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The Binding of TAPBPR and Tapasin to MHC Class I Is Mutually Exclusive

Clemens Hermann,* Lisa M. Strittmatter,* Janet E. Deane,† and Louise H. Boyle*

The loading of peptide Ags onto MHC class I molecules is a highly controlled process in which the MHC class I–dedicated chaperone tapasin is a key player. We recently identified a tapasin-related molecule, TAPBPR, as an additional component in the MHC class I Ag-presentation pathway. In this study, we show that the amino acid residues important for tapasin to interact with MHC class I are highly conserved on TAPBPR. We identify specific residues in the N-terminal and C-terminal domains of TAPBPR involved in associating with MHC class I. Furthermore, we demonstrate that residues on MHC class I crucial for its association with tapasin, such as T134, are also essential for its interaction with TAPBPR. Taken together, the data indicate that TAPBPR and tapasin bind in a similar orientation to the same site on MHC class I. In the absence of tapasin, the association of MHC class I with TAPBPR is increased. However, in the absence of TAPBPR, the interaction between MHC class I and tapasin does not increase. In light of our findings, previous data determining the function of tapasin in the MHC class I Ag–processing and presentation pathway must be re-evaluated. The Journal of Immunology, 2013, 191: 5743–5750.

The MHC class I Ag-processing and presentation pathway ensures the efficient and stable presentation of peptide Ag at the cell surface for immunological monitoring, resulting in the elimination of viral infections and tumorigenic cells. Folding and peptide loading of MHC class I H chain/β2m heterodimers in the endoplasmic reticulum (ER) are assisted by the MHC class I–specific chaperone tapasin, as well as a number of generic chaperones, such as calnexin and calreticulin. Tapasin simultaneously binds to peptide-receptive MHC class I heterodimers and to TAP, localizing MHC class I to a concentrated source of newly degraded antigenic peptides (1–5). There is also evidence that tapasin optimizes or edits the peptides presented on MHC class I by facilitating exchange of suboptimal peptides for higher-affinity cargo (6–10).

The binding interface between tapasin and MHC class I is emerging. The N-terminal domain of tapasin is essential for association and peptide loading of MHC class I (11). By comparing the sequences of tapasin from different species and screening mutant tapasin molecules, Dong et al. (12) identified a region of the N-terminal domain of tapasin that interacts with MHC class I. This cluster of residues on tapasin includes E185, R187, Q189, H190, L191, K193, L250, and Q261 defined by the panel of tapasin TN mutants (TN3, TN4, TN5, TN6, TN7). This region of tapasin is predicted to bind a loop consisting of residues 128–136 below the α2-1 helix of the MHC class I heterodimer (12–15). Residues in the predicted contact site in MHC class I (e.g., T134) are essential for incorporation of MHC class I into the peptide loading complex and efficient peptide loading (13–17). A second interaction point between tapasin and the MHC class I H chain involves residues 333–342 in the C-terminal Ig-like domain of tapasin (18–21), which are predicted to bind residues 222–229, situated in a β strand in the α3 domain of the MHC class I heterodimer (20, 22–25).

A tapasin-related protein (TAPBPR) is encoded on chromosome 12 in an MHC paralogous region by the TAPBPL gene (26). Although the amino acid sequence of TAPBPR is only 22% identical to tapasin, TAPBPR also binds to MHC class I H chain/β2m heterodimers in the ER (27). However, in contrast to tapasin, human TAPBPR does not associate with TAP, ERp57, or calreticulin and is not essential for peptide loading onto MHC class I molecules. TAPBPR decreases the rate at which MHC class I molecules mature through the secretory pathway (27). Although it is not a component of the peptide loading complex, TAPBPR is necessary to maintain prolonged contact of MHC class I with the peptide loading complex, a role that might be important for peptide selection by MHC class I molecules.

Given our recent identification of TAPBPR as a second MHC class I–specific component in the Ag-presentation pathway, our aim was to investigate how TAPBPR interacts with MHC class I.

Materials and Methods

Homology modeling of TAPBPR

A model for the structure of TAPBPR was generated using the Fold and Function Assignment System based on a profile–profile matching algorithm (28, 29). Tapasin was identified as the closest structural homolog available in the Protein Data Bank, and its structure (PDB-ID 3F8U) (12) was used as a template to generate a model for TAPBPR using the program SCWRL4 to predict and optimize side-chain conformations (30). The model was built for only the luminal domains of TAPBPR. Figures were generated with PyMOL Molecular Graphics System, Version 1.3 (Schrodinger).

