Cutting Edge: Tapasin Is Retained in the Endoplasmic Reticulum by Dynamic Clustering and Exclusion from Endoplasmic Reticulum Exit Sites

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Tapasin retains empty or suboptimally loaded MHC class I molecules in the endoplasmic reticulum (ER). However, the molecular mechanism of this process and how tapasin itself is retained in the ER are unknown. These questions were addressed by tagging tapasin with the cyan fluorescent protein or yellow fluorescent protein (YFP) and probing the distribution and mobility of the tagged proteins. YFP-tapasin molecules were functional and could be isolated in association with TAP, as reported for native tapasin. YFP-tapasin was excluded from ER exit sites even after accumulation of secretory cargo due to disrupted anterograde traffic. Almost all tapasin molecules were clustered, and these clusters diffused freely in the ER. Tapasin oligomers appear to be retained by the failure of the export machinery to recognize them as cargo. The Journal of Immunology, 2002, 168: 1538–1541.

The MHC class I heterotrimer, heavy chain, β2-microglobulin, and peptide, is assembled in the endoplasmic reticulum (ER) lumens via interactions with ER-resident chaperones and TAP and is subsequently transported to the plasma membrane (1). One of the chaperones, tapasin, increases local peptide concentration by tethering heavy chain/β2-microglobulin dimers to the TAP complex (2, 3), stimulates peptide transport by stabilization of the TAP heterodimer (4, 5), stabilizes empty MHC dimers in a peptide-receptive conformation (3, 6), retains empty class I molecules until peptide loading (7–9), and optimizes the affinity of class I-bound peptides by acting as a peptide editor (8, 10, 11).

Understanding the multiple roles of tapasin in MHC assembly requires understanding the mechanism of its retention in the ER. Tapasin bears a C-terminal dilysin (KKXXX) motif that could mediate its retrieval from post-ER compartments via associations with the coat protein I (COPI) vesicle coat (13–18). However, KKAA retains a CD4 chimera in the ER in the absence of COPI; therefore, the motif could also effect direct ER retention (19). Several molecular mechanisms have been proposed to account for such retention, including direct binding to microtubules (20), formation of large oligomers that cannot be included into transport vesicles (21), or interactions with a matrix of ER-resident proteins (22–25).

To determine the mechanism of tapasin-mediated class I retention, we investigated the intracellular organization of tapasin, tagged with yellow fluorescent protein (YFP) or cyan fluorescent protein (CFP) at the N terminus, by deconvolution fluorescence microscopy, fluorescence recovery after photobleaching (FRAP), and fluorescence resonance energy transfer (FRET). Tapasin did not appear to reach the medial Golgi or to cycle rapidly between the ER and the ER-to-Golgi intermediate compartment (ERGIC); it was excluded from ER exit sites under conditions favoring cargo accumulation in those regions of the ER. Furthermore, whereas FRET showed that all tapasin molecules were clustered, FRAP showed that these oligomers were freely diffusing and highly mobile, making unlikely the possibility that tapasin is retained by association with a static protein matrix. The data indicate that tapasin, and possibly class I molecules bound to tapasin, form diffusing oligomers that are not recognized by the ER export machinery.

Materials and Methods

Cells, Abs, and reagents

HeLa Tet-On cells (Clontech Laboratories, Palo Alto, CA) were maintained and transfected as described previously (26). The 721.220.B8 (.220) cell line, a gift from Dr. P. Cresswell (Yale University, New Haven, CT), was maintained as described (4). It was transfected with YFP-tapasin using electroporation, selected, and sorted three times. mAbs HC10 (27), BB7.2 (28), and W6/32 (29) were purified as described (26). mAb Gl1/93, recognizing human ERGIC-53 (30), was a gift of Dr. H.-P. Hauri, University of Basel (Basel, Switzerland). Other Abs were purchased from commercial suppliers. Oligonucleotides were synthesized and purified by Integrated DNA Technologies (Coralville, IA).

Gene construction

To generate N-terminal fusions of tapasin to YFP and CFP, the signal sequence of the tapasin cDNA (3), a gift of Dr. P. Cresswell, was deleted by PCR. The YFP linker fragment from the positive control for FRET (26) was excised and ligated into the tapasin construct above, which had been cloned into vector pEGFP-N3 (Clontech) lacking the green fluorescent protein (GFP). CFP and YFP were fused to the folate receptor signal sequence and the myc tag from a GFP-CDI construct, a gift of Dr. S. Lacey (University of Texas Southwestern Medical Center, Dallas, TX), using PCR. These fusions were then ligated into the leaderless tapasin construct above lacking the YFP tag. The inserts were also introduced in the pBI vector (Clontech).
Pulse-chase, immunoprecipitations, and Western blotting

Cells were incubated in cysteine- and methionine-free medium containing 10% dialyzed FBS for 30 min, labeled with 500 μCi/ml Tran 35S-label (ICN Pharmaceuticals, Costa Mesa, CA) for 90 min and chased in complete medium for 16 h as described (26). They were lysed in 0.5% Triton X-100 (Sigma, St. Louis, MO) and precleared, and tapasin was immunoprecipitated with anti-tapasin antiserum (StressGen Biotechnologies, Victoria, Canada) and treated with endoglycosidase H (endo H), as described (26).

For sequential immunoprecipitations with anti-TAP1 and anti-tapasin antiserum, 3 million cells were treated with 400 U/ml human IFN-γ for 48 h before labeling. Cells were starved in methionine- and cysteine-free medium containing 5% dialyzed FBS for 60 min, pulsed with 1.25 mCi/ml Tran 35S-label for 5 min, and chased for 75 min in medium containing 5 mM methionine and cysteine. Solubilization, preclearing, and immunoprecipitation were all performed as previously described (4, 31).

For Western blotting, cells were lysed in buffer containing 1% 3-(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS). All immunoprecipitations and protein transfers were performed as described (32). Membranes were incubated with anti-tapasin serum and HRP-conjugated anti-rabbit Ab and visualized by ECL (Amersham Pharmacia Bio- tech, Piscataway, NJ).

Deconvolution fluorescence microscopy

Cells fixed in 4% paraformaldehyde in PBS were washed with 0.25% NH4Cl in PBS and permeabilized with 0.2% saponin in PBS-1% BSA. Coverslips were mounted in SlowFade Light (Molecular Probes, Eugene, OR) and imaged on a DeltaVision deconvolution system (Applied Precision), using a 1.4 numerical aperture 100× Zeiss Plan-apochromat objective. Images were collected with a 12-bit CH300-cooled charge-coupled device (Roper Scientific, Trenton, NJ) and out-of-focus light was subtracted using the softWoRx software (Applied Precision, Issaquah, WA).

Flow cytometry, FRAP measurements, and FRET microscopy

Cells were stained with mAb W6/32 at 4°C and analyzed by flow cytometry, as described (26). The lactacystin and peptide treatments, lateral diffusion measurements at 37°C by FRAP, and FRET measurements were as detailed in our earlier papers (26, 32). FRET was calculated from five 10 × 10-pixel (680 × 680 nm) nuclear envelope regions per cell as the percent increase in CFP fluorescence after YFP photobleaching, as described (26).

Results and Discussion

Because tapasin contains a C-terminal KKKXX motif (3, 12), we fused YFP to its N terminus. The YFP tag did not interfere with tapasin function. Expression of the chimeric molecule in tapasin-deficient .220 cells restored MHC class I surface expression (Fig. 1A) and association of MHC class I heavy chains with TAP (Fig. 1B). The level of MHC class I expression was proportional to the level of YFP-tagged tapasin over a 10- to 20-fold range of tapasin expression (data not shown). In digitonin lysates of HeLa cells, most molecules of YFP-tapasin and endogenous tapasin were bound to TAP (Fig. 2A), similarly to endogenous tapasin in non-transfected cells (31). There was a small population of TAP-free molecules; this was also observed for native tapasin in overexposed gels (Fig. 2A, inset). In addition to TAP, YFP-tapasin also associated with calnexin, calreticulin (Fig. 2B) and MHC class I heavy chains (Fig. 2C).

Deconvolution microscopy showed that YFP-tapasin localized exclusively to the ER (Fig. 3A). In pulse-chase experiments, the YFP-tapasin carbohydrates remained fully sensitive to endo H digestion, indicating that they were never processed by medial Golgi enzymes (Fig. 3B, upper gel). At steady state, endogenous and YFP-tagged tapasin molecules were also fully sensitive to endo H digestion (Fig. 3B, lower gel). To see whether tapasin localization to the ER was due to retrieval from earlier post-ER compartments (i.e., ERGIC, cis-Golgi), the transfected cells were incubated at 15°C or treated with nocodazole. Incubation at 15°C leads to the formation of a nonphysiological hybrid compartment resulting from the fusion of ER exit sites with the ERGIC (33). Nocodazole
YFP-tapasin (green) and ERGIC-53 (red) labeled with [35S]methionine and chased in nonradioactive medium for 16 h. B, Upper gel, HeLa cells expressing YFP-tapasin were metabolically labeled with [35S]methionine and chased in nonradioactive medium for 2 h at 37°C (D). The images are overlays of YFP-tapasin (green) and ERGIC-53 (red) fluorescence. Scale bar, 10 μm. B, Upper gel, HeLa cells expressing YFP-tapasin were metabolically labeled with [35S]methionine and chased in nonradioactive medium for 16 h. The cells were lysed in 0.5% Triton X-100, immunoprecipitated with anti-tapasin serum and digested with Endo H. R and S refer to the expected endo H-resistant and the endo H-sensitive forms of the proteins, respectively. Lower gel, HeLa cells expressing YFP-tapasin were lysed in 0.5% Triton X-100. Mock treated and endo H-treated lysates were run on SDS-PAGE and blotted with anti-tapasin antiserum.

causes the accumulation of secretory cargo and proteins that cycle between the ER and the ERGIC at ER exit sites consequent to microtubule depolymerization (33). YFP-tapasin was excluded from the ER exit sites, even after incubation at 15°C (Fig. 3C) or treatment with nocodazole (Fig. 3D), whereas the ERGIC marker ERGIC-53 accumulated in the hybrid compartment after incubation at 15°C (Fig. 3C) or at discrete ER exit sites after nocodazole treatment (Fig. 3D). These data are not consistent with ER retention of tapasin by tight binding to microtubules or by continuous retrieval from a post-ER compartment. They also argue against rapid tapasin cycling between the ER and the ERGIC as a possible mechanism for retrieving suboptimally loaded class I molecules.

YFP-tapasin diffusion was high (3.2 ± 0.5 × 10−9 cm² s⁻¹), comparable with the diffusion of A2-YFP (Table I). Because treatment with lactacystin decreased A2-YFP diffusion as a result of an increased A2-YFP fraction bound to TAP (Table I; Ref. 26), its effect on YFP-tapasin was also investigated. Lactacystin treatment did not affect YFP-tapasin diffusion (Table I). Because FRAP of lactacystin-treated cells detected TAP binding by a fraction of the total A2-YFP, it should have detected an increased fraction of TAP binding by YFP-tapasin in these cells. The lack of effect of lactacystin shows that at steady state, all tapasin-binding sites at TAP are already occupied and that this association is not static but involves rapid cycles of association and dissociation. Besides associating with the peptide-loading complex (TAP1/2, MHC class I, calreticulin, ERp57), tapasin molecules are also known to form complexes with TAP alone and with TAP bound to calnexin and ERp57 (31). Lactacystin would not change the diffusion coefficient (D) observed for YFP-tapasin if most tapasin molecules are dynamically bound to subcomplexes of TAP that remain free of MHC dimers, whereas only a small fraction forms a stable bridge between TAP and MHC class I. Overall, the diffusion data indicate that the mechanism of tapasin retention in the ER is dynamic and does not appear to involve stable interactions with TAP or an ER matrix. Similar observations have been made for the ER retention of misfolded vesicular stomatitis virus G protein-GFP (25).

FRET between fluorescent tapasin molecules depended on the YFP:CFP ratio and was insensitive to the YFP surface density both at steady state (Fig. 4, A and C) and after treatment with lactacystin (Fig. 4, B and C). These characteristics are hallmarks of a clustered

Table 1. Diffusion coefficients (D) mobile fraction (R) and 95% confidence limits (CL) for YFP-tapasin and A2-YFP

<table>
<thead>
<tr>
<th>Molecule</th>
<th>Treatment</th>
<th>D (× 10⁻⁹ cm² s⁻¹)</th>
<th>R (%) ± 95% CL</th>
<th>95% CL</th>
</tr>
</thead>
<tbody>
<tr>
<td>YFP-tapasin only</td>
<td></td>
<td>3.2 ± 0.5</td>
<td>88 ± 4</td>
<td></td>
</tr>
<tr>
<td>YFP-tapasin + untagged</td>
<td>Lactacystin</td>
<td>3.1 ± 0.3</td>
<td>80 ± 3</td>
<td></td>
</tr>
<tr>
<td>HLA-A2</td>
<td></td>
<td>3.1 ± 0.3</td>
<td>85 ± 4</td>
<td></td>
</tr>
<tr>
<td>A2-YFP</td>
<td>Lactacystin</td>
<td>2.1 ± 0.3</td>
<td>78 ± 6</td>
<td></td>
</tr>
</tbody>
</table>

FIGURE 4. All tapasin molecules are clustered. In cells expressing tagged tapasin, FRET was largely insensitive to YFP concentration but depended on the ratio of YFP to CFP. YFP:CFP = 1:1 (□), YFP: CFP = 2:1 (○), YFP: CFP = 3:1 (●) at steady state (A) and after treatment with 100 μM lactacystin (B). C, The mean FRET (%) for YFP concentration in the range 1500–2500 fluorescence units for YFP:CFP ratios of 2:1 and 3:1, as well as the 98% confidence limits (bars) were plotted for untreated cells (□; a) and for cells treated with lactacystin (□; b).
fluorophore population (26, 34). The lack of effect of lactacystin suggests that at least some of the clusters are located at TAP.

In conclusion, tapasin retention in the ER does not involve continuous retrieval from post-ER compartments and is not mediated by static associations with microtubules or with an ER protein matrix. If tapasin is excluded from ER-budding vesicles by forming large clusters >60–80 nm in diameter (the size of a small coated vesicle; Ref. 35), then D should be significantly lower than that of export-competent MHC class I molecules (see Ref. 36). However, in HeLa cells, D of tapasin (3.2 ± 0.5 × 10⁻⁹ cm² s⁻¹) closely resembles D of peptide-loaded HLA-A2 molecules (2.5–3.5 × 10⁻⁹ cm² s⁻¹; Ref. 26). Therefore, we believe that tapasin forms dynamic oligomers that are not recognized by the ER export machinery. This interpretation is consistent with lack of phenylalanine at positions −1 and −2 of the C'-terminal sequence (KKKAE) of tapasin. Phenylalanine-dependent interactions with COPII have been shown for ERGIC-53 and the p24 protein family (36, 37); alanine substitution (KKA) results in ER retention (19). The potential clustering of tapasin and empty or suboptimally loaded MHC class I molecules might ensure class I retention in the ER until loading with high affinity peptides. However, such complexes could not be directly demonstrated because FRET between YFP-tapasin and N-terminally tagged A2-CFP or A2-T134-CFP was below 10% even at the highest YFP:CFP ratio (data not shown), possibly because direct binding involves the tapasin N terminus (5) and might place the two fluorescent tags out of FRET range. Because MHC class I molecules cluster after peptide loading (26), perhaps as part of the mechanism for their export from the ER, the clamping of tapasin in such clusters may reduce the multivalency of potential interactions between COPII and the cytoplasmic tails of MHC class I molecules or of their cargo receptors (38).

Acknowledgments
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References