to the cell surface loaded with a high affinity (low dissociation rate) peptide that remains bound during transit and for some time afterward. The molecular mechanisms of these two processes are not well understood, but control of both editing and transport involves the MHC class I loading complex, an assembly of TAP, the protein-disulfide isomerases ERP57 and PDI (the protein-disulfide isomerase P4Hβ), and the chaperones tapasin and calreticulin, which bind directly to class I (5, 6). Tapasin, on one hand, is required for peptide editing by most (but not all) alleles (4, 7). On the other hand, intracellular retention functions in the absence of tapasin but depends on the other members of the loading complex, except TAP (6, 8–11).

It is unknown how members of the loading complex identify peptide-receptive class I molecules and, once associated with them, how they prevent them from trafficking to the cell surface (12). In general, proteins can be held in the early secretory pathway in two ways (13). Some folded proteins, for example P450 cytochromes (14) and ribophorins (15, 16), as well as many misfolded mutant proteins, do not enter the COPII vesicles that bud from the ER and travel to the ERGIC; as a consequence, such proteins are restricted to the ER. In contrast, other predominantly ER-localized proteins, such as Sec61α (a subunit of the ER protein translocon (17)) and the ts045 mutant of the vesicular stomatitis virus glycoprotein (VSV-G (18)), can also be found in the ERGIC and/or the cis-Golgi, which implies that...
they enter COPII vesicles but are then retrieved from a more distal compartment. Both mechanisms of localization, stringent retention and retrieval, act upon fully folded as well as misfolded proteins, and it is unknown how a given protein is “assigned” to one mechanism. In fact, some localization signals on folded proteins, such as a cytosolic C-terminal -K(X)KXX sequence on transmembrane proteins, support both retention and retrieval (19, 20).

To understand whether retention or retrieval localize peptide-receptive class I molecules to the early secretory pathway, investigators have resorted to using conditions where no peptides are available in the ER lumen, either because the TAP transporter is inactive or because the proteasome is chemically inhibited. In such cells, most class I alleles rapidly bind exogenous peptide added at the time of lysis (21), and they do not become EndoH-resistant. In one such study, the green fluorescent protein (GFP) fusion of H-2Kb did not exit the ER upon proteasome inhibition but remained clustered with the loading complex as judged by fluorescence microscopy (22). Others, in apparent contradiction, have found class I molecules in the ERGIC and/or the cis-Golgi in TAP-deficient cells (23–26) and proposed that peptide-receptive class I molecules cycle between the ER and more distal compartments in a manner similar to Sec61α and VSVG-ts045.

In addition to this unresolved controversy, recent data have raised the question whether these results, obtained from mutant cells and/or overexpressed GFP fusions of class I molecules, indeed represent the behavior of endogenous peptide-receptive class I molecules in wild-type cells. Especially the overexpression of an ER-retained protein may lead to its artificial escape in yeast and mammalian cells (27–30), even though for the GFP fusions of HLA-A2, traffic similar to the wild-type protein has been demonstrated in pulse-chase experiments (31). Indeed, the authors of the VSVG-ts045 study cited above suggest that this mutant protein exits the ER because its high rate of production saturates the ER quality control system. Thus, in studies where class I molecules were overexpressed, or where no peptide was available, ER quality control may have become overwhelmed to allow the nonphysiological exit of peptide-receptive class I molecules from the ER.

Another point of controversy regarding the above studies is whether class I molecules in TAP-deficient cells are indeed biochemically equivalent to immature class I molecules in wild-type cells, i.e., whether they have low affinity peptides bound (32) or not (33). This difference is significant because even low affinity peptides induce a conformational change in the peptide binding groove of class I (34, 35). Such conformational variants can have different fates in the secretory pathway; in a recent study, out of eight disease-related mutants of the V2 vasopressin receptor, three did not leave the ER, but five (which had a different conformation) cycled through the ERGIC and the cis-Golgi prior to degradation (28). Thus, the localization of class I molecules in TAP-deficient cells may follow different pathways and may be governed by different factors compared with peptide-receptive class I molecules in wild-type cells.

We have now investigated the trafficking of endogenous peptide-receptive class I molecules in wild-type cells, both lymphocytes and fibroblasts. In addition to determining their steady-state distribution by fluorescence microscopy, we have assessed their rates of exit from the ER using a novel in vitro COPII vesicle formation assay. We demonstrate that under wild-type conditions, peptide-occupied and peptide-receptive class I molecules leave the ER at the same rate, but the latter are returned from the cis-Golgi. Our results suggest that under wild-type conditions peptide editing and class I exit from the ER are mechanistically independent, and they suggest a role for the ERGIC and the cis-Golgi in MHC class I quality control.

