The transporter associated with antigen processing (TAP) is active in a post-ER compartment

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Summary

The translocation of cytosolic peptides into the lumen of the endoplasmic reticulum (ER) is a crucial step in the presentation of intracellular antigen to T cells by major histocompatibility complex (MHC) class I molecules. It is mediated by the transporter associated with antigen processing (TAP) protein, which binds to peptide-receptive MHC class I molecules to form the MHC class I peptide-loading complex (PLC). We investigated whether TAP is present and active in compartments downstream of the ER. By fluorescence microscopy, we found that TAP is localized to the ERGIC (ER-Golgi intermediate compartment) and the Golgi of both fibroblasts and lymphocytes. Using an in vitro vesicle formation assay, we show that COPII vesicles, which carry secretory cargo out of the ER, contain functional TAP that is associated with MHC class I molecules. Together with our previous work on post-ER localization of peptide-receptive class I molecules, our results suggest that loading of peptides onto class I molecules in the context of the peptide-loading complex can occur outside the ER.

Key words: ABC transporter, Antigen presentation, Endoplasmic reticulum to Golgi transport

Introduction

Major histocompatibility complex (MHC) class I molecules present intracellular peptides to cytotoxic T cells. The peptides are generated in the cytosol and transported into the lumen of the ER by TAP, the transporter associated with antigen processing. TAP is bound to class I molecules via the TAP-associated protein tapasin (Ortmann et al., 1997; Sadasivan et al., 1996). Class I, TAP and tapasin associate with the protein disulfide isomerase ERP57 and the lectin chaperone calreticulin to form the peptide loading complex (PLC), which is important in the retention of peptide-empty class I molecules in the early secretory pathway, and in the binding of high-affinity peptides (Cabrera, 2007; Elliott and Williams, 2005; Howe et al., 2009; Koch and Tampe, 2006; Wearsch and Cresswell, 2008; Wright et al., 2004).

TAP belongs to the superfamily of ABC (ATP-binding cassette) transporters. It consists of two homologous subunits, TAP1/ABCB2 and TAP2/ABCB3. Each subunit comprises a hydrophobic transmembrane domain (with ten and nine transmembrane helices, respectively) and a hydrophilic conserved cytoplasmic nucleotide binding domain (Beisemann-Driemeyer and Tampe, 2004; Koch et al., 2004; Schrodt et al., 2006).

The function of TAP in the ER has been studied extensively, but there is little information about its activity in post-ER compartments. Importantly however, peptide-receptive class I molecules (i.e. class I molecules without or with low-affinity peptides) can exit the ER freely and move to the Golgi complex, usually to be returned from there to the ER (Garstka et al., 2007; Howe et al., 2009; Hsu et al., 1991). Indeed, class I molecules in the cis-Golgi can be stained with fluorescent peptides, indicating that they are peptide receptive (Day et al., 1995). Thus, if TAP were to exit the ER – perhaps bound to class I and/or the PLC – and travel to the Golgi, then class I molecules could be loaded with peptide in post-ER compartments.

In the literature, different methods have so far resulted in differing evidence on the localization of TAP. Initially, TAP was detected mainly in the ER by immuno-electron microscopy, with a few gold particles visible in Golgi-like membranes (Kleijmeer et al., 1992). Most subsequent investigations report an ER localization of antibody-stained stained or GFP-tagged TAP subunits by fluorescence microscopy (Kobayashi et al., 2004; Reits et al., 2000; Ritz et al., 2003; Russ et al., 1995). By contrast, two biochemical studies that use cell fractionation and immunoprecipitation have detected small amounts of TAP in post-ER membranes (Bresnahan et al., 1997; Paulsson et al., 2002).

In mammalian cells, there are few examples of steady-state ER proteins that are stringently retained in the ER (Fu and Kreibich, 2000). Instead, many ER proteins seem to cycle through the Golgi from where they are retrieved by: (1) a dilysine motif that binds to the coat of COPII retrograde vesicles, (2) a KDEL motif that binds to the KDEL receptor, or (3) an unknown provision. This is in stark contrast to the yeast Saccharomyces cerevisiae, where many ER proteins never enter the COPII vesicles that bud from the ER and...
deliver proteins to the ERGIC. For example, the protein translocon Sec61p in yeast is stringently excluded from COPII vesicles and post-ER compartments in yeast (Bednarek et al., 1995), whereas its mammalian homolog, Sec61α, can access the ERGIC (Greenfield and High, 1999). Interestingly, TAP lacks any known COPII packaging, ER retention or Golgi-to-ER retrieval signal.

Recently, we have developed a novel method to study export rates of proteins from the ER by generating COPII (coat protein complex II) ER–Golgi transport vesicles in vitro and analyzing their contents (Baker et al., 1988; Garstka et al., 2007; Nohturfft et al., 1999). In this work, we have used this vesicle generation system, together with fluorescence microscopy, to investigate whether TAP accompanies peptide-receptive MHC class I molecules to the cis-Golgi, and we have examined for the first time whether peptide transport and class I association can take place outside the ER by using a novel peptide-loading assay. Our results suggest that functional TAP exits the ER in COPII vesicles in association with MHC class I, and that peptide translocation by TAP and binding to class I can occur in post-ER compartments.

**Results**

**TAP is packaged into COPII vesicles and accumulates in the ERGIC and the cis-Golgi**

To investigate the location of the TAP complex, we first expressed in COS cells a fusion of the green fluorescent protein (GFP) to the C terminus of human TAP1 (TAP1–GFP, Fig. 1A). In addition to the strong reticular ER signal, which was seen in all cells, TAP1–GFP fluorescence accumulated close to the nucleus in 60% of the cells (arrow). This accumulation varied in its dimensions between cells, possibly mirroring the variability of organelle structures in COS cells. When we examined the accumulation of TAP in confocal serial sections, we found it very close to the nucleus, in a structure that was reminiscent of the ERGIC or the Golgi complex (Fig. 1B). The signal was clearly different from the distribution of the ER marker protein disulfide isomerase (PDI), which never showed such accumulation, indicating that TAP1–GFP had access to a post-ER compartment (Fig. 1C).

We next asked whether overexpression of the protein or the fused GFP domain were influencing the localization of the TAP complex. COS cells were transfected with TAP1–GFP and stained with the monoclonal antibody 429.3 against TAP2 (Fig. 1D, top and center panel). GFP and antibody stain were very similar in transfected cells, suggesting that TAP1–GFP was associated with endogenous TAP2. The antibody stain showed very similar accumulations in transfected and untransfected cells, which suggests that the TAP complex was not redistributed upon TAP1–GFP expression. In a three-color experiment, the distributions of TAP1–GFP, total TAP1 (stained with the antiserum R.RING4c) and TAP2 were also very similar (Fig. 1D, bottom panel). Since in some images, staining of TAP1–GFP outside TAP2-staining areas was visible, we considered the possibility that TAP1–GFP was stable without association to TAP2 [as described for TAP1 (Leonhardt et al., 2005)] and that as a result of overexpression, TAP1–GFP might reach post-ER compartments on its own. We therefore studied the localization of TAP1–GFP in COS cells as a function of its expression by comparing its intracellular localization in a population of cells with the lowest distinguishable expression level versus a population of strongly expressing cells (supplementary material Fig. S1). We found that the percentage of cells that showed post-ER accumulation was the same in both groups, suggesting that in our experiments, there were no localization effects that depended on the expression level of TAP1–GFP.

We next investigated whether the TAP transporter leaves the ER in COPII vesicles, the standard mode of transport between the ER and the Golgi complex. When COS cells were transiently transfected with the dominant-negative inhibitor of COPII vesicular transport, Sar1(T39N) (Ward et al., 2001), followed by TAP1–GFP, the percentage of cells that accumulated TAP1 dropped from 60% to 30% (Fig. 1E).

To directly demonstrate COPII-dependent exit of TAP from the ER, we generated COPII vesicles using an in vitro reaction from microsomal membranes (Garstka et al., 2007). In this assay, microsome-enriched membranes are incubated with cytosol, Sar1 (the GTPase that drives COPII vesicle formation), and a source of energy, which leads to the formation of COPII vesicles; after sedimenting the donor membranes, the vesicles are harvested from the supernatant by high-speed centrifugation and analyzed for their content by western blotting. We used donor membranes from the lymphocyte cell line 721.220 stably transfected with tapasin; in a control reaction, COPII vesicle generation was suppressed by adding the dominant inhibitor, Sar1(T39N) protein (Fig. 1F). We found that the ERGIC protein ERGIC-53 was specifically packaged into the COPII vesicles, whereas packaging of the ER chaperone, calnexin, was very low, suggesting that the assay faithfully reconstitutes the cargo selection process occurring at ER exit sites in vivo (Lee and Miller, 2007). In the same reaction, we also found endogenous TAP1 in the COPII vesicles, even though its budding efficiency (i.e. the percentage of the protein packaged into the vesicles) was significantly lower than that of ERGIC-53. Taken together, these data demonstrate that TAP can leave ER membranes in COPII vesicles, but that it is not recognized for efficient export by the sorting machinery.

ER export and recycling from the Golgi complex is a common feature for proteins that reside predominantly in the ER at steady state (Pelham, 1991). This also applies to peptide-receptive class I molecules, which bind to TAP via tapasin; they travel to the cis-Golgi and return from there to the ER (Garstka et al., 2007; Howe et al., 2009; Hsu et al., 1991). To find out which post-ER compartments the TAP transporter is able to access, we first performed colocalization experiments in COS cells. The extra-ER accumulation of TAP1–GFP always colocalized well with the ERGIC marker, ERGIC-53 (Fig. 2A). The TAP1 signal was usually broader than that of the Golgi marker GM130, and their overlap was less pronounced than that of TAP1 with ERGIC-53, even though the signals were close. When TAP1–GFP, ERGIC-53 and GM130 were simultaneously imaged in a three-color experiment, it was found that the extra-ER accumulation of TAP1 showed a distribution similar to both compartment markers but resembled that of ERGIC-53 more closely. In addition, there were individual Golgi elements that did not show TAP1 localization at all (Fig. 2A, bottom panel, arrow), suggesting that the access of TAP to the Golgi is limited.

To investigate more thoroughly whether TAP has access to the Golgi, or is instead returned to the ER from the ERGIC, we applied low-temperature blocks to vesicular traffic at different stages of the early secretory pathway, and imaged the two TAP subunits with different compartment markers. At 15°C, where exit from the ERGIC is inhibited (Lippincott-Schwartz et al., 1990), both transfected TAP1–GFP and endogenous TAP2 accumulated together with ERGIC-53, as previously (Fig. 2B). When exit from the Golgi was blocked at 20°C to force accumulation of cycling proteins (Matlin and Simons, 1983), both now colocalized fairly well with the Golgi marker, GM130 (Fig. 2B). We conclude that the TAP
complex has access to both the ERGIC and – perhaps to a lesser extent – to the cis-Golgi.

**TAP colocalizes with class I molecules outside the ER**

Since peptide-receptive class I molecules can leave the ER and accumulate in the ER and the cis-Golgi, we asked whether TAP and class I were colocalized in such structures. COS Cells that were stained with the ERGIC marker, ERGIC-53, or the cis-Golgi marker, p27, showed good colocalization with the accumulation signal for HC10, an antibody that recognizes all forms of the B allotypes of MHC class I; the remainder of class I staining was at the plasma membrane and/or the ER (Fig. 3A). When the same staining experiment was performed in TAP1–GFP transfected cells with 15°C or 20°C temperature blocks to enhance the accumulation

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**Fig. 1. TAP1 leaves the ER in COPII vesicles and accumulates in a post-ER compartment.**

(A) Confocal fluorescence micrographs of COS cells transfected with TAP1–GFP. The percentages of cells with and without visible TAP accumulation are shown (n=150). TAP was considered accumulated if a concentrated signal was visible next to the nucleus (arrow). All other phenotypes, for example even distributions throughout the ER as seen in the two cells of the right-hand panel, were considered not accumulated. More cells, and the independence of the degree of accumulation on the expression levels of TAP1–GFP, are shown in supplementary material Fig. S1. (B) Confocal serial sections (plane thickness, 1 μm) of a COS cell transfected with TAP1–GFP. The juxtanuclear character of the accumulation is visible. (C) TAP1–GFP distribution is different from that of the ER marker, protein disulfide isomerase (PDI). Confocal images of a COS cells transfected with TAP1–GFP and stained for PDI. (D) TAP1–GFP expression does not alter the localization of the TAP complex. Confocal images of COS cells transfected with TAP1–GFP and stained with anti-TAP2 antibody 429.3, the anti-TAP1 antiseraum R.RING4c and the early endosomal marker EEA1, as indicated. The first three rows show colocalization plots with Manders colocalization coefficients; in the top row, the colocalization plot encompasses the two cells transfected with TAP1–GFP, and the colocalizing pixels of the highest intensity are highlighted in a copy of the original image (on the far right). (E) TAP accumulation depends on COPII-mediated ER exit. COS cells were co-transfected with the dominant inhibitory Sar1(T39N) and mRFP (as a transfection marker) and, after 12 hours, either fixed and stained with anti-TAP2 antibody, 429.3 (right column, ‘untransfected’), or retransfected with TAP1–GFP and fixed after 8 hours (left and center columns). The chart shows the percentage of untransfected cells (–) and cells expressing Sar1(T39N) (detected by the mRFP signal) that showed TAP1-GFP accumulation in cells, with the s.e.m. from three experiments (for each of which, n=30). (F) Endogenous TAP1 enters COPII vesicles. Lysates of the COPII vesicle fraction from an in vitro reaction with microsomal membranes from human lymphocytes (721.220 + tapasin) were analyzed for calnexin, ERGIC-53 and TAP1 by western blotting. Controls include addition of the dominant inhibitory Sar1(T39N) protein, 4°C incubation of the reaction, and reactions performed without COPII components or membranes, as indicated. For the diagram, protein bands from four independent experiments done on different days were quantified, and the amount in the Sar1(T39N) lane (non-COPII vesicles and fragmentation) was subtracted from the amount in the complete reaction. TAP1 and calnexin values were then standardized to the amount of ERGIC-53 in the vesicles (=100). The amount of TAP1 is significantly greater than that of calnexin (**P<0.01). Scale bars: 10 μm.
of the proteins in the ERGIC and the cis-Golgi, respectively, we found good colocalization between class I, TAP and ERGIC-53, suggesting that the TAP complex and class I are found together in the ERGIC (Fig. 3B).

While the localization of class I in COS cells was not influenced by the expression of exogenous TAP1–GFP (double arrow in Fig. 3B), we asked, as a control experiment, whether this was also true in human cells. We therefore stained HeLa fibroblasts with the antibodies HC10 and W6/32 (specific for peptide-bound HLA molecules; supplementary material Figs S5, S6). While the HC10 and W6/32 stains did not overlap completely, both showed – in addition to the extensive ER stain common to all cells–juxtanuclear accumulations in the majority of cells both at 37°C and 20°C, which were not significantly influenced by exogenous TAP1–GFP, whatever the expression level (arrows in supplementary material Fig. S6A,B). In these cells, the W6/32 stain shared the pronounced signal for the nuclear envelope that was seen with TAP (supplementary material Fig. S6C).

### TAP1 partially colocalizes with Bap31

The Bap31 protein is supportive but not obligatory for the export of peptide-bound class I molecules to the cell surface (Abe et al., 2009; Ladasky et al., 2006; Paquet et al., 2004; Spiliotis et al., 2000). To investigate whether it is present in the post-ER accumulations of class I and TAP, we stained TAP1–GFP-transfected HeLa cells with W6/32 and an anti-Bap31 antiserum (supplementary material Fig. S6). While Bap31 showed clear post-ER accumulation (arrows in supplementary material Fig. S6) as
found previously by other groups (Annaert et al., 1997; Paquet et al., 2004; Wakana et al., 2008), it colocalized better with W6/32-reactive class I than with TAP, lacking especially the characteristic nuclear envelope stain of the latter (supplementary material Fig. S6C). Similarly to that of class I, the intracellular distribution of Bap31 was not influenced by exogenous TAP1–GFP. Thus, Bap31 is probably available in those intracellular locations where TAP and class I are also present.

**Endogenous TAP in lymphocytes accumulates in a post-ER compartment**
To confirm that the extra-ER accumulation of TAP was not only occurring in fibroblasts, we decided to investigate endogenous TAP in lymphocytes by microscopy. Both Raji and Jurkat cells express large amounts of endogenous TAP (Gorbulev et al., 2001). In a field of Raji cells stained with a TAP1 antibody, about 20% showed a clear accumulation (Fig. 4A, arrows). The fraction of cells accumulating TAP is probably considerably higher, because in a microscopy experiment, spherical cells must become immobilized in a particular orientation for the extra-ER accumulation to become visible (Garstka et al., 2007). We next stained Jurkat cells with antibodies against TAP1 and TAP2. Again, we observed a strong ER signal, most likely representing mainly the nuclear envelope, but also extra-ER accumulations in many cells; at high resolution, they were observed to underlie the nucleus, as seen frequently for ERGIC and Golgi compartments (Saraste et al., 2009), and they did not colocalize with the ER marker, PDI (Fig. 4B, third row). The stains for TAP1 and for TAP2 were nearly identical. At 15°C, this accumulation of endogenous TAP1 or transfected TAP1–GFP colocalized with the ERGIC, and at 20°C, an overlap (but, like in the fibroblasts, again no complete colocalization) with GM130 was seen (Fig. 4B). The results suggest that in lymphocytes as well as in fibroblasts, endogenous TAP can leave the ER and traffic to the ERGIC and the cis-Golgi.

**TAP is associated with class I in COPII vesicles**
In the ER, TAP is associated with peptide-receptive class I molecules as part of the PLC. To see whether this association remained intact in post-ER compartments, we generated COPII vesicles from microsomal membranes as described for Fig. 1F, lysed them with detergent, immunoprecipitated TAP with the antiseraum R.RING4c and tested for specific interaction of the TAP complex with MHC class I by immunoblotting with the monoclonal antibody HC10. We found that class I co-precipitated with TAP

![Fig. 4. Endogenous TAP leaves the ER in lymphocytes in complex with class I.](image)
that was precipitated from the vesicle lysates (Fig. 4C, lane 4). This result indicates that the complex of TAP and peptide-receptive class I persists upon exit from the ER.

**TAP is active in the Golgi**

Finally, we wanted to determine within which post-ER compartments the TAP complex was enzymatically active, so we developed a novel assay that allowed us to study TAP activity at the single-cell level in spatial resolution. We made use of our previous observation that in monkey cells, murine class I molecules are inefficiently loaded with peptides, and that they populate mainly ER, ERGIC and cis-Golgi, with only a minority progressing to the cell surface (Garstka et al., 2007). We hypothesized that these class I molecules would efficiently trap fluorescently labeled peptides that would become translocated into any of these compartments during a classic TAP activity assay with permeabilized cells (van Hall et al., 2007), and that this would enable us to directly view the compartments with active TAP under the fluorescence microscope. For this, we decided to use scKb-GFP, a single chain variant of H-2Kb-GFP, in which the C-terminus of β2m was linked to the N-terminus of the heavy chain to ensure β2m remains bound to the class I heavy chain as a stable complex even in the absence of peptide. Thus, COS cells were transfected with scKb-GFP, and the plasma membrane was permeabilized with saponin, which leaves internal membranes intact (Wassler et al., 1987). The cells were then incubated with ATP and the H-2Kb binding SIINFEKL peptide that was labeled with the fluorescent dye TAMRA, and green and red fluorescence were observed (Fig. 5). We saw binding of SIINFEKL only to those cells that also showed a signal for H-2Kb, demonstrating that the peptide specifically bound to the transfected class I molecule. In unpermeabilized cells, a surface stain of SIINFEKL was visible, which indicates the presence of some peptide-receptive class I molecules at the cell surface. When the cells were permeabilized with saponin, there was a striking colocalization of the SIINFEKL stain with the accumulation of H-2Kb, suggesting that the peptide had access to the ERGIC and/or the cis-Golgi. This ERGIC or Golgi stain of SIINFEKL was abolished at low temperatures and in the absence of ATP, suggesting that it originated from an energy-dependent process, not from random permeabilization. We then performed analogous experiments with a low concentration of saponin (0.005%), where not all cells became permeabilized, and added an antibody against the Golgi matrix protein GM130 to the cells to stain the Golgi of the permeabilized cells only. It again became evident that only permeabilized cells showed an accumulation of the SIINFEKL stain, and that it coincided with the Golgi (supplementary material Fig. S2). Addition of the soluble viral TAP inhibitor ICP47 (Ahn et al., 1996; Neumann et al., 1997; Tomazin et al., 1996) abolished SIINFEKL accumulation, demonstrating that it is due to TAP-mediated transport. It is extremely unlikely that the accumulated SIINFEKL–TAMRA/H-2Kb complex originated in the ER and become transported to the Golgi during the 1 minute incubation with peptide, because ER–Golgi transport of proteins in permeabilized cells requires cytosol (Beckers et al., 1987), which was not present in our assay. A similar microscopic translocation assay, which was performed with the same fluorescent peptide but with RMA lymphocytes (in which endogenous H-2Kb molecules served to trap the peptides) yielded the same results (supplementary material Fig. S3). We therefore conclude that unmodified endogenous TAP can transport peptides into ERGIC and Golgi membranes, which are loaded onto class I molecules present in these organelles.

**Discussion**

ABC transporters are found at all stations of the secretory pathway in yeast and mammals (Higgins, 2001). For example, the closest relative of the TAP complex in humans TAP-L/ABCB9, translocates unfolded polypeptides into lysosomes (Demirel et al., 2010; Wolters et al., 2005). In the work reported here, we now show that TAP itself can leave the ER and function in peptide loading in post-ER compartments. ABC transporters are found at all stations of the secretory pathway in yeast and mammals (Higgins, 2001). For example, the closest relative of the TAP complex in humans TAP-L/ABCB9, translocates unfolded polypeptides into lysosomes (Demirel et al., 2010; Wolters et al., 2005). In the work reported here, we now show that TAP itself can leave the ER and function in peptide loading in post-ER compartments.

Our results are in agreement with the first published localization of TAP. By immunogold labeling, Trowsdale and collaborators detected endogenous TAP in the ER and the ERGIC (labeled by the marker ERGIC-53) of LCL721 lymphoblastoid cells (Kleijmeer et al., 1992; Schweizer et al., 1990). Upon closer investigation, other reports that have used microscopy might also have seen post-ER localization for TAP, or if not, they might have missed it for technical reasons, or because it was visible in the majority of their cells (Reits et al., 2000; Russ et al., 1995). Indeed, in many fibroblast cell lines, the ERGIC and the cis-Golgi can be difficult to discern from the strong background of the ER, leading researchers to perhaps overlook the post-ER appearance of the
TAP complex. Additionally, it is likely that the efficiency of the ER exit of TAP varies between cells, especially for fibroblasts that are not stimulated with interferon-γ for efficient antigen presentation; this is why we used lymphocytes in our investigation (Fig. 4).

Interestingly, Brodsky and collaborators fractionated extracts of the human B-lymphoblastoid cell line JY and found a weak signal for TAP2 in the α-mannosidase-II-positive fraction that presumably contained the cis and medial Golgi membranes (Bresnahan et al., 1997). In a similar experiment, Wang and co-workers lysed microsomes from 721.221 lymphocytes that were transfected with HLA-A2 and found that TAP, tapasin and HLA-A2 coprecipitated with coatomer, which coats the Golgi to ER retrograde COPI vesicles (Paulsson et al., 2006; Paulsson et al., 2002). Their results suggest that the PLC is recycled from the Golgi to the ER; however, an alternative explanation, namely association of a portion of coatomer with ER membranes, cannot be fully excluded (Mironov et al., 2003).

Which signals direct TAP to exit the ER and to return from the Golgi? Because TAP does not contain any of the known trafficking signals, one might speculate that the TAP-associated proteins in the PLC influence the localization of TAP. Tapasin, which links TAP to peptide-receptive class I, has a cytosolic KKKXX tail, whereas the luminal soluble lectin chaperone calreticulin, which is bound to class I molecules, ends in KDEL. However, in tapasin-deficient 220 cells, we observed a distribution that was similar to that in wild-type lymphocytes (not shown). This suggests that the TAP complex indeed carries some transport determinants of its own that are yet to be elucidated.

The most straightforward interpretation of the presence of functional TAP with class I in the ERGIC and the cis-Golgi, in the context of the PLC, suggests that the loading of class I molecules with antigenic peptides takes place not only in the ER, but in the entire early secretory pathway. This view agrees well with our previous result that peptide-receptive class I molecules can access the cis-Golgi (Garstka et al., 2007). Following peptide binding, class I molecules might then dissociate from the PLC (Ortmann et al., 1994) and proceed to the medial Golgi to become resistant to endoglycosidase H, whereas TAP and the chaperones of the PLC are retrieved to the ER, either individually or in association. However, it is important to state that our results, including those from the spatially resolved translocation assay, do not prove that peptide loading to class I occurs mainly outside the ER; for a quantitative assessment, substantial further work is required, and the result might differ between class I allotypes, cell types and even activation states of a single cell.

Alternatively, the chemical environment in the ERGIC and/or the cis-Golgi might be different from that of the ER, and ER–Golgi cycling might be significant for antigen presentation. Peptide binding to class I is an iterative process, where under the influence of tapasin, class I molecules first bind low-affinity peptides directly after their synthesis, which are then exchanged for higher-affinity ligands that have the proper length and sequence to form highly thermostable complexes (Schneeweiss et al., 2009; Williams et al., 2002). We have previously suggested that this process, termed ‘peptide editing’, depends on the repeated association and dissociation of the PLC, allowing tapasin and the peptide to compete for binding to class I (Wright et al., 2004). Perhaps altered pH or redox conditions in the later compartments of the secretory pathway favor the dissociation of the PLC, which is held together by the stable disulfide bond between tapasin and ERp57 (Peaper et al., 2005), enabling or supporting this binding and release process. To elucidate this question, substantial work is still required.

A third hypothesis concerns the antiviral immune response itself. Viruses use various strategies to escape immune surveillance, among them the destruction of class I molecules and even TAP in the ER (Loch and Tampe, 2005; Vossen et al., 2002; Wiertz et al., 1996a; Wiertz et al., 1996b). In the cis-Golgi, class I and TAP might be out of the immediate reach of viral factors such as US2, US6, US11 and UL49.5 that block their function and/or target them for degradation.

Our results have general implications for the understanding of the early secretory pathway of mammalian cells. In the yeast Saccharomyces cerevisiae, where ER–Golgi transport is well investigated, some proteins, for example the translocon Sec61p, are strictly barred from entering COPII vesicles, and are thus excluded from post-ER compartments (Bednarek et al., 1995). This puts a strong emphasis on the regulation of uptake into COPII vesicles as the decisive sorting procedure in the early secretory pathway. In mammalian cells, by contrast, we and others have found few examples of membrane proteins that are localized to the ER by such stringent retention, the most notable one perhaps being the transcription factor precursor SREBP/SCAP (Radhakrishnan et al., 2007). Remarkably, the mammalian homolog of Sec61p, SEC61α, is found prominently in the ERGIC (Greenfield and High, 1999). Thus, retrieval from the Golgi might have a greater role in the localization of ER proteins for mammalian cells than for yeast. A direct comparison of the mechanisms by which TAP is localized to the ER in mammals and – when expressed heterologously – in yeast (Urlander et al., 1997) might further elucidate this question.

Finally, the exit of the TAP complex from the ER is consistent in principle with the idea that it travels further along the secretory pathway, at least in some cell types, to participate in the import of peptides into late endosomes (Ackerman et al., 2006; Burgdorf et al., 2008; Johnstone and Del Val, 2007; Raghavan et al., 2008; Saveanu et al., 2009). Substantial further investigation is required to clarify this issue.

Materials and Methods

Antibodies and cell lines

The mAb HC10 is specific for unfolded HLA-B and HLA-C alleles (Stam et al., 1990). The anti-TAP1 (148.3) and anti-TAP2 (429.3) monoclonal antibodies were described earlier (Meyer et al., 1994; van Endert et al., 1994). The anti-TAP1 rabbit antiserum R.RING4c (Dick et al., 2002) was a kind gift from Peter Cresswell (Yale University, New Haven, CT). As compartment markers, we used: the protein disulfide isomerase PDI (ER; both rabbit antiserum and mouse monoclonal were a gift from Irina Majoul and Rainer Duden, University of Lübeck, Germany), a p58 antisera (from Ralf Pettersson, Karolinska Institute, Stockholm, Sweden), which also reacts with the human and monkey homologs (called ERGIC-53 for the ERGIC, and GM130 (Golgi, mAb from BD Biosciences). COS-1 and Raji cells were from the German Collection of Microorganisms and Cultured Cells (DSMZ). T1 and T2 cells were a gift from Peter Cresswell. LCL721.220 transfected with tapasin was a gift from Anthony Williams and Tim Elliott (University of Southampton Medical School, UK).

DNA constructs, cell transfection and microscopy

The single-chain fusion of H-2Kb, termed scKb–GFP, consists of β2m fused to the N-terminus of H-2Kb. To generate it, the gene for human β2m (including the signal sequence) was cloned into pCR-TOPO (Invitrogen), cut with NcoI and XhoI, and cloned into the Ncol and SalI sites of pASK-IBA5 (IBA, Göttingen, Germany). The oligonucleotides CAT GGG TGG CGG AGG TAG TGG TGG CTC CGG TGG TGG CG and GAT CCG CCA CCA CCG GAG CCA CCG CCA CCA CCA GGC CCA CAC TCG CTG and CCC GTC GAC TTA TCA CGC TAG AGA TCC GCC CAC AAC TGG CGG TGG CTG CTG and CCC GGC CCA CAC TCG CTG and CCC GTC GAC TTA TCA CCG TGC TAG AGA ATG AG and cloned into the pTM1 (Moss et al., 1990) was removed by filling in with Pfu polymerase and blunt ends. The entire 2m–H-2Kb fusion vector. The gene encoding H-2Kb was amplified with the primers CCC GGA TCC GGC CCA CAC TCG CTG and CCC GTC GAC TTA TCA CCG TGC TAG AGA ATG AG and cloned into the BamHI and SalI sites of that vector. The gene encoding H-2Kb was amplified with the primers CCC GGA TCC GGC CCA CAC TCG CTG and CCC GTC GAC TTA TCA CCG TGC TAG AGA ATG AG and cloned into the BamHI and SalI sites of that vector. The entire β2m–H-2Kb fusion gene was then amplified with the primers CCC CGT CCA CGA CCA TGG TGG CTG CTC GCT
Saponin was removed with cold PBS on ice, then samples were incubated with either

COPII vesicle formation assay

Membranes were prepared as described (Garstka et al., 2007). Briefly, cells were harvested, resuspended in a low osmolarity buffer, and broken with 20 passes through a 22G syringe needle. After sedimenting nuclei and unbroken cells, the high-speed pellet (15,000 g, 5 minutes) was washed twice and used as donor membranes. For the vesicle formation assay, donor membranes were incubated with an ATP regenerating system, GTP, cytosol and recombinant Sar1 at 30°C for 30 minutes, and the donor membranes were removed by high-speed centrifugation. Vesicles in the supernatant fraction were centrifuged at 100,000 g for 30 minutes, washed and lysed with SDS buffer for SDS-PAGE and western blotting. Preparation of mammalian cytosol and Sar1 wild type or Sar1(T39N) protein was carried out as described (Garstka et al., 2007). Proteins were detected by western blotting using standard techniques.

Microscopic TAP activity assay

The peptide SINFEKL-TAMRA, with TAMRA dye coupled to the lysine side chain, was prepared by Biosyntan (Berlin, Germany). COS cells were transfected with the scKα-GFP and, after overnight expression, rinsed with PBS at room temperature and permeabilized: cells were washed with cold PBS and incubated on ice with or without saponin (0.05% or 0.005%; Sigma) for PBS in 3 minutes. Saponin was removed with cold PBS on ice, then samples were incubated with either an ATP regenerating system (60 μM) for 5 minutes at 20°C, apyrase (10 μg/ml; Sigma) for 15 minutes at 37°C, or ICP47 (50 μM) for 30 minutes at 15°C. Immediately afterwards, SINFEKL-TAMRA peptide (2 μM) was added for 1 or 2 minutes at 20°C. Cells were then washed twice at 20°C and fixed with 3% paraformaldehyde for 10 minutes at 20°C. To differentiate permeabilized from unpermeabilized cells, the side-chain fluorescence of anti-GM130 and Alexa-Fluor-633-coupled secondary antibody for 40 minutes at room temperature was carried out. The cells were then washed with PBS and mounted onto microscopic slides using Mowiol. For all the washes, PBS was supplemented with 100 mM glycine.


References

the ER in the presence of brefeldin A suggests an ER recycling pathway. Cell 60, 821-836.