Cell culture

HEK-293T, HeLa, and KBM-7 cells were maintained in DMEM, RPMI 1640, and IMDM media (Life Technologies), respectively, supplemented with 10% FCS, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37°C and 5% CO2. To induce expression of endogenous TAPBPR, cells were treated with 50 U/ml IFN-γ (Roche) at 37°C for 48 h.

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The online version of this article contains supplemental material.

Abbreviations used in this article: ER, endoplasmic reticulum; shRNA, short hairpin RNA; WT, wild-type.

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Constructs

PKI-A2 encoding an N-terminally GFP-tagged HLA-A2 molecule was described previously (31). Full-length untagged TAPBPR and untagged HLA-A2 were cloned into pCR-Blunt II-TOPO. Site-directed mutagenesis was performed to mutate specific residues in TAPBPR or HLA-A2 using Quik-Change II mutagenesis (Stratagene) along with the primers listed in Tables I and II. TAPBPR and its variants were subsequently cloned into the lentiviral vector pHRSIN-C56W-UHEM, producing TAPBPR under the SFFV promoter and the GFP derivative protein melanin under a ubiquitin promoter. GFP-A2 or untagged HLA-A2 and their variants were cloned into the lentiviral vector pHRSIN-PPT-SGW. For RNA interference, lentiviral short hairpin RNA (shRNA) plasmid V2LHS 155531 on the pGIPZ backbone (Open Biosystems) was purchased from Open Biosystems. The lentiviral plasmids were transfected into HEK293T cells using TransIT-293 (Mirus) along with pCMVRS.91 packaging vector and pMD-G envelope vector. These supernatants were used to produce stable transduced HeLa, KBM-7, and 721.221 cells. Cell sorting was performed using a BD Influx cell sorter to generate equally expressing transduced HeLa cells lines on the basis of their GFP expression levels. TAPBPR shRNA-depleted transduced cell lines were selected with puromycin. Tapasin-deficient KBM-7 cells were produced as described previously (32).

Abs

The following Abs were used: rabbit anti-TAPBPR R014 raised against aa 22–406 of human TAPBPR (27); rabbit anti-TAPBPR R021 raised against the cytoplasmic tail of human TAPBPR; a conformational specific mAb raised against aa 22–406 of human TAPBPR (PeTe4) (27); a mouse anti-TAPBPR raised against aa 23–122 of full-length human TAPBPR (absbutton411; Abcam); the tapasin-specific mAbs Pasta1 and Rgp48N (both kind gifts from Peter Cresswell, Yale University School of Medicine, New Haven, CT); rabbit anti-GFP (Ab290; Abcam); mouse anti-GFP (Roche); rabbit anti-calnexin (Enzo Life Sciences); mAb HC10, which recognizes HLA-A, HLA-B, and HLA-C containing a PxxWDR motif at aa 57–62 in the α1 domain (33, 34); mAb specific for conformational HLA-A2 and HLA-A68 (One Lambda); and HLA-A2-specific mAb BB7.2 (35). IgG1 and IgG2a isotype-control Abs were also used (Dako). Species-specific fluorescent and HRP-conjugated secondary Abs were from Molecular Probes and Dako, respectively.

Radiolabeling and pulse chase

Cells were starved in methionine and cysteine-free RPMI 1640 for 30 min at 37°C and labeled with [35S]methionine/cysteine Promix (Amersham Pharma) at 37°C for the indicated time. In TAPBPR half-life experiments, samples were chased at 37°C for 0–72 h in medium containing excess methionine/cysteine.

Immunoprecipitation and gel electrophoresis

Cells were lysed in methionine and cysteine-free RPMI 1640 for 30 min at 37°C and labeled with [35S]methionine/cysteine Promix (Amersham Pharma) at 37°C for the indicated time. In TAPBPR half-life experiments, samples were chased at 37°C for 0–72 h in medium containing excess methionine/cysteine.

Immunoprecipitation and gel electrophoresis

Cells were lysed in either 1% Triton X-100 (Sigma) or 1% digitonin (Calbiochem) in TBS (20 mM Tris-HCl, 150 mM NaCl, 5 mM MgCl2, 1 mM EDTA) containing 10 mM EDTA (pH 7.4) together with a TAPBPR polyclonal antiserum, followed by Western blotting with the primary Abs and the appropriate secondary Abs. For immunoblotting, proteins were transferred onto an Immobilon transfer membrane (Millipore) and then blocked with the indicated Abs. For radiolabeled samples, gels were fixed and dried and then images were obtained using a phosphor screen (Perkin-Elmer) or on film. PhosphorImager analysis was performed using Typhoon Trio variable mode imager (GE Healthcare) together with ImageQuant TL software. Graphs were generated using GraphPad Prism 6.

