Tapasin Increases Efficiency of MHC I Assembly in the Endoplasmic Reticulum but Does Not Affect MHC I Stability at the Cell Surface

Maya W. Everett and Michael Edidin

*J Immunol* 2007; 179:7646-7652; doi: 10.4049/jimmunol.179.11.7646

http://www.jimmunol.org/content/179/11/7646

References This article cites 41 articles, 21 of which you can access for free at:

http://www.jimmunol.org/content/179/11/7646.full#ref-list-1

Why *The JI*? Submit online.

- **Rapid Reviews!** 30 days* from submission to initial decision
- **No Triage!** Every submission reviewed by practicing scientists
- **Fast Publication!** 4 weeks from acceptance to publication

*average

Subscription Information about subscribing to *The Journal of Immunology* is online at:

http://jimmunol.org/subscription

Permissions Submit copyright permission requests at:

http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts Receive free email-alerts when new articles cite this article. Sign up at:

http://jimmunol.org/alerts

*The Journal of Immunology* is published twice each month by The American Association of Immunologists, Inc., 1451 Rockville Pike, Suite 650, Rockville, MD 20852
Copyright © 2007 by The American Association of Immunologists All rights reserved.
Print ISSN: 0022-1767 Online ISSN: 1550-6606.
Tapasin Increases Efficiency of MHC I Assembly in the Endoplasmic Reticulum but Does Not Affect MHC I Stability at the Cell Surface

Maya W. Everett and Michael Edidin

Cell surface expression of MHC I molecules reports the internal health of a cell by presenting an 8–10 amino acid peptide to surveillance by CD8+ T cells and NK cells. Tapasin is a chaperone dedicated to MHC I assembly within the endoplasmic reticulum (ER) and is critical to MHC I cell surface expression. Tapasin-knockout mice have reduced ability to mount a recovery after photobleaching. In contrast to earlier reports, all three constructs localize to the endoplasmic reticulum (ER), though soluble tapasin is more mobile than wild type and N300 tapasin, which does not interact with MHC I. In contrast to earlier reports, all three constructs localize to the endoplasmic reticulum (ER), though soluble tapasin is more mobile than wild type and N300. Soluble tapasin does not increase MHC I surface levels to the same extent as wild type, which suggests that proximity to TAP is necessary for full tapasin function. N300 acts as a dominant-negative perhaps by blocking wild-type tapasin access to TAP. None of the constructs affects MHC I stability at the cell surface, although stability of ER resident MHC I is decreased in tapasin-negative cells. We propose that tapasin acts primarily to increase efficiency of assembly of MHC I within the ER. The Journal of Immunology, 2007, 179: 7646–7652.

Materials and Methods

Cell lines

HeLa cells were maintained in DMEM (Mediatech) supplemented with 10% heat-inactivated FBS (HyClone) and 2 mM l-glutamine (Invitrogen Life Technologies). T2 cells lack TAP whereas T1 cells are TAP positive (23). The 721.220 cell line lacks tapasin and has been stably transfected with HLA-B8 (24), HLA-B2705, and HLA-B4402 (7).
DNA constructs

We previously made N-terminal-tagged cyan fluorescent protein (CFP)-wild-type and yellow fluorescent protein (YFP)-wild-type tapasin (17). We generated monomeric CFP and YFP by introducing A243K, which corresponds to the A206K mutation that abolishes dimerization of these fluorescent proteins at high concentrations (25). Fluorescent deletion tapasin constructs were designed by PCR amplification of the desired regions of the tapasin construct and reinsertion into the original plasmid with the tapasin sequence removed via restriction digest. The tandem tapasin construct was engineered by PCR amplification of the CFP plus linker sequence from the CFP-tapasin construct and then inserted into the YFP-tapasin construct so that the YFP is linked to the CFP, which is then linked to the tapasin sequence. All primers were synthesized by Integrated DNA Technologies. All constructs were sequenced by Johns Hopkins University Core DNA Analysis Facility. YFP-labeled HLA-A2 has been described (26).

Microscopy and colocalization measurements

HeLa cells were plated on coverslips and transfected using Fugene 6 transfection reagent (Roche). Cells were fixed and stained as described (17). The Golgi complex was visualized with a rabbit polyclonal Ab against the Golgi marker giantin (Covance Research Products) and a goat anti-rabbit IgG conjugated to Alexa Fluor 633 (Molecular Probes). Cells were mounted using the SlowFade antifade kit (Molecular Probes).

Images were collected using a Zeiss LSM 510 confocal microscope with a 63X objective. Colocalization analyses were performed with MIPAV software (27). Background subtraction was performed before colocalization analysis using the orthogonal regression tool. Percent colocalization refers to the percentage of the fluorophores at high concentrations (25). Fluorescent deletion tapasin constructs were designed by PCR amplification of the desired regions of the tapasin construct and then inserted into the YFP-tapasin construct so that the YFP is linked to the CFP, which is then linked to the tapasin sequence. All primers were synthesized by Integrated DNA Technologies. All constructs were sequenced by Johns Hopkins University Core DNA Analysis Facility. YFP-labeled HLA-A2 has been described (26).

Flow cytometry, MHC I surface expression, and stability measurements

721.220 cell lines were transiently transfected by electroporation (BTX Electro Cell Manipulator 600). MHC I surface molecules were labeled with the mouse mAbs KE2 (29) or W6/32 (30), purified from hybridoma supernatants using Protein-A affinity chromatography, and directly conjugated to Cy5 with the Amersham Cy5 mono-reactive dye pack (GE Healthcare). Cells were washed in cold PBS before staining with saturating levels of Ab (determined by titration curves). Cells were washed again in cold PBS to remove excess Ab. For measurements with a secondary Ab, after staining cells with saturating levels of unlabeled W6/32, cells were washed with cold PBS and then stained with goat anti-mouse IgG conjugated to Alexa Fluor 633 (Molecular Probes) and then washed in cold PBS. Data were collected on a FACSCalibur (BD Biosciences). For MHC I stability measurements, cells were treated with 5 μM brefeldin A (BFA) (Sigma-Aldrich). Release of unstable and/or empty MHC I to the cell surface was measured mobility by FRAP. YFP-soluble tapasin had a higher mobility than YFP-wild type and YFP-N300 in the TAP-positive cell lines HeLa and T1, but all three constructs had the same mobility in the TAP-negative cell line T2 (Table I). Although mobility fraction values were different between the cell lines, the trend was the same in TAP-positive cells and different in TAP-negative cells. Although YFP-N300 had a lower mobility than YFP-wild-type tapasin in HeLa cells, this trend was not seen in T1 or T2 cells and may be a cell-specific effect.

Results

Wild-type and soluble tapasin colocalize in the ER and neither construct colocalizes with a Golgi complex marker

It has been reported that soluble tapasin is secreted whereas wild-type tapasin maintains an ER localization (16–18, 22). Using our fluorescent tapasin constructs, we found no evidence for secretion of soluble tapasin. Both colors of wild-type and soluble tapasin colocalize in the ER (Fig. 1) and have the same colocalization coefficients (80–85%) as tandem tapasin, which has CFP and YFP physically linked (data not shown). Neither wild-type nor soluble tapasin colocalized with a Golgi complex marker (Fig. 2, A and B). In contrast, there was significant colocalization of a YFP-tagged MHC I molecule HLA-A2 with the Golgi (Fig. 2C), consistent with the traffic of this molecule through the Golgi to the cell surface. Whereas most YFP-tagged HLA-A2 expressing cells have 20–30% of the YFP localized to the Golgi, there is <10% colocalization of YFP-wild-type or YFP-soluble tapasin with the Golgi marker (Fig. 2D). Thus most soluble tapasin is retained in the ER.

Soluble tapasin is more mobile than wild type and N300 in the presence of TAP

Because soluble tapasin does not bind TAP (22), we expected the mobility of soluble tapasin in the ER to differ from that of wild type and N300, which do bind TAP (4, 21). Although soluble tapasin is not a membrane protein, measurements of its mobile fraction should be independent of membrane attachment. We measured mobility by FRAP. YFP-soluble tapasin had a higher mobility than YFP-wild type and YFP-N300 in the TAP-positive cell lines HeLa and T1, but all three constructs had the same mobility in the TAP-negative cell line T2 (Table I). Although mobility fraction values were different between the cell lines, the trend was the same in TAP-positive cells and different in TAP-negative cells. Although YFP-N300 had a lower mobility than YFP-wild-type tapasin in HeLa cells, this trend was not seen in T1 or T2 cells and may be a cell-specific effect.

Tapasin proximity to TAP increases MHC I surface expression

The mobility differences we observed for the YFP-wild-type and YFP-soluble tapasin constructs corresponded to differences in their function. YFP-wild-type, YFP-soluble, or YFP-N300 tapasin were transfected into the tapasin-negative cell line 721.220, stably expressing the tapasin-dependent allele HLA-B4402. YFP-wild-type and YFP-soluble tapasin transfections increased MHC I surface levels in proportion to the amount of tapasin expressed. At higher levels of expression, both constructs reached a level of saturation.
in which increased expression did not result in increased MHC I surface levels (Fig. 3A). This saturating level of YFP expression was used for all additional experiments. YFP-soluble tapasin did not increase MHC I surface levels to the same extent as YFP-wild-type tapasin (Fig. 3A). As expected from previous work, transfection with YFP-N300 tapasin did not significantly increase MHC I surface levels (Fig. 3A) (21). We repeated this experiment with two other MHC I alleles: B2705 and B8 (Fig. 3B). We observed the same trends for B2705 and B8 as for B4402 (Fig. 3B). Consistent with its reported tapasin independence (7), B2705 showed the smallest increase in MHC I surface expression after transfection of YFP-wild-type tapasin and had the highest levels of surface MHC I after transfection of YFP-N300 or mock transfection (Fig. 3B).

Table 1. YFP-wild-type and YFP-N300 tapasin have limited mobility due to interactions with TAP

<table>
<thead>
<tr>
<th>Transfection</th>
<th>HeLa Mf (%)</th>
<th>T1 Mf (%)</th>
<th>T2 Mf (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>YFP-WT</td>
<td>52.3 ± 1.6</td>
<td>34.8 ± 0.9</td>
<td>30.4 ± 1.2</td>
</tr>
<tr>
<td>YFP-Sol</td>
<td>63.8 ± 1.8***</td>
<td>42.4 ± 1.0***</td>
<td>35.3 ± 1.3</td>
</tr>
<tr>
<td>YFP-N300</td>
<td>42.8 ± 1.3</td>
<td>33.3 ± 1.0</td>
<td>33.7 ± 1.3</td>
</tr>
</tbody>
</table>

*Mobile fractions of YFP-wild-type, YFP-soluble, or YFP-N300 tapasin in HeLa, T1, or T2 cells were measured by FRAP. Data are combined from 3 experiments with N = 30 and shown with standard errors. (**p, < 0.0001).

N300 is a dominant-negative when coexpressed with wild-type tapasin

N300 tapasin does not interact with MHC I but does interact with TAP (21). This interaction with TAP correlates with our observation of its reduced mobility in TAP-positive cells (Table 1). We expected that binding of N300 to TAP would block binding of wild-type tapasin and interfere with its function. YFP-N300 tapasin transfection of 721.220 cells stably transfected with unlabeled wild-type tapasin decreased surface expression of B4402, B2705, and B8 (Fig. 4). Transfection of YFP-N300 does not alter MHC I surface expression in tapasin-negative cells (Fig. 3). Expression of B2705, which is reported to be the least tapasin-dependent of the three HLA alleles (7), was least affected when YFP-N300 was coexpressed with wild-type tapasin (Fig. 4).

**Tapasin does not affect stability of surface MHC I molecules**

Though the wild-type, soluble, and N300 tapasin constructs have different effects on levels of surface MHC I expression, we found that they do not affect the surface lifetimes of these molecules at 37°C (Fig. 5). Although B4402 has low expression levels in the absence of tapasin (Fig. 3), these levels were still significantly above background and allowed for accurate measurements of decay curves (data not shown). These stability measurements were in contrast to previous work which has reported that MHC I surface lifetimes are reduced in the absence of wild-type tapasin or the presence of soluble tapasin (1, 8, 9, 11, 18, 31). This discrepancy may lie in the fact that previously reported data were collected using indirect immunofluorescence, whereas we used directly conjugated primary Ab. Additionally, we used the KE2 mouse mAb, which we have found to bind the same epitope as the W6/32 mouse mAb that was primarily used in previous work with human cell lines. Repeating our stability measurements using directly conjugated W6/32 showed no difference in surface stabilities between YFP-wild-type tapasin and mock-transfected 721.220 cells (data not shown). However, use of unlabeled W6/32 followed by fluorescent secondary Ab verified that this technique could qualitatively yield opposite results, although we observed higher experimental error, which revealed these results as not statistically significant (data not shown).
Tapasin reduces the fraction of empty and/or unstable MHC I molecules in the ER

Incubation of cells at 25°C stabilizes empty MHC I molecules and allows them to reach the cell surface (32). We used this treatment to investigate whether tapasin reduced the fraction of empty MHC I molecules in the ER. After transfection with YFP-wild-type tapasin, cells were cooled to 25°C for 5 h before measuring the stability of the MHC I surface molecules over time at 37°C. All three MHC I alleles had longer lifetimes on the cell surface when assembled in the presence of YFP-wild-type tapasin than when assembled in the absence of tapasin (Fig. 6). These data show that tapasin-negative cells have a larger population of poorly assembled and/or empty MHC I within the ER compared with tapasin-positive cells. At biological temperatures, these molecules do not reach the cell surface because there is no difference between lifetimes of surface MHC I molecules from tapasin-positive or tapasin-negative cells that were not previously treated at 25°C (Fig. 5).

Discussion

We used fluorescent constructs to investigate how tapasin localization correlates with its function. Our data highlight the importance of tapasin proximity to TAP and its local peptide supply for optimal MHC I surface expression. Tapasin increases TAP stability and TAP peptide translocation (2, 21, 22, 33, 34), which indicates that tapasin contributes to increased peptide concentrations near the tethered MHC I. Wild-type tapasin, which tethers MHC I to TAP, enhanced MHC I surface expression. N300 tapasin, which binds TAP but not MHC I, reduced the effect of wild-type tapasin and acted as a dominant-negative apparently by competing for TAP binding sites. Soluble tapasin, which binds MHC I but not TAP, also enhances MHC I surface expression, although not to the same extent as wild type. Consistent with this finding, mutations in TAP that diminished tapasin binding are reported to reduce surface expression of MHC I (35). Other laboratories have also found that soluble tapasin seems to work less efficiently by taking more time to either assemble equivalent amounts of thermally stable MHC I molecules (31) or rescue MHC I surface levels after acid strip of existing surface MHC I (11). Recombinant tapasin in collaboration with ERp57 has been shown to act on the peptide binding groove to allow for peptide binding and editing (13–15); because soluble tapasin is ER localized, it may be that binding of soluble tapasin to MHC I allows for peptide binding, although this process is limited due to lower concentrations of local peptide supply.

Our imaging analyses of tapasin and its mutants differ from earlier reports. We find that a significant population of soluble tapasin is in the ER, and we cannot detect soluble tapasin in the Golgi. Previous work using pulse-chase methods reported secretion of soluble tapasin (18, 22). Pulse-chase measurements are more sensitive than microscope image analysis, and a small portion of the soluble tapasin population escaping into the secretory pathway could be missed by colocalization experiments. Wang and colleagues (19) have reported on similar work with a fluorescent tapasin construct in which the ER retention-retrieval motif was mutated by alanine substitution and showed that their construct colocalizes with the Golgi. Our contrasting observations may be due to differences in cell line, Golgi marker used, expression levels of the fluorescent construct, or the need for a transmembrane domain and cytoplasmic tail for ER export. We also note that image collection with a confocal microscope using large pinhole settings can lead to spurious colocalization due to thickness of optical sections (F. Abe and M. Edidin, unpublished observations); we maintained the smallest pinhole sizes possible for all colocalization measurements.

We also present the novel finding that tapasin does not affect the lifetime of surface MHC I molecules. This work contradicts a number of studies that have reported reduced cell surface lifetime of MHC I molecules assembled in the absence of tapasin or presence of soluble tapasin (1, 8, 9, 11, 18, 31). We propose that the difference in observations may be a result of use of a secondary Ab for measuring levels of MHC I surface expression. We found that
labeling with directly conjugated primary Abs yielded more accurate measurements of MHC I surface levels. Additionally, although one report noted similar MHC I surface expression levels between tapasin-positive and -negative cells (9), it has been shown that 6–12 times expression levels of MHC I in tapasin-deficient cells are needed to achieve similar MHC I surface levels in tapasin-positive cells (36). This level of MHC I expression may cause the peptide pool to be limiting and affect MHC I stability; hence, differences in expression levels of MHC I may also have contributed to the observed discrepancies between our data and some previous reports.

Another method that is used to measure stability of MHC I is immunoprecipitation of the native MHC I peptide complex from

**FIGURE 5.** YFP-tapasin constructs do not affect MHC I surface stability. 721.220.B4402 (A), 721.220.B8 (B), or 721.220.B2705 (C) cells were transiently transfected with YFP-wild-type, YFP-soluble, or YFP-N300 tapasin. Cells were treated with 5 μg/ml BFA for varying lengths of time. MHC I surface molecules were stained with Cy5-conjugated KE2 and surface levels were measured by flow cytometry. Decay curves are standardized to each transfection at time zero. Rate constants are shown with SEs (D).

**FIGURE 6.** Tapasin-negative cells have a higher percentage of unstable MHC I that do not reach the cell surface at steady state. 721.220.B4402 (A), 721.220.B8 (B), or 721.220.B2705 (C) cells were transiently transfected with YFP-wild-type tapasin or mock transfected. Cells were cooled to 25°C for 5 h before treatment with 5 μg/ml BFA for varying lengths of time. MHC I surface molecules were stained with Cy5-conjugated KE2, and surface levels were measured by flow cytometry. Decay curves are standardized to each transfection. The rate constants for the decay curves are shown with SEs (D).
heated cell lysates (1, 18, 31). In these experiments, molecules from both the cell surface and the ER are captured. If tapasin acts to assemble the MHC I subunits, assembly would be limited in tapasin-negative cells, and a large portion of unstable heterodimers lacking peptide would be present in the ER. These molecules would contribute to thermal instability in immunoprecipitation experiments, but they do not represent MHC I stability at the surface. Consistent with this idea, we found that when we stabilized ER resident MHC I complexes by cooling cells to 25°C and allowed their export to the cell surface, we observed a difference in MHC I surface stabilities at 37°C between tapasin-positive and tapasin-negative cells, indicating that there is a larger population of unstable MHC I in the ER of tapasin-negative cells than in the ER of tapasin-positive cells.

Although it has been proposed that retention of MHC I molecules in the ER is an important aspect of tapasin function (2, 8, 16), H2-M3, a MHC class Ib molecule, is efficiently retained in the ER in the absence of tapasin (37, 38). Similarly, we have found a large population of unstable ER resident MHC I in tapasin-negative cells that fail to reach the cell surface, which argues against tapasin functioning to retain MHC I in the ER. A number of past reports have provided evidence that tapasin expression alters the peptide repertoire bound by MHC I (13–15). Interestingly, although peptide editing should confer higher stability of the resulting MHC I complex, we do not observe differences in MHC I lifetimes at the cell surface between tapasin-positive and tapasin-negative cells. It may be that in the absence of tapasin, unloaded or suboptimally loaded MHC I are rapidly degraded before or during ER export (2). Tapasin function may also be compensated by other proteins that assist in MHC I peptide assembly and/or prevent unstable MHC I from reaching the cell surface. Protein disulfide isomerase may stabilize a peptide-receptive state of the MHC I heterodimer and allow for peptide editing (39). Additionally, we have identified Bap31 as a cargo receptor for export of MHC I to the secretory pathway (40). Recent work suggests that Bap31 contributes to quality control of MHC I peptide complexes by selectively retrieving MHC I destabilized by the lower pH of the ER-Golgi intermediate compartment (41). Although Williams and colleagues (42) have shown that Bap31 does not affect MHC I surface lifetimes, these measurements were performed after treatment at 26°C, which would allow the release of unstable MHC I and may bypass our proposed mechanism for Bap31. Protein disulfide isomerase, Bap31, and other unidentified proteins may ensure that only stable MHC I molecules reach the cell surface. Although our data do not show that tapasin increases MHC I stability at the cell surface, the efficiency of stable MHC I peptide assembly and subsequent cell surface expression cannot be matched by other proteins in the absence of tapasin. As mentioned, loss of tapasin has many detrimental effects on immune system mechanisms dependent on MHC I in mice and humans (1–3). Our data suggest that these effects are caused by a decrease in MHC I surface levels and not by a decrease in MHC I stability.

Acknowledgments

We thank Dr. Peter Cresswell (Yale University) for providing the 721,220 cell lines, Edward Perkins (Johns Hopkins University Integrated Imaging Center), and Drs. David Fooksman, Saame Raza Shaih, and Jason Weil for helpful discussions and technical expertise.

Disclosures

The authors have no financial conflict of interest.

References