**EXPERIMENTAL PROCEDURES**

**Cells and Transfections**—Chinese hamster ovary (CHO) and TAP-deficient CHO fibroblasts (stably transfected with H-2Db or H-2Kb), kindly provided by K. Gould (London, UK), were grown in 10-cm cell culture dishes in Ham’s F-12 medium supplemented with 5% FCS and PSG (100 units/ml penicillin, 100 μg/ml streptomycin, and 2 mM glutamine). Human T1 and T2 lymphoblastoid cell lines, a kind gift from A. Townsend (Oxford, UK), were maintained in RPMI 1640 medium with 10% FCS and PSG. Vero (African green monkey kidney) cells were grown in 10-cm cell culture dishes in Dulbecco’s modified Eagle’s medium with 10% FCS and PSG. All cell lines were grown at 37°C in an atmosphere of 5% CO2, T1, T2, CHO, TAP-deficient CHO, and Vero cells were transfected by electroporation following Ref. 36. Briefly, cells were washed twice with PBS and transferred into transfection medium (120 mM KCl, 10 mM KH2PO4, 5 mM MgCl2, 0.15 mM CaCl2, 25 mM HEPES, pH 7.2, 2 mM EGTA, 2 mM ATP, and 5 mM oxidized glutathione). About 1–2 million cells were transfected in 400 μl final volume with 20 μg of maxiprep DNA (Qiagen, Hilden, Germany). CHO, TAP-deficient CHO, and Vero cells were transfected at 700 V and 50 microfarads with H-2Kb-GFP and H-2Dβ-GFP constructs. T1 and T2 cells were transfected at 240 V for 40 ms.

**Recombinant DNA Constructs and Expression Plasmids**—The plasmid expression vector pEGFP-N1 (Clontech), which uses the cytomegalovirus immediate early promoter for expression of the cloned insert and G418 resistance for selection of stable transfectants, and its derivative pECFP-N1 were used in all transfection experiments. A cDNA encoding wild-type H-2Kb (H-2-K1) and H-2Db (H-2-D1) was amplified by the PCR with a mutagenic 5’ primer containing a Sall site (H-2Kb, CCC CTG CGA CCA TGG TAC CGT CGA CGC TG; H-2Db, CCC CTG CGA CCA TGG GGG CGA CTA CGT) and a 3’ primer that replaces the stop codon with a BamHI restriction site (H-2Kb, GTG GAT TGC TCT GAG AAT GAG GGT CA, H-2Db, GTG GAT TGC TCT GAG AAT GAG GGT CA). The fragments were inserted in-frame into the polylinker of pEGFP-N1. Both constructs were verified by DNA sequencing. To obtain a Sec22-CFP expression construct, Sec22-GFP (obtained from R. Scheller, Stanford, CA) was subcloned into pECFP-N1. The ECFP fusion of rat p23 (Timed10) was described in Ref. 36. GalT-ECFP was obtained from J. Lippincott-Schwartz (Bethesda). Sec13-EGFP was obtained from D. Stephens (Bristol, UK). CD63-CFP was obtained from P. Luzio (Cambridge, UK), and the CD63 gene was moved into the pECFP-N1 vector.
Antibodies—For immunoblot analysis, rabbit anti-calnexin serum was purchased from Nventa (formerly StressGen Bioreagents, San Diego). For p58 in CHO cells, a serum kindly provided by J. Saraste (Bergen, Norway) was used. Human ERGIC-53 was detected with an antisera that was a generous gift from R. Pettersson (Stockholm, Sweden). Mouse H-2D\textsuperscript{b} was detected with rabbit T18 antisera (kindly provided by T. Elliott, Southampton, UK). Rabbit anti-HLA serum was purchased from Santa Cruz Biotechnology (Heidelberg, Germany). Secondary antibodies conjugated to alkaline phosphatase were from Dianova (Hamburg, Germany). For immunoprecipitation, the mAb W6/32, which recognizes HLA molecules associated with \( \beta_2 \)-microglobulin (37), and the mAb 28.14.85, which recognizes the \( \alpha_2 \) domain of H-2D\textsuperscript{b} (38), were kindly provided by A. Townsend. The mAb a6F antibody against the \( \alpha \)-subunit of the plasma membrane Na\textsuperscript{+}/K\textsuperscript{+} transporter (39) was purchased from the Developmental Studies Hybridoma Bank (University of Iowa). For cell staining, rabbit anti-calnexin serum was kindly provided by D. Williams (Toronto, Canada); rabbit anti-Sec31 serum was obtained from F. Gorelick (Yale University); rabbit anti-Sar1 serum was from Abcam (Cambridge, UK); and ERGIC-53 and mouse p58 were detected with the serum from R. Petersson. The HC10 monoclonal antibody, which binds the cytosolic domain of HLA-B and HLA-C (40), was obtained from A. Townsend. Secondary antibodies labeled with Cy2, Cy3, or Cy5 came from Jackson Immunoresearch (Soham, UK).

Pure Peptides and Proteins—SIINFEKL (from ovalbumin, 257–264; H-2\textsuperscript{D} and H-2\textsuperscript{K}\textsuperscript{b}), ILKepVGhV (from human immunodeficiency virus polymerase, residues 476 – 484, HLA-A*0201), and QPRAPRPI (from Epstein-Barr virus, EBNA 3C protein, 881–889, HLA-B*5101), purified by high pressure liquid chromatography, were from Biosyntan (Berlin, Germany). Hamster SAR1B (both wild-type and T39N mutant) bacterial expression plasmids were obtained from R. Schekman (Berkeley, CA); the proteins were purified according to the published protocol (41).

Buffers—Phosphate-buffered saline (PBS) contained 1.5 mM KH\textsubscript{2}PO\textsubscript{4}, 2.5 mM KCl, 10 mM Na\textsubscript{2}HPO\textsubscript{4}, 130 mM NaCl. TBS (Tris-buffered saline) is 10 mM Tris-Cl, pH 7.5, 150 mM NaCl. Buffer G consisted of 20 mM HEPES-KOH, pH 7.2, 250 mM sorbitol, 150 mM K行事, 0.5 mM Mg(OAc)\textsubscript{2}. Buffer E contained 50 mM HEPES-KOH, pH 7.2, 250 mM sorbitol, 70 mM KO行事, 5 mM potassium EDTA, and 2.5 mM Mg(OAc)\textsubscript{2}.

Mammalian Cytosol—The cytosol preparation was adopted from a published protocol (16). All work was performed at 4 °C. Freshly excised livers from rats or Djungarian hamsters were washed three times with ice-cold PBS and cut into small pieces. One to two ml of Buffer E plus protease inhibitors (PI: 1 mM phenylmethylsulfonyl fluoride, 2 \( \mu \)g/ml aprotinin, 10 \( \mu \)g/ml leupeptin, 5 \( \mu \)g/ml pepstatin A) and 1 mM dithiothreitol were added per g of liver, and the tissue was disrupted using a mortar and pestle. Homogenization was performed by 10 strokes in a Dounce homogenizer with a loose pestle. The crude extract was spun at 25,000 \( \times \) \( g \) for 15 min at 4 °C, and the supernatant was filtered through one layer of gauze. After centrifugation at 186,000 \( \times \) \( g \) for 1 h, the supernatant was transferred to fresh tubes, and the centrifugation was repeated. For the preparation of pig brain cytosol, brains (without meninges and cerebellum) were cut into pieces, washed with PBS, homogenized in 0.6 ml/g Buffer E plus protease inhibitors in a stainless steel blender, and centrifuged consecutively at 1,000 \( \times \) \( g \) (10 min), 25,000 \( \times \) \( g \) (10 min, with subsequent filtration through gauze), and 100,000 \( \times \) \( g \) (1 h). The final supernatant usually had a protein concentration of 15 – 25 \( \mu \)g/ml as determined by the Bradford assay with a bovine serum albumin standard. Cytosol aliquots were frozen in liquid nitrogen and stored at −80 °C.

Preparation of Semi-intact Cells and Microsome-enriched Membranes—To prepare semi-intact cells, 2 million cells were washed three times with 5 ml of ice-cold PBS. Adherent cells (80 – 90% confluent 10-cm dishes) were scraped from the dishes in the third wash. Cells were spun at 800 \( \times \) \( g \) for 5 min at 4 °C. The pellet was resuspended in 200 \( \mu \)l Buffer G, spun at 800 \( \times \) \( g \) for 5 min at 4 °C, and again resuspended in Buffer G + PI. The cell suspension was shock-frozen in liquid nitrogen for 1 min and thawed in a 40 °C water bath. This procedure was carried out three times in total. After the freeze-thaw, semi-intact cells were pelleted at 800 \( \times \) \( g \) for 5 min at 4 °C, washed once in 200 \( \mu \)l of Buffer G + PI, and transferred to siliconized tubes. After another 800 \( \times \) \( g \) spin for 5 min at 4 °C, the final pellet was resuspended in 30 \( \mu \)l of Buffer G + PI, and included in one budding reaction. Microsome-enriched membranes were prepared in Buffer G + PI as described (41).

In Vitro Vesicle Formation Assay for Nonlabeled Proteins (Immunoblot Analysis)—Each reaction contained Buffer G + PI, 30 \( \mu \)l of semi-intact cells, 8 mg/ml of cytosol, 0.2 mM GTP, an ATP-regenerating system (1 mM ATP, 40 mM creatine phosphate, and 0.2 mg of creatine phosphokinase), and 500 nm Sar1 (where indicated), in an 80-ml final volume in siliconized tubes. After a 5-min preincubation on ice, budding was carried out at 25 °C for 30 min and terminated by transferring the tubes on ice. Except for the 100% (total) sample, fractions were spun at 14,000 \( \times \) \( g \) for 20 min at 4 °C to separate vesicles from donor membranes. Vesicles were then sedimented at 100,000 \( \times \) \( g \) at 4 °C for 25 min. The pellets were washed once with 100 \( \mu \)l of Buffer G, and the 100,000 \( \times \) \( g \) spin was repeated. The pellets were resuspended in sample buffer and heated for 5 min at 95 °C. The samples were frozen at −20 °C or directly loaded onto 10% SDS-acrylamide gels. Nonlabeled proteins were transferred to polyvinylidene difluoride membranes and analyzed by immunoblotting with the indicated antibodies.

Radiolabeling of Cells and Immunoprecipitation—for pulse-labeling experiments, cells were washed twice at room temperature with methionine-free RPMI 1640 medium supplemented with 2% FCS and incubated for 1 h at 37 °C. 200 \( \mu \)Ci of \( { }^{35} \)S)methionine per 6 million cells were added, and the cells were incubated for a further 30 min at 37 °C. The labeled cells were washed in ice-cold Buffer G prior to microsome preparation. For pulse-chase experiments, cells were labeled for 15 min as above and then incubated at 37 °C in a complete RPMI 1640 medium supplemented with 2% FCS for indicated periods of time. The cells were harvested, washed in PBS, and lysed in lysis buffer containing 1% Triton X-100 followed by centrifugation for 20 min at 16,000 \( \times \) \( g \). Labeled proteins were then immunoprecipitated with antibodies prebound to protein A-agarose beads. Immune complexes were washed three times with 1%
Triton X-100 in TBS. Endoglycosidase H₄ treatment, where indicated, was done according to the manufacturer's instructions (New England Biolabs, Frankfurt, Germany). Samples were then resuspended in 2× sample buffer, incubated at 95 °C for 5 min, and loaded onto 11% SDS-acrylamide gels. After Coomassie Blue staining and destaining, gels were dried, and autoradiography was performed with a Fuji FLA-3000 imager (Fujifilm, Düsseldorf, Germany).

In Vitro Vesicle Formation Assay for Labeled Proteins—For one budding reaction, ~2 million cells were used. Cells were radiolabeled for 30 min, and radioactive microsomes were incubated with the budding reaction components as described above at 34 °C for 30 min. Vesicles were lysed overnight at 4 °C with TBS containing 1% Triton X-100, 2 mM phenylmethylsulfonyl fluoride, and 5 mM iodoacetamide. Immunoprecipitation was performed as above.

In Vitro Transcription, Translation, and Translocation—Full-length H-2Kd protein was produced in an in vitro transcription/translation system (Clontech) from pTM1 (42) in the presence of [35S]methionine (PerkinElmer Life Sciences) and cotranslationally translocated into microsome-enriched membranes prepared (as described above) from Raji cells. Following translocation, COPII vesicle generation, lysis of vesicles and donor membranes, and immunoprecipitation (using the conformation-specific mAb B22.249 (43)) were carried out as described above.

Immunofluorescence Microscopy and Compartment Markers—Where indicated, cells were transfected with Sec22-CFP, p23-CFP, GalT-CFP, and MHC class I (H-2Kb-GFP or H-2Db-GFP, CHO cells only) constructs 24 h before fixation and staining. T1 and T2 cells were attached to coverslips with L-polylysine for 20 °C and then fixed with 4% paraformaldehyde in PBS for 30 min at room temperature. To arrest trafficking of class I molecules in the ERGIC, cells were incubated for 2 h at 37 °C and then fixed with 4% paraformaldehyde in PBS for 30 min at 15 °C. To accumulate class I molecules in the Golgi apparatus, cells were incubated for 2 h at 20 °C and then fixed with 4% paraformaldehyde in PBS for 30 min at 20 °C. Live cell images were taken at 37 °C, 24 h after transfection, with an Olympus FY1000 confocal microscope, with excitation at 440 nm for CFP, 488 nm for GFP, and 514 nm for yellow fluorescent protein. Fixed cells were observed with a Zeiss LSM 510 confocal microscope.

Calnexin is a transmembrane protein that is localized to the ER at steady state (44). Antibodies against Sec31, a COPII protein, stain ER exit sites (45). The transport receptor p58 (rodents) and its human ortholog ERGIC-53 localize to the ERGIC (46, 47), whereas the CFP fusion of p23, a member of the p24 family of transmembrane proteins, is mostly present in the cis-Golgi at steady state (36). In addition, we used galactosyltransferase (GalT)-CFP for the trans-Golgi (48) and CD63-CFP for lysosomes (49).

Electroporation of Peptide—CHO or Vero cells (2–6 × 10⁶) were washed twice in PBS, transferred to the transfection medium, then transiently transfected at 700 V and 50 microfarads with 20 μg of H-2Db-GFP maxiprep DNA (Qiagen), spotted onto glass microscope slides in Dulbecco's modified Eagle's medium containing 300 nM FAPGNYPAL peptide, incubated for 24 h, and observed.

RESULTS

Peptide-receptive Class I Molecules Accumulate in the cis-Golgi—T1 (TAP-proficient) and T2 (TAP-deficient) cells are derived from a fusion of the B lymphoblastoid cell line 721.174 and the T cell line CEM⁸.3. Both express HLA-B*5101 and A*0201, with T1 expressing additional class I alleles from CEM⁸.3 (50). We first analyzed the intracellular distribution of total class I by treating lysates of T1 and T2 with EndoH followed by SDS-PAGE and Western blotting with anti-HLA antisemur, and then the dynamics of class I transport by pulse-chase analysis (Fig. 1, A and B). In T1 cells, class I molecules acquired EndoH resistance with a half-time of about 30 min, and at steady state, about 50% of class I was EndoH-resistant. In contrast, in T2 cells, no EndoH-resistant class I was detectable at steady state, and very little maturation to EndoH resistance during the 4-h chase occurred. Even though HLA-A*0201 can bind to signal peptides and exit the ER to a small extent in T2 (24, 50, 51), it seems that in our experiments, this fraction was too small to be detected.

We then assessed the steady-state distribution of endogenous HLA-B molecules in T1 and T2 cells by colocalization with known markers in immunofluorescence microscopy, using the monoclonal antibody HC10 that recognizes denatured HLA-B molecules after fixation (52) (Fig. 2). In T2 cells, B*5101 partially colocalized with the ER transmembrane protein, calnexin, and with protein-disulfide isomerase (not shown). In
addition, we saw them in punctate structures that were closely adjacent to, but not congruent with, ER exit sites (detected by the marker protein Sec31) and the ERGIC (ERGIC-53) and that colocalized very well with the cis-Golgi marker, p23, at 37 and 20 °C and relocalized to the ERGIC at 15 °C (Figs. 3 and supplemental Fig. S2). We speculate that this intracellular accumulation represents a pool of peptide-receptive class I molecules in wild-type cells that await their retrieval to the ER.

Peptide-receptive Class I Molecules Enter COP II Vesicles in TAP-deficient Lymphocytes—If peptide-receptive MHC class I molecules can reach post-ER compartments, then it should be possible to detect them in the COP II vesicles that bud from the exit sites of the ER and deliver their cargo to the ERGIC. Because these vesicles cannot be isolated from cells because of their short lifetime, we developed an in vitro vesicle formation assay based on a published procedure (41). T1 and T2 cells were permeabilized by freeze-thaw cycles and incubated with cytosol, GTP, and an ATP-regenerating system. The vesicles were isolated by differential centrifugation, and vesicle-associated proteins were separated by SDS-PAGE and detected by immunoblotting (Fig. 4). Our assay faithfully reconstituted the sorting of cargo proteins because the ER-resident protein, calnexin, was excluded from the vesicles, whereas ERGIC-53 was packaged into the vesicles with high efficiency (about 30% of the amount present in the donor membranes). In agreement with the microscopy data, we found that in both T1 and T2, class I molecules (detected by a pan-class I antiserum) were specifically packaged into COP II vesicles, albeit much less efficiently (around 1%) than ERGIC-53. Class I packaging in T2 was usually slightly better than in T1 (see the figure). Thus, inclusion of class I into COP II vesicles occurs irrespective of peptide loading.

Peptide-receptive Class I Molecules Enter COP II Vesicles in Wild-type Lymphocytes—Next, we wished to directly compare simultaneous COP II packaging of peptide-occupied and peptide-receptive class I molecules in wild-type cells. T1 (and T2 as a control) cells were labeled for 30 min with [35S]methionine, and microsome-enriched membranes were prepared and used for COP II vesicle generation. We lysed the vesicles with detergent and then isolated the class I molecules by immunoprecipitation, treated them with EndoH, cells also showed a faint intracellular accumulation of class I (arrow in Fig. 3), which, just as in T2 cells, colocalized best with the cis-Golgi marker, p23, at 37 and 20 °C and relocalized to the ERGIC at 15 °C (Figs. 3 and supplemental Fig. S2). We speculate that this intracellular accumulation represents a pool of peptide-receptive class I molecules in wild-type cells that await their retrieval to the ER.

FIGURE 2. HLA-B*5101 molecules in T2 cells cycle through the ERGIC and the cis-Golgi. Cells were grown at 37 °C and incubated at 15 °C for 2 h where indicated and stained with mAb HC10. Compartment markers were calnexin (ER), Sec31 (ER exit sites), p58 (ERGIC), p23-CFP (cis-Golgi), and GalT-CFP (trans-Golgi). Representative individual cells are shown. Where two rows are shown for one compartment marker, the lower row images are partial enlargements of the upper row. Bar, 10 μm. Cells grown at 37 °C show an accumulation in the cis-Golgi, whereas an incubation at 15 °C leads to a redistribution of the cycling molecules to the ERGIC.

In T1 cells, most class I molecules were found on the cell surface at 37 °C. Coincident with the biochemical analysis, T1...
and detected them by SDS-PAGE and autoradiography. COPII vesicles from both T1 and T2 cells contained class I molecules, and their inclusion into vesicles depended on cytosol and ATP, just as in the previous experiments (Fig. 5A, lane 1). Packaging of class I was increased by addition of the small GTPase Sar1 (which drives COPII vesicle formation (56); data not shown) and inhibited by the dominant negative GDP-restricted mutant of Sar1, Sar1(T39N) (57) (Fig. 5A, lane 7), which shows that protein recruitment into the vesicles was COPII-dependent. The packaging efficiency for total class I was up to 30%, significantly higher than with the Western blotting assay; this may reflect a higher export efficiency for freshly synthesized class I molecules, or it may be due to the differences in the membrane preparation protocols in the two assays.

To detect peptide-receptive class I molecules, we next incubated the detergent lysates of the vesicles overnight in the presence or absence of specific high affinity peptides (ILKEPVGHV for A*0201 and QPRAPIRPI for B*5101). During overnight incubation, peptide-receptive class I molecules dissociate into heavy chain and \( \beta_2 \)-microglobulin unless peptide is added to stabilize them (21). By immunoprecipitation with the \( \beta_2 \)-microglobulin-dependent monoclonal antibody W6/32, we then isolated all \( \beta_2 \)-microglobulin/peptide-associated heavy chains. Fig. 5A shows that in T2 cells, there was a striking increase of the class I signal when specific peptide was added (compare lanes 2–4 to lane 1), whereas the signal for the control protein, plasma membrane Na\(^+\)/K\(^+\)-ATPase, did not change. The amount of the increase thus corresponds to the peptide-receptive class molecules that were present in the COPII vesicles. Intriguingly, in T1 cells, there was also a significant signal increase upon peptide addition (Fig. 5B). For both cell lines, the A*0201 peptide caused a stronger increase than the B*5101 peptide, suggesting that more peptide-receptive A*0201 molecules were present in the donor membranes.

Interestingly, when we related the packaging efficiency of peptide-occupied (determined by immunoprecipitation without peptide) and total (determined by addition of peptide upon lysis) class I to the amount present in the donor membranes, we found that both populations were packaged with very similar efficiencies (Fig. 5C). We thus conclude that in wild-type lymphocytes, peptide-receptive and pep-
ER-Golgi Cycling of Empty MHC Class I Molecules

A

| Lane | Vessel formation | Fraction | Peptides added at lysis | Vesicles | 25% of total | Sarl T39N | Sarl T39N ves. |
|------|------------------|----------|------------------------|----------|-------------|-----------|--------------|
| 1    | complete reaction|          | no cyto                |          |             |           |              |
| 2    | complete reaction|          | no cyto                |          |             |           |              |
| 3    | complete reaction|          | no cyto                |          |             | no ATP    |              |
| 4    | complete reaction|          | no cyto                |          |             | T39N      |              |
| 5    | complete reaction|          | no cyto                |          |             | T39N      |              |
| 6    | complete reaction|          | no cyto                |          |             | T39N      |              |
| 7    | complete reaction|          | no cyto                |          |             | T39N      |              |
| 8    | complete reaction|          | no cyto                |          |             | T39N      |              |
| 9    | complete reaction|          | no cyto                |          |             | T39N      |              |
| 10   | complete reaction|          | no cyto                |          |             | T39N      |              |

B

FIGURE 5. Peptide-receptive and peptide-occupied HLA-A*0201 and HLA-B*5101 molecules are packaged into COPII vesicles with similar efficiencies in wild-type lymphocytes. A, in vitro COPII vesicle formation assay from microsomes of radiolabeled T1 and T2 cells. Peptides specific for HLA-A*0201 (A2) and HLA-B*5101 (BS) were added upon lysis of the COPII vesicles and total donor membranes where indicated. MHC class I molecules (mAb W6/32) and the α-subunit of Na+/K+ ATPase (mAb α6F) in the vesicle fractions were detected by immunoprecipitation. In lanes without added peptide, only class I bound to endogenous peptide is precipitated. The 25% total membranes control was taken from a reaction that was not centrifuged. No cyto = no cytosol. The experiment shown is representative for five independent repeats. B, quantification of data in A. Recovery of proteins relative to complete reaction without peptide added at lysis (100%). C, quantification of data in A. Packaging efficiency of peptide-occupied forms only (from samples without peptide added at lysis) and all forms of class I (from samples with both A2 and BS peptides added at lysis) in T1 (filled bars) and T2 cells (open bars) relative to the total membrane controls.

tide-associated class I molecules simultaneously leave the ER in COPII vesicles.

To more stringently define the origin of the isolated vesicles, we modified a published in vitro transcription-translation assay (58). The murine class I molecule, H-2Kb, was produced in a reticulocyte lysate by coupled transcription, translation, and translocation in the presence of microsome-enriched membranes. Translocation of H-2Kb was assessed by protease protection assay, EndoH digest, and concanavalin A precipitation (Fig. 6A). We performed our in vitro vesicle formation assay with membranes that contained freshly translocated H-2Kb, lysed the vesicles in the presence or absence of added peptide, incubated overnight, and precipitated with the peptide-dependent monoclonal antibody Y3 (Fig. 6B). Both peptide-receptive and peptide-bound H-2Kb were taken up into COPII vesicles with very high efficiency (between 30 and 60% in different experiments).

High Affinity Peptide Allows Exit from the ER-Golgi Cycle and Surface Transport of Class I in Fibroblasts—We hypothesized that class I molecules might be induced to leave their ER-Golgi cycle upon binding of high affinity peptides. To investigate this, we used wild-type Chinese hamster ovary (CHO) fibroblasts (59). In these cells, only a very small fraction of the stably expressed mouse class I allele H-2Db is EndoH-resistant at steady state (Fig. 7A, arrow). In a CHO cell line that is deficient for TAP2 function (TAP2d CHO, derived by Shastri and co-workers (60)), there was no EndoH-resistant H-2Db at steady state. In both CHO and TAP2d CHO cells, transiently transfected H-2Db-GFP was almost exclusively localized to the ER, but small accumulations outside the ER were visible especially with the 15 and 20 °C temperature blocks (supplemental Figs. S3–S5), indicating that class I molecules did indeed cycle between ER and Golgi. Remarkably, colocalization of Dα-GFP with p23 was consistently stronger in TAP-deficient than in wild-type CHO cells, possibly indicating a greater abundance of recycling class I molecules in the former. Identical results were obtained with H-2Kb-GFP in both cell lines (not shown). Our in vitro vesicle formation essay showed very efficient export of H-2Db from the ER in both cell lines (Fig. 7B), which suggests that fast retrieval from the cis-Golgi may be the reason for the low steady-state concentration of class I in the cis-Golgi in these cells.

To assess the effect of peptide on class I cycling, we next electroporated these CHO cells with a plasmid that carries the gene for H-2Db-GFP and immediately thereafter added the Dα-specific peptide, FAPGNYPAL, to the medium. Strikingly, a large population of H-2Db-GFP was almost exclusively localized to the ER, but small accumulations outside the ER were visible especially with the 15 and 20 °C temperature blocks (supplemental Figs. S3–S5), indicating that class I molecules did indeed cycle between ER and Golgi. Remarkably, colocalization of Dα-GFP with p23 was consistently stronger in TAP-deficient than in wild-type CHO cells, possibly indicating a greater abundance of recycling class I molecules in the former. Identical results were obtained with H-2Kb-GFP in both cell lines (not shown).
allow them to proceed to the cell surface. A model that summarizes our findings is shown in Fig. 9.

**DISCUSSION**

Using a novel *in vitro* vesicle formation assay, we have made the surprising observation that peptide-receptive and peptide-occupied endogenous HLA-A*0201 and B*5101 enter COPII vesicles and leave the ER in wild-type T1 lymphocytes with approximately the same packaging efficiencies. In support of the conclusion that the peptide-receptive molecules cycle between ER and Golgi, we show by microscopy that in the TAP-deficient cell line T2, a large population of class I molecules accumulates at the cis-Golgi and that they relocalize to the ERGIC when a 15 °C block is applied. Intriguingly, a similar accumulation (albeit to a smaller extent) is visible in T1 cells, and we propose that these molecules represent the recycling pool of peptide-receptive class I in wild-type cells that we have observed in the vesicle formation assay.

To confirm that class I molecules can exit from their cycle between ER and Golgi upon binding of high affinity peptides, we have investigated murine class I molecules in CHO cells. In these cells, whether TAP deficient or not, GFP fusions of H-2Db and H-2Kb cycle between ER and cis-Golgi in the same manner as in T2 cells. Indeed, we find that electroporation of peptide relieves their intracellular retention and leads to surface expression in a TAP-dependent and allele-specific manner.
ER-Golgi Cycling of Empty MHC Class I Molecules

An interesting question is whether the peptide-receptive molecules that cycle between ER and Golgi are bound to low affinity peptides or whether they are devoid of peptides altogether. Because they efficiently express GFP fusions of hamster class I molecules at the cell surface (59), we suggest that wild-type CHO cells can generate high affinity peptides and transport them into the ER but cannot load them onto Db-GFP and Kb-GFP. This may be due to an incompatibility of the hamster tapasin with the murine class I molecules, which probably retain low affinity peptides in their binding groove, like in tapasin-deficient cells, and in this state enter COP II vesicles (Fig. 7). In conditions where high affinity peptide loading is more efficient, such as for endogenous class I molecules in T1 cells, the fraction of cycling molecules is smaller, but they may likewise be bound to low affinity peptides and not be completely empty, because the concentration of low affinity peptides in the ER probably exceeds that of high affinity peptides by a large factor (61). In addition, the fact that we can detect cis-Golgi accumulations of class I in both T1 and T2 cells suggests that the trafficking of peptide-receptive endogenous class I molecules in TAP-proficient and in TAP-deficient cells underlies the same principles, even if it is not entirely clear whether class I molecules in the absence of TAP contain low affinity peptides or no peptides at all (32, 33).

We demonstrate here that the export of peptide-receptive class I molecules from the ER is not an artifact of the overexpression or GFP fusion of class I nor of the saturation of class I-specific retention mechanisms in TAP-deficient cells. Our findings are supported by earlier studies that have found class I molecules in the ERGIC or Golgi apparatus of TAP-deficient cells (23–26). Thus, it appears that peptide-receptive class I molecules belong to the group of mammalian membrane pro-

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protein determine whether it will be stringently retained in the ER or cycle between the ER and more distal compartments. In a recent study, five disease-related mutants of the V2 vasopressin receptor were shown to cycle through the ERGIC, whereas three were stringently retained in the ER (28). Likewise, the N153D mutant of the tissue nonspecific alkaline phosphatase accumulates in the cis-Golgi, whereas the R54C mutant cannot leave the ER; the latter has a shorter half-life (about 1 h) than the ERGIC-localized variant (3 h) (74). If the degradation of immature proteins, which often involves membrane extraction, cytosolic ubiquitination, and destruction by the proteasome (75), takes place only in the ER but not in the cis-Golgi, then the intermittent escape of a protein out of the ER would extend its lifetime. Indeed, peptide-receptive class I molecules in TAP-deficient cells are quite stable (Fig. 1) (76), and keeping some of them ready for peptide binding but protected from degradation may allow a rapid response to changes in the proteome without protein synthesis, which is often targeted by an invading virus.

An additional consequence of class I cycling between ER and Golgi may be a periodical change of the chemical environment of class I. It has been suggested that binding of high affinity peptide is accompanied by opening and closing of disulfide bonds either within class I (6, 77–79) or between ERp57 and tapasin in the loading complex (12). Because Ero1, the source of disulfide oxidizing equivalents, is restricted to the ER, conditions in the Golgi may be more reducing (80, 81), and this could induce class I molecules to release their low affinity peptides prior to their return to the ER. Further work is needed to test these hypotheses.

It is important to state that the exact course of events in protein traffic between ER and Golgi in mammalian cells is still a matter of dispute (55, 82, 83). Especially, it is unclear whether ERGIC elements progress to the Golgi region to form a new cis-Golgi cisterna, or whether they remain stationary but emit anterograde carrier vesicles that transport cargo to the Golgi apparatus. If the latter is true, then it is possible that we have isolated anterograde carriers in our vesicle formation assay instead of, or in addition to, COPII vesicles. Likewise, we cannot exclude that peptide-occupied and peptide-receptive class I molecules are packaged into different vesicle species in our assays. However, these possibilities do not influence the principal conclusions from our experiments.

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