Flow cytometry

Following trypsinization, cells were incubated at 37°C in RPMI 1640 supplemented with 10% FCS to allow membrane recovery from trypsinization. Cells were stained at 4°C with MHC class I–specific Abs anti-A60 or BB7.2. Isotype-control Abs were used as negative controls. Ab were subsequently detected with species-specific Alexa Fluor 647 secondary Abs (Molecular Probes). Cells were analyzed on a BD Biosciences FACSCalibur four-color analyzer.

Results

MHC class I binding sites defined on tapasin are conserved on TAPBPR

A series of tapasin mutants (TN3, TN4, TN5, TN6, TN7) identified an MHC class I binding site on tapasin (12). To determine whether a similar MHC class I binding site is conserved on TAPBPR, we compared amino acid sequence alignments of human tapasin with human TAPBPR (Fig. 1A). Although tapasin and TAPBPR are only ∼22% identical, a number of the key residues on tapasin critical for binding to MHC class I are well conserved on TAPBPR (Fig. 1A). MHC I binding residues defined by the tapasin TN6 mutant (E185, R187, Q189, Q261) are completely conserved on TAPBPR, whereas those identified by the tapasin TN7 mutant (H190, L191, K193) are relatively conserved, sharing the histidine and a charged residue. A leucine at residue 250, characterized by the tapasin TN5 mutant, also contributes to MHC class I binding. This is an isoleucine in TAPBPR and is localized in a region with high sequence identity with the two molecules sharing the preceding residues EGTY.

An additional MHC class I interaction site has been suggested on the membrane proximal domain of tapasin consisting of residues 333–342 (18–21). The amino acid alignment suggests that, although R333 and S341 are conserved between the two proteins, there is considerable variation between the two molecules in this region. However, it is possible that Q334 and S335 in TAPBPR form similar hydrogen bonds with MHC class I and, therefore, could contribute to the binding.

We used the crystal structure of human tapasin obtained by Dong et al. (12) to create a homology model for TAPBPR. The residues that were shown to be important for MHC class I binding by tapasin lie on a well-conserved and highly ordered face of tapasin. Our homology model indicates that this face is structurally conserved in TAPBPR, allowing us to predict that MHC class I will bind TAPBPR in the same manner as it does tapasin (Fig. 1B, 1C). Many of these residues are highly conserved in TAPBPR across different species, supporting the possibility that they make up a functionally important region of TAPBPR (Supplemental Fig. 1).

Residues in the N-terminal and C-terminal domains of TAPBPR are involved in the association with MHC class I

To determine whether the conserved residues described above form a MHC class I binding site on TAPBPR, a panel of eight mutant TAPBPR molecules was made in which charge alterations were applied following the same concept as Dong et al. (12) (Table I). This panel of TAPBPR molecules was cloned into a lentiviral expression vector with a bicistronic GFP reporter and transduced into HeLa cells. Because endogenous TAPBPR expression is undetectable in HeLa cells (27), this cell line provides an ideal system for testing the effect of alterations to TAPBPR on its ability to bind to MHC class I. To produce stable HeLa cells expressing comparable TAPBPR levels, cell lines were sorted based on emerald expression encoded bisontrically from the TAPBPR protein. Western blotting for transduced TAPBPR revealed that all TAPBPR mutant molecules were stably expressed (Fig. 2A).

To analyze the interaction between the TAPBPR molecules and MHC class I, TAPBPR was immunoprecipitated from cell lysates using a TAPBPR polyclonal antiserum, followed by Western blotting for the MHC class I H chain using HC10. A strong association between TAPBPR and the MHC class I H chain was observed with wild-type (WT) TAPBPR (Fig. 2A). The TN4 mutant also bound strongly to MHC class I (Fig. 2A). However, no association was observed between TAPBPR and MHC class I using the TN5 or TN6 TAPBPR mutants (Fig. 2A). The TN7 mutant exhibited a reduced capacity to interact with MHC class I. Therefore, like tapasin, a conserved patch on the N-terminal domain of TAPBPR constitutes...
a major binding domain for MHC class I, with residues I261 (TN5), E205, R207, Q209, Q272 (TN6) