Novel Peptide-Binding Proteins and Peptide Transport in Normal and TAP-Deficient Microsomes

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ABSTRACT: Most major histocompatibility complex (MHC) class I-binding peptides are translocated by TAP heterodimers, but some enter the ER lumen by alternative pathways. To further define mechanisms of peptide handling, we developed a system for the analysis of peptide-binding components in the ER membrane and lumen using iodinated cross-linkable peptide derivatives. Here we demonstrate that at least three proteins bind peptides in the ER lumen. Peptide cross-linking to these lumenal proteins can be used as an alternative method to monitor peptide transport. TAP and one other protein bind peptides on the cytoplasmic face of the ER. The presence of multiple peptide-binding proteins necessitates caution in interpreting traditional peptide-binding and transport assays. Finally, we demonstrate sequence-specific peptide transport in TAP-deficient cells transfected with only rat TAP1.

Peptide transport from the cytosol into the lumen of the endoplasmic reticulum is an essential step in the major histocompatibility complex (MHC)[1] class I antigen processing pathway. Transport of most peptides is mediated by the products of two MHC-encoded gene products, TAP1 and TAP2 (Heemels & Ploegh, 1995; Marusina & Monaco, 1996; Monaco, 1995). TAP1 and TAP2 form a heterodimer that resides in the ER and cis-Golgi membranes (Kleijmeer et al., 1992), and TAP-dependent peptide binding and transport reportedly occur only in the presence of both (Androlewitcz & Cresswell, 1994). Accordingly, the TAP2-deficient cell line RMA-S (Attaya et al., 1992; Karre at al., 1986; Powis et al., 1991; Townsend et al., 1989) fails to present most antigens to $CD8^+$ T cells. However, a number of peptides derived from viral proteins can be detected on the surface of RMA-S by antigen-specific CTL, at levels indistinguishable from those in wild-type cells (Gabathuler et al., 1994; Hosken & Bevan, 1992; Ossevoort et al., 1993). One potential mechanism of peptide translocation in RMA-S is a putative TAP1 homodimer (Gabathuler et al., 1994). However, other TAP-independent pathways, such as one mediated by secretory signal sequences (Anderson et al., 1991; Henderson et al., 1995), or other uncharacterized pathways may be also involved in peptide transport.

The most common approach for measuring peptide transport across the ER membrane involves the use of radioiodinated peptides that contain an NX(S/T) glycosylation signal sequence. Since glycosylation occurs only if the peptide gains access to the ER lumen, transport can be quantitated by measuring the recovery of radioactive glycosylated peptides on Con A−Sepharose (Momburg et al., 1994). Glycosylation also traps the reporter peptide in the ER lumen, which enhances the sensitivity of the assay; nonglycosylatable peptides fail to accumulate unless they are capable of binding to endogenous MHC class I molecules, which may also serve as a peptide trap (Heemels & Ploegh, 1995). In the latter case, quantitation of radioactivity associated with the membrane fraction after microsomal lysis is equated with peptide transport (Heemels & Ploegh, 1995; Shepherd et al., 1993; Schumacher et al., 1994a,b; Van Endert et al., 1994; Yang & Braciale, 1995). For peptides that neither contain glycosylation signals nor bind efficiently to class I, transport is evaluated in competition assays, by measuring the ability to inhibit transport of a reporter peptide (Momburg et al., 1994; Shepherd et al., 1993; Yang & Braciale, 1995). This approach relies on the assumption that peptide binding by TAP is necessarily followed by its transport.

We developed an alternative peptide-binding/transport system, utilizing iodinated cross-linkable peptide derivatives (Androlewitcz & Cresswell, 1994), to look at peptide-binding components in the ER, and also to follow the fate of translocated peptides. We demonstrate that at least three other proteins, one of them being the ER resident heat shock protein gp96, rapidly bind translocated peptides inside the ER lumen. This intralumenal binding may serve as a convenient molecular marker of peptide translocation into the ER, and may be particularly useful for directly assessing the transport of peptides that neither bind to class I nor contain glycosylation signal sequences. We also identified a novel 100 kDa membrane-associated protein (p100) which binds peptides in a sequence-specific manner on the cytoplasmic side of the ER membrane in several cell lines of different origin. Peptide binding by this protein may complicate the interpretation of some studies where microsome-associated peptide is assumed to have been transported. Using this assay, we also demonstrate TAP1-

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[1] Abbreviations: CTL, cytotoxic T-lymphocyte; ER, endoplasmic reticulum; HSAB, hydroxysuccinylamido benzoate; INF-γ, γ interferon; MHC, major histocompatibility complex; TAP, transporters associated with antigen processing.

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dependent and sequence-specific peptide transport in microsomes from a TAP2-deficient cell line.

MATERIALS AND METHODS

Mice and Cell Lines. C57BL/6 mice were purchased from Jackson Laboratories (Bar Harbor, ME). TAP1$^{-/-}$ knockout mice were the kind gift of Dr. Luc van Kaer (van Kaer et al., 1992). CMT.64 (small cell lung carcinoma) transfectants were received from Dr. Wilf Jefferies (Vancouver, Canada), and have been described previously (Gabathuler et al., 1994). Human B-lymphoblastoid cell lines 721.220 and 721.221, and the X2 hybrid (721.174 × 721.220, transfected with HLA-B8), were received from Dr. Thomas Spies (Seattle, WA) (Grandea et al., 1995). RBL-5 was a gift from Dr. Nakamura (Buffalo, NY). Other cell lines, RMA, RMA-S (Karre et al., 1986), 18-81.A20 (Alt et al., 1991), RAW 264.7 (Ralph & Nakoinz, 1977), EL-4 (Martin & Wunderlich, 1972), H6 (Nandi et al., 1996), T2, 721.174, 721.45, and 721.134 (DeMars et al., 1985), were maintained in our laboratory. All cell lines were grown in 2 L roller bottles in RPMI-1640 medium, supplemented with 10% fetal calf serum, penicillin, streptomycin, and L-glutamine. CMT.64 transfectants and H6 cells were induced with 100 units/mL and 20 units/mL mouse INF-γ (Boehringer Mannheim), respectively.

Peptide Labeling. All peptides were synthesized and purified in our laboratory by F-moc chemistry on a Synergy peptide synthesizer (Applied Biosystems, Inc.). Natural peptide sequences were modified to introduce a tyrosine residue for iodination and/or a lysine residue for conjugation to the UV-inducible cross-linker hydroxysuccinylsulfoamido benzoate (HSAB). HSAB modification was performed as described (Androlewicz & Cresswell, 1994). HSAB-peptides were purified by HPLC and iodinated using the chloramine-T method. Briefly, 20 nmol of the peptide was incubated with 1 mCi of $Na^{125}I$ in the presence of chloramine-T (20 μL of 1 mg/mL stock) in a total volume of 50 μL for 5 min (Hunter & Greenwood, 1962). The reaction was stopped by addition of sodium metabisulfate (20 μL, 7 mg/mL). Unincorporated iodine was removed using DEAE-MC spin columns (Millipore) according to the manufacturer's directions.

Preparation of Microsomes. Preparation of microsomes from mouse livers was conducted as described by Walter and Blobel (1983). This protocol was modified for preparation of microsomes from cell lines. Briefly, 2×10^{10} cells were pelleted, washed in HBSS, and resuspended in 10 mL of buffer A (Walter & Blobel, 1983). The cell suspension was sonicated in an Ultrasonic Processor W-385 (Heat Systems-Ultrasonics, Inc.) with probe no. C3 (2 s pulses, for 10 s total), and cell debris was pelleted for 10 min at 1000*g*. The supernatant was transferred into clean tubes, and nuclei and Golgi were pelleted for 10 min at 7500*g*. Finally, microsomes were pelleted for 30 min at 90000*g*. The microsomal pellet was resuspended in buffer B (Walter & Blobel, 1983) by homogenizing, diluted to a concentration of $A_{280} = 0.5$, frozen in 50 μL aliquots, and stored at −80 °C.

Binding and Transport Assays. Transport assay mixtures, modified from Schumacher et al. (1994a,b), were assembled on ice in 96-well plates. Each reaction contained 50 mM HEPES, 150 mM KOAc, 5 mM MgOAc, 250 mM sucrose, 1 mM DTT, and 1% aprotinin (7.7 TIU/mL). An ATP-regenerating system (50 μM ATP, 250 μM UTP, 2.5 mM creatine phosphate, and 8 units of rabbit muscle creatine phosphokinase) was added where indicated. Reactions were started by adding 0.1 nmol of labeled peptide and 5 μL of microsomes, and carried out at room temperature for 5 min. After incubation, reactions were placed on ice and irradiated with 254 nm shortwave UV light for 5 min to induce cross-linking. Next, the reaction mixtures were overlaid on a cushion containing 500 mM sucrose in buffer A. Microsomes were pelleted for 10 min at 14000*g*, the supernatant was aspirated, and the pellet was lysed in SDS sample buffer. Proteins were separated by SDS−PAGE, and iodinated peptide−protein complexes were visualized by PhosphorImager (Molecular Dynamics) or by exposure to X-ray film. For tunicamycin experiments, microsomes were preincubated with tunicamycin (Sigma) in transport buffer at a final concentration of 1.5 mg/mL for 45 min. Next, radiolabeled peptides were added, and the reaction was allowed to proceed as described above.

Antibodies. Anti-mouse TAP1 (no. 548) and anti-mouse TAP2 (no. 706) were produced in our laboratory by immunizing rabbits with GST−TAP fusion protein (D. Nandi and J. J. M., manuscript in preparation). Anti-gp96 antiserum was a gift from Dr. P. Srivastava (Buffalo, NY). Anti-PDI antiserum was a gift from Dr. B. Noiva (University of South Dakota). Other antibodies to heat shock protein families were purchased from Stressgen. For immunoprecipitations, after peptide cross-linking, microsomes were lysed in 5 mM CHAPS, 150 mM NaCl, and 50 mM Tris (pH 8.0) buffer. Debris was removed by centrifugation at 10000*g* for 10 min, and supernatants were incubated for 30 min with antiserum, followed by addition of fixed, heat-killed *Staphylococcus aureus* (Pansorbin, Calbiochem) and another 30 min incubation, on ice. Immunoprecipitates were pelleted, washed 3 times with lysis buffer, and resuspended in SDS sample buffer. Samples were boiled for 4 min prior to SDS−PAGE.

RESULTS

A 100 kDa Protein Binds Peptides on the Cytoplasmic Side of the ER. The peptides used in this study are based on the sequence either of a naturally occurring H-2K^b-restricted peptide derived from ovalbumin (SIINFEKL) that is not presented by TAP2-defective RMA-S cells or of a vesicular stomatitis virus nucleoprotein (VSV-NP) peptide (RGYVYQGL) that is presented by these cells. The ovalbumin peptide was modified to incorporate an iodinatable tyrosine residue, and the VSV peptide was altered to include a lysine residue suitable for attachment of the photoactivatable cross-linker, HSAB. UV-induced cross-linking of iodinated peptide enabled us to identify peptide-binding proteins in the ER membrane.

ATP is not required for peptide binding by TAP (Androlewicz & Cresswell, 1994; Van Endert et al., 1994), but it releases peptides from TAP, promoting their translocation into the ER (Van Endert et al., 1994). As expected, in the absence of ATP, iodinated SIINYEKL bound to 70−72 kDa proteins in C57BL/6 microsomes (Figure 1a). These proteins can be immunoprecipitated with anti-TAP antiserum (D. Nandi, manuscript in preparation, and data not shown), indicating that they represent the TAP1/2 heterodimer. Unlabeled competitor peptide specifically competed for binding of the iodinated peptide to TAP. In RMA mi-

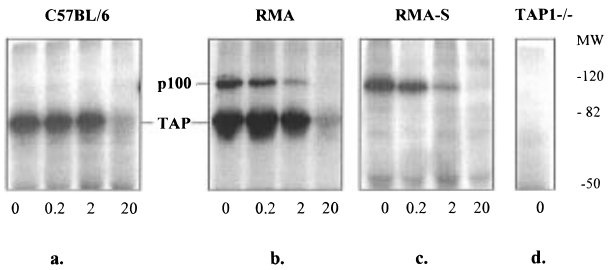

FIGURE 1: Peptides bind to TAP1/2 and p100 in the absence of ATP. Iodinated HSAB-SIINYEKL peptide was incubated with C57BL/6 (a), RMA (b), RMA-S (c), and TAP1−/− knockout mouse liver (d) microsomes. Unlabeled SIINYEKL was added at the indicated concentrations (in μM) prior to cross-linking. Molecular weight markers are shown on the right. Bands corresponding to TAP and p100 are marked.

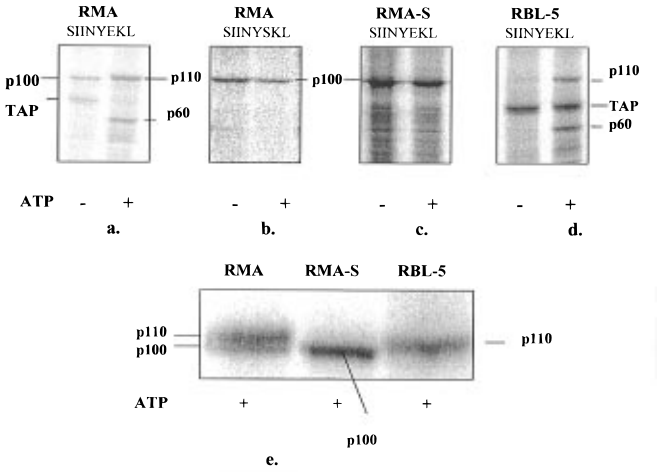

FIGURE 2: In the presence of ATP, peptides bound to p60 and p110 (gp96). Iodinated HSAB-SIINYEKL was incubated with RMA (a), RMA-S (c), and RBL-5 (d) microsomes in the absence (−) or presence (+) of an ATP-regenerating system, followed by cross-linking. Bands corresponding to p110, p100, TAP, and p60 are marked. In (e), the portion of the gel around 100 kDa is shown at higher resolution in order to distinguish p100 and p110. In the presence of ATP, both p100 and p110 are visible in RMA (lane 1), only p100 in RMA-S (lane 2), and only p110 in RBL-5 microsomes. In (b), iodinated HSAB-SIINYSKL was used with RMA microsomes under conditions identical to those in (a).

crosomes (Figure 1b), SIINYEKL bound to TAP as well as to another protein with an apparent molecular mass of approximately 100 kDa, hereafter referred to as p100. Peptide binding to p100 is also specifically competed by addition of unlabeled peptide (Figure 1b) . In TAP2-deficient RMA-S microsomes (Figure 1c), SIINYEKL bound only to p100, further supporting the identification of the 70-72 kDa bands as TAP. Neither TAP nor p100 binds SIINYEKL in microsomes from TAP1−/− knockout mice (Figure 1d). Preincubation of microsomes with anti-TAP2 antiserum significantly reduced peptide binding to TAP but not to p100 (data not shown).

Translocated Peptides Bind to ER Resident Proteins. Addition of ATP promotes TAP-dependent peptide translocation into the ER (Androlewicz & Cresswell, 1994). To investigate the fate of translocated peptides, we repeated the cross-linking experiments in the presence of ATP. Under these conditions, iodinated SIINYEKL bound to two other proteins with apparent molecular masses of 110 kDa (p110) and 60 kDa (p60) in TAP-expressing wild-type cells (Figure 2a,d,e). At the same time, binding to TAP was generally decreased. Binding to p110 and p60 is not observed in the

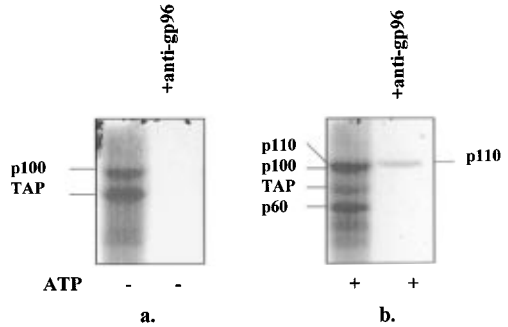

FIGURE 3: P110 but not p100 can be precipitated with anti-gp96 antisera. Iodinated SIINYEKL was incubated with RMA microsomes in the absence (a) or presence (b) of ATP, followed by cross-linking and lysis. Protein−peptide complexes were immunoprecipitated with anti-gp96 antiserum (lanes 2 and 4). Bands corresponding to p110, p100, TAP, and p60 are marked.

absence of ATP (Figure 2a,d, Figure 3), nor is it seen in TAP2-defective RMA-S microsomes, either in the presence or in the absence of ATP (Figure 2c), suggesting that these are lumenal proteins. Moreover, a peptide (SIINYSKL) which fails to bind to TAP in RMA microsomes, and hence presumably cannot be transported, also fails to bind either p110 or p60 (Figure 2b). Interestingly, similar to what is observed in microsomes from normal mouse tissue (Figure 1a,d), peptide binding to p100 is observed in microsomes from RBL-5 (Figure 2d,e), the cell line from which both RMA and RMA-S were derived.

The p110−peptide complex, but not the p100−peptide complex, could be immunoprecipitated (Figure 3) with antiserum to the ER-resident heat shock protein gp96 (Suto & Srivastava, 1995). In the absence of ATP, peptides bound to p100 (Figure 3a), but these complexes could not be immunoprecipitated with anti-gp96 antisera. In the presence of ATP, peptides bound to both p100 and p110. Although the resolution of the gel is not always sufficient to separate these proteins, the p110−peptide complex could be immunoprecipitated with anti-gp96 antisera (Figure 3b).

Peptide binding to p110 (gp96) and p60 was observed in all cell lines with functional TAP1/2 heterodimers, although p110 was harder to visualize in human cell lines (see below). Preincubation of microsomes with anti-TAP antisera significantly decreased binding to both p110 and p60 (data not shown). Overall, these data indicate that peptide binding to p110 (gp96) and p60 occurs after peptides have been translocated, and it is consistent with the known intralumenal localization of gp96 (Suto & Srivastava, 1995). Thus, binding of peptides to p110 (gp96) and p60 may serve as a convenient assay for peptide translocation, and may be used to assess translocation of peptides that do not possess a glycosylation motif.

Using peptides that do contain an N-linked glycosylation signal sequence, a group of intralumenal peptide-binding proteins was observed in addition to p110 and p60 (Figure 4). These proteins had apparent molecular masses of about 36 kDa, and are collectively referred to as p36 complex. SIINYEKL does not have a glycosylation motif, and therefore was released from microsomes over a period of time, as evidenced by decreased cross-linking to p110 and p60 (Figure 3a). In contrast, SNITYEKL was rapidly glycosylated and retained in the ER, accumulating primarily on the p36 complex (Figure 4b), again with binding to p110 and p60 decreasing over time. Binding to the p36 complex

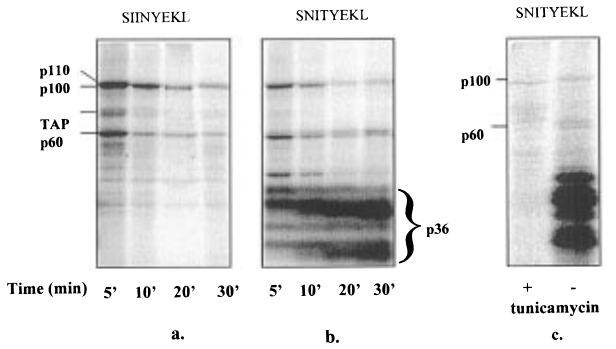

FIGURE 4: p36 complex binds glycosylated peptides in the ER lumen. Iodinated HSAB-SIINYEKL (a) and HSAB-SNITYEKL (b, c) were incubated with RMA microsomes in the presence of an ATP-regenerating system for the indicated time periods before cross-linking. Bands corresponding to p110, p100, TAP, p60, and p36 complex are marked. In (c), microsomes were preincubated with (+) or without (−) tunicamycin prior to peptide addition.

is not observed in TAP-defective microsomes, or in the absence of ATP. Pretreatment of microsomes with tunicamycin or anti-TAP antisera completely abrogated binding to the p36 complex (Figure 4c), supporting the proposed specificity for carbohydrate and intralumenal location, respectively. The double band noted for p60 with the SNITYEKL peptide probably results from binding to either unglycosylated or glycosylated forms of the peptide (Figures 4b and 7).

Tissue Distribution of p100. Other than TAP, p100 was the only protein we could visualize that binds peptides on the cytoplasmic side of the ER. However, although it is present in both RMA and RMA-S, it is curiously absent from the parental T cell line, RBL-5, from which both RMA and RMA-S were derived. It is also not seen in microsomes derived from normal mouse tissues (spleen and liver). We were therefore interested in assessing its presence in other cell lines and tissue types. Microsomes were prepared from a variety of cells, and peptide binding to p100 was assessed using the ovalbumin derivatives SIINYEKL and SNITYEKL. Peptide binding to p100 was seen in only 4 of 15 cell lines: RMA and RMA-S (murine T cell lymphoma), 721.220 (human B-lymphoblastoid), and CMT.64 (lung carcinoma) (Table 1). Thus, p100 is not restricted to T cell lines—it is in fact absent in the T cell line EL4, which is derived from the same strain, C57BL/6, as RMA.

The family of human lymphoblastoid cell lines shown in Table 1 is of particular interest. The 721.45 cell line is an MHC hemizygous B-lymphoblastoid cell line, selected for loss of one MHC haplotype after γ-irradiation (DeMars et al., 1985). This line is the parent of 721.134, in which the TAP1 gene in the remaining MHC haplotype is nonfunctional (Spies & DeMars, 1991), and 721.174, which has lost the second MHC haplotype (including both TAP genes) by deletion. Fusion of 721.174 with the T cell line CEM, followed by loss of the CEM-derived MHC alleles, resulted in the T2 cell line, carrying the same homozygous MHC deletion as 721.174. γ-Irradiation of the hemizygous .184 cell line (derived from .45), followed by selection for the loss of HLA-C expression, resulted in two cell lines, 721.220 and 721.221. The latter has lost the class I structural genes by deletion, while the former has an as yet incompletely characterized defect in the class I antigen processing pathway that results in a failure of TAP1 to associate with class I/β2 microglobulin (Grandea et al., 1995) and impaired class I cell surface expression. Interestingly, p100 binding to both SIINYEKL and SNITYEKL is observed only in 721.220 (Table 1 and Figure 5a−d). Furthermore, p100 binding is extinguished in a hybrid (X2; Grandea et al., 1995) between 721.174 and 721.220 in which the TAP defect of 721.174 is complemented by the TAP genes of 721.220, and the unknown defect in class I antigen processing in 721.220 is complemented by genes from 721.174.

Ineffective peptide translocation in 721.220 could be also visualized in these experiments. Both SIINYEKL and SNITYEKL peptides were able to bind to human TAP (Figure 5) as was confirmed by immunoprecipitation (data not shown). SNITYEKL was translocated into the ER in all TAP$^+$ human cell lines, including .221, 220, and X2, as judged by its binding to p60 (Figure 5b,d,f). In contrast, SIINYEKL was translocated in .221 (Figure 5a) but not in .220 (Figure 5c), and its transport is restored in the X2 hybrid (Figure 5e).

Peptide Transport and Binding in CMT.64 and Its Derivatives. The CMT.64 cell line was derived from a spontaneous lung carcinoma, and fails to express TAP genes unless treated with IFN-γ (Franks et al., 1976). IFN-induced, but not uninduced, CMT.64 cells process and present both the Ova-derived peptide SIINFEKL and the VSV-derived peptide RGYVYQGL to CD8$^+$ T cells. It has been reported (Gabathuler et al., 1994) that CMT.64 transfected with only the rat TAP1 gene can present the VSV peptide to CTL, similar to what is seen with the TAP2-defective RMA-S cell line (Hosken & Bevan et al., 1992). These results suggest that TAP1 (homodimers?) may function to transport selected peptides into the ER. We addressed this issue using our *in vitro* peptide transport system.

The SIINYEKL (Figure 2a) and RGYVYKGL (Figure 6a) peptides bound to both p100 and TAP in RMA, and, in the presence of ATP, were transported, as evidenced by reduced binding to TAP and strong binding to p110 and p60. In RMA-S, both peptides bound to p100, but no binding to TAP1 was observed, nor was there any evidence of transport (Figures 2c and 6b).

All of the Ova-related peptides bound strongly to p100 in uninduced CMT.64 (SIINYEKL is shown in Figure 6c). However, despite the fact that RGYVYKGL was able to bind to p100 in RMA and RMA-S, no binding was detected in uninduced CMT.64 (Figure 6d). Neither these peptides nor the other ovalbumin derivatives (SIINYSKL and SNITYEKL) were translocated, consistent with the lack of TAP expression in these cells. Good TAP binding and transport of both SIINYEKL and RGYVYKGL were observed in CMT.64 induced with INF-γ (Figure 6e,f), similar to what is observed in RMA. CMT.64 cells expressing only rat TAP1 displayed strong p100 binding with all ovalbumin peptide derivatives (SIINYEKL is shown in Figure 6i), but no TAP binding, and no transport. Binding of RGYVYKGL to p100 and TAP in these cells was essentially undetectable (Figure 6i). However, RGYVYKGL clearly bound to p60 in the presence of ATP (Figure 6j), indicating that it was translocated into the ER. These data correlate with the reported ability of TAP1-transfected CMT.64 cells to present VSV to CD8$^+$ T cells (Gabathuler et al., 1994).

Table 1: P100−Peptide Binding Distribution

name	source	binding to p100[a]
	Mouse Tissue	
C57BL/6	mouse liver	−
TAP1−/− knockout	mouse liver	−
	Mouse Cell Lines	
RBL-5	T-lymphoma, Rauscher virus induced	−
RMA	T-lymphoma, Rauscher virus induced,EMS mutagenized	+
RMA-S	T-lymphoma, Rauscher virus induced, EMS mutagenized	+
CMT.64 (pHBA neo)[b]	spontaneous small lung carcinoma	+
CMT.64 (rTAP1)[b]	spontaneous small lung carcinoma	+
CMT.64 (rTAP1/2)[b]	spontaneous small lung carcinoma	+
18−81.A20	pre-B cell line, Abelson virus transformed	−
EL-4	T-lymphoma, chemically induced	−
RAW 264.7	macrophage	−
H6[b]	hepatoma	−
	Human Cell Lines	
.45	B-LCL, γ-irradiated	−
.174	B-LCL, γ-irradiated	−
T2	B-LCL, γ-irradiated	−
.134	B-LCL, γ-irradiated	−
.221	B-LCL, γ-irradiated	−
.220	B-LCL, γ-irradiated	+
X2	(.174×.220(B8)) cell hybrid	−

[a] Microsomes prepared from different cell lines and mouse livers were analyzed for the ability of p100 to bind SIINYEKL and SNITYEKL peptides in the absence of ATP as described under Materials and Methods. [b] The results are identical for γ-INF-induced cell lines.

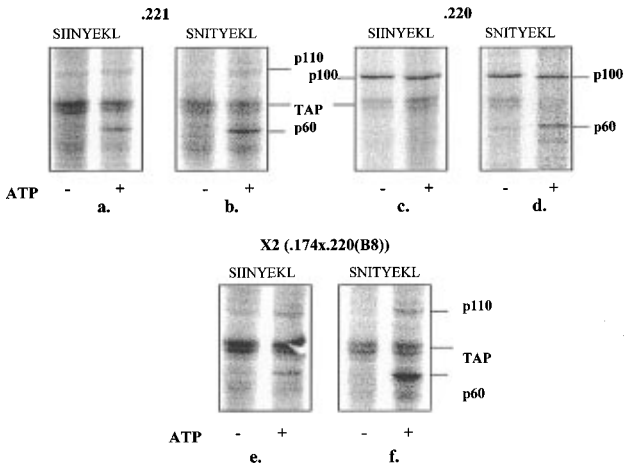

FIGURE 5: Transport of SIINYEKL peptide is inefficient in .220 microsomes. Iodinated HSAB-SIINYEKL or HSAB-SNITYEKL peptides were incubated with 721.221 (a, b), 721.220 (c, d), or X2 (e, f) microsomes, in the absence (−) or presence (+) of ATP. Bands corresponding to TAP, p60, and p100 are marked.

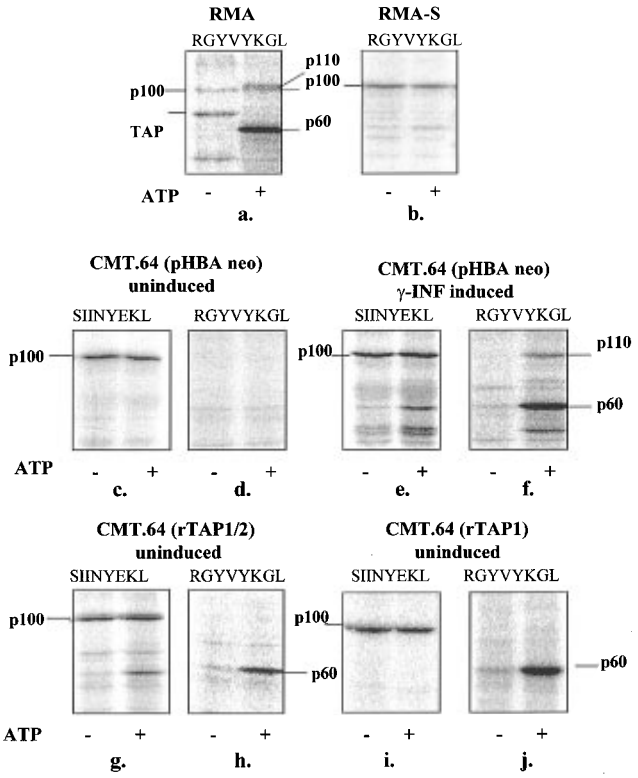

FIGURE 6: The VSV-N peptide derivative RGYVYKGL is transported in CMT.64 (rTAP1). Iodinated VSV-derived (HSAB-RGYVYKGL) and Ova-derived (HSAB-SIINYEKL) peptides were incubated with RMA (a), RMA-S (b), CMT.64 (pHBAneo) (c, d), CMT.64 (pHBAneo) INF-γ (e, f), CMT.64 (rTAP1/2) (g, h), or CMT.64(rTAP1) (i, j) microsomes, in the absence (−) or presence of ATP (+). Bands corresponding to p110, p100, TAP, and p60 are marked.

DISCUSSION

We developed an assay system, utilizing iodinated HSAB-peptide derivatives to identify peptide-binding proteins in the membrane and lumen of the ER, and to characterize peptide transport in class I antigen presentation mutant cell lines. Three lumenal peptide-binding components were identified—p110, p60, and p36 complex. One group of proteins, referred as the p36 complex, bound only peptides that contained N-linked glycosylation signal sequences, and is therefore likely to be specific for the carbohydrate moiety attached to such peptides. This was confirmed by demonstrating inhibition of peptide binding to p36 complex by tunicamycin treatment. Peptide binding to p36 complex increases with time of incubation, and may at least in part be responsible for the trapping of such peptides within the ER lumen that has been described previously [for a review, see Heemels and Ploegh et al. (1995)]. Binding to p36 complex was most pronounced in mouse cell lines, and less so in human cells, which may be due to interspecies differences. Interestingly, binding to p36 complex was negligible in mouse cell lines treated with INF-γ (CMT.64 and H6, data not shown), suggesting that either the peptide glycosylation machinery or more likely the p36 complex

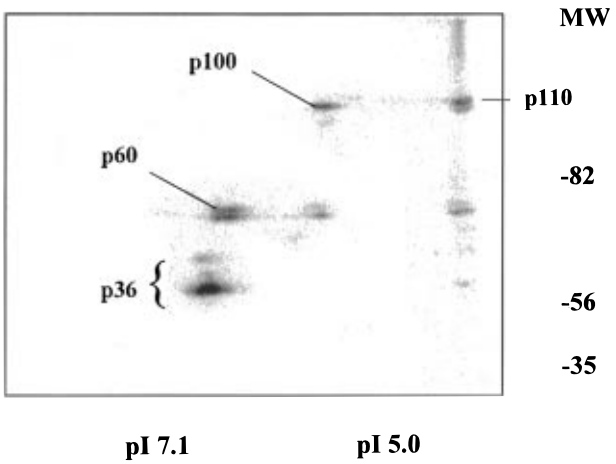

FIGURE 7: Two-dimensional separation of ER peptide-binding proteins. After cross-linking with HSAB-SNITYEKL, RMA microsomes were dissolved in NEPHGE sample buffer. Proteins were separated according to p*I* by nonequilibrium pH gradient electrophoresis (NEPHGE) in the first dimension and molecular weight (second dimension, 10% SDS−PAGE). Positions of molecular weight markers and p*I* markers are indicated. Spots corresponding to p110, p100, p60, and p36 are marked.

itself is down-regulated by INF-γ. Proteins in p36 complex may belong to the family of mannan-binding proteins residing within the rough ER and Golgi (Mori et al., 1984). Members of this family share a conserved C-type lectin domain which confers mannose and *N*-acetylglucosamine binding properties.

The two other lumenal proteins which bind translocated peptides are p110 and p60. We considered it likely that these represented members of the heat shock family of proteins, and tested for reactivity with anti-heat shock protein antibodies. Commercially available (Stressgen) polyclonal antibodies against Hsp 25, Hsp 73, Grp 78 (BiP), Hsp60, and Hsp 90 failed to precipitate any of the proteins described in this report (data not shown). Similar results were obtained using antiserum against p100/110, described by Feuerbach and Burgert (1993), and protein disulfide isomerase (Noiva et al., 1991). However, antibodies to the ER-resident heat shock protein gp96 recognized the p110 protein. Based on the known properties of gp96 (i.e., molecular size, peptide binding, and intralumenal localization) (Suto & Srivastava, 1995), it is likely that it is identical to p110. However, we cannot rule out the possibility that p110 is closely related, but not identical, to gp96.

Being unable to identify p36 complex, p60, or p100 by immunoprecipitation with known antisera, we determined their isoelectric points by two-dimensional gel electrophoresis (Figure 7), and searched the Swiss-Prot database for ER/Golgi proteins with similar molecular weights and p*I*s. However, no obvious matches were found, and hence the identity of these proteins remains unknown. Nevertheless, peptide cross-linking to p110 (gp96), p60, and/or p36 complex may serve as a useful method of assessing peptide translocation. They are present in all cell lines tested (Table 1), and several lines of evidence argue that peptide cross-linking to these proteins occurs only intralumenally. First, in TAP^+ microsomes, binding to these proteins occurs only in the presence of ATP, i.e., only under conditions which allow for peptide translocation. In contrast, no binding is observed in TAP^- microsomes, either in the presence or in the absence of ATP. Second, whereas all peptides demonstrating good TAP binding, i.e., peptides that are substrates for TAP-mediated transport (Van Endert et al., 1994), bind to both p110 and p60, one peptide that is not a good substrate for TAP-mediated transport (SIINYSKL, Figure 2b) is not cross-linked to these proteins either with or without ATP. Finally, cross-linking to p110 and p60 is not an artifact that occurs subsequent to lysis of the microsomes, since intentional lysis of the microsomes prior to UV-induced activation of the cross-linker results in dramatically reduced binding. Binding to both proteins appears transient, and decreases with time of incubation prior to cross-linking (Figure 6), consistent with previous observations of rapid loss of (nonglycosylatable) peptides from the ER lumen over time (Anderson et al., 1991). While the panel of peptides tested here is small, both proteins appear to have fairly broad specificity, and we have observed binding to several other peptides, including peptides up to 18 amino acids in length. This is consistent with the promiscuous peptide binding observed for gp96, and suggests that cross-linking to these proteins can serve as a convenient marker of peptide translocation, and may be particularly useful for the direct assessment of transport of peptides that do not contain glycosylation sites. Although the exact function of these proteins is not known, gp96, p60, and p36 complex may participate in peptide trafficking inside the ER. We are in the process of isolating and characterizing these proteins, which would aid in understanding their function.

Curiously, we did not observe peptide cross-linking to class I molecules in these experiments. Either this assay is not sensitive enough to detect class I binding or the modifications (amino acid substitutions, iodination, covalent coupling of HSAB) made to the naturally occurring peptides on which our experiments are based resulted in decreased class I binding ability as compared to the naturally occurring peptides. Since these peptides by themselves poorly stabilized empty class I molecules on RMA-S cells, as demonstrated by FACS (not shown), we favor the latter possibility.

At the cytoplasmic face, two microsomal proteins were found to bind peptides, both in the presence and in the absence of ATP. As expected, one of these was identified as the TAP transporter by immunoprecipitation with anti-TAP antibodies. Most of the synthetic peptides used in these studies bound to TAP in TAP^+ (e.g., RMA) microsomes. However, the Ova-related peptide SIINYSKL fails to bind to TAP and fails to be transported (Figure 2b). This peptide differs from the TAP-binding, transport-competent peptide SIINYEKL only at position 6, where it contains serine in place of glutamic acid. This position has not been observed to have a dramatic influence on peptide transport in other studies, and thus demonstrates the importance of flanking residues on the on the ability of single substitutions to cause differences in the specificity of TAP-mediated transport.

Neither TAP binding nor transport was detected for any tested peptide, including the VSV-N derivative RGYVYKGL, in TAP2-deficient RMA-S cells. This peptide differs from the naturally occurring VSV peptide (which is processed and presented normally by RMA-S cells) by a glutamine → lysine substitution at position 6, which may account for our inability to observe transport. Alternatively, it may be that the naturally transported peptide containing this epitope is longer than the nominal peptide, and is trimmed subsequent to transport, or that the iodination and/or HSAB conjugation inhibited transport in our system. In this regard, it is

somewhat surprising that transport of this peptide was clearly observed in CMT.64 cells transfected with rat TAP1 only (Figure 6h). Although TAP binding was not observed, we have found that rat TAPs are more difficult to visualize as compared with mouse or human TAPs in this system, for unknown reasons (unpublished observations). These data are consistent with the observation that the corresponding VSV epitope is presented to T cells by the TAP1-transfected, but not control, CMT.64 cells (Gabathuler et al., 1994), and with the possibility of transport mediated by TAP1 homodimers in these cells. It should be noted that, despite the transport of the VSV epitope, none of the Ova derivatives transported by TAP^+ cells is transported by CMT.64(rTAP1). However, IFN-γ-treated CMT.64 cells, in which the endogenous mouse TAP1 and TAP2 proteins are expressed, do transport the Ova peptides (not shown). It is not clear why RMA-S is different from CMT.64(rTAP1) with respect to transport of RGYVYKGL, since both of these mouse cell lines are derived from the same strain and express TAP1 but not TAP2. This may reflect a difference in specificity between mouse and rat TAP1.

Clearly, the most enigmatic and potentially interesting protein visualized in these studies is p100. This protein binds peptides at the cytoplasmic face of the ER membrane, and both TAP binding and p100 binding survive centrifugation through sucrose prior to cross-linking. Thus, in peptide transport assays which rely solely on determination of microsome-associated radioactivity, binding to p100 will influence the results. Indeed, we originally interpreted such assays done in our laboratory as indicative of non-ATP-dependent transport of peptide in RMA-S cells (data not shown). Moreover, in competition assays, unlabeled peptides compete for binding to both p100 and TAP (Figure 1), and p100 binding also displays sequence specificity (Figure 6, and data not shown). Therefore, caution must be exercised in the performance and interpretation of such transport assays to ensure that it is indeed only transport that is being measured, exclusive of contributions from p100 binding.

Peptide binding by p100 was detected only in 4 of the 15 cell lines tested in this study, and was not observed in microsomes from normal mouse liver (Table 1). It is observed in RMA and RMA-S, but not the parental cell line from which they were derived (RBL-5), and similarly is observed in 721.220, but not in the parental 721.45 or other related cell lines. It is possible that p100 expression is the result of mutation, and that similar mutations occurred independently in both of these cell lines, and probably in CMT.64 as well (although the normal tissue counterpart of CMT.64 was not tested for p100). Under this hypothesis, p100 expression would be expected to be a dominant phenotype. However, p100 expression appears to be recessive in the hybrid between 721.174 and 721.220. Although chromosome loss is a possible alternative explanation to the results in the hybrid, taken together these results suggest that p100 may be present in most or all cells, but that the ability to observe cross-linking of peptide to it is secondary to another defect.

Interestingly, there appears to be some correlation between the ability to observe peptide cross-linking to p100 and defects in the class I antigen processing pathway. While the molecular basis of the defect in 721.220 is still unknown, MHC class I molecules fail to associate with TAP in these cells, and peptide transport is inefficient. Fusion with 721.174 corrects the antigen processing defect, and simultaneously results in the apparent loss of p100. RMA-S cells have a nonsense mutation in TAP2 which renders it nonfunctional. However, transfection of TAP2 into RMA-S fails to completely correct class I surface expression (Attaya et al., 1992; Powis et al., 1991), suggesting that these cells may also have additional, more subtle defects in class I antigen processing. These more subtle defects may be shared with RMA cells. CMT.64 cells fail to express the TAP genes, and are therefore also antigen processing deficient.

Perhaps most surprisingly, while p100 binds to all of the Ova-related peptides in CMT.64 cells, it fails to bind RGYVYKGL in the same cells. This is in striking contrast to RMA-S cells, where all of these peptides (including RGYVYKGL) bind p100. Although RMA-S and CMT.64 are both derived from the same strain of mice, it is possible that their sequence differs because of mutation. However, we have been unable to detect differences in p100 derived from RMA-S and CMT.64 by two-dimensional gel electrophoresis (data not shown). Moreover, RGYVYKGL (but not any of the Ova-related peptides) is transported in CMT.64 expressing rat TAP, while this peptide is not transported in RMA-S (which expresses mouse TAP1), again suggesting some relationship between peptide transporting ability and p100 binding. If p100 is expressed in all cells, its peptide-binding function may be transient and therefore undetectable in the absence of peptide transport defects.

Nevertheless, p100 was not observed in several other cell lines which also have defects in class I antigen processing (e.g., 721.174, T2, 721.134, and H6; Table 1), and we have been unable to detect any physical or functional interaction between p100 and TAP. These proteins do not coimmunoprecipitate, and anti-TAP1 or anti-TAP2 antibodies fail to inhibit (or promote) peptide binding to p100 (data not shown). However, peptide binding to p100 was not detected if microsomes were lysed prior to peptide cross-linking. Thus, p100 may require interactions with other ER membrane components in order to bind peptides.

Our system utilized iodinated cross-linkable peptide derivatives to characterize ER peptide-binding elements. It enables us to visualize a complex set of sequence-specific interactions of peptides with several ER proteins. Together, these results demonstrate that there may be more components in the class I pathway than are currently recognized. Our data further suggest that the RMA cell line may possess an unidentified defect in the class I pathway, one that results in peptide binding to p100 and unites RMA with RMA-S, 721.220, and CMT.64. Purification and identification of these components will enable us to clarify their potential interaction with TAPs and class I molecules.

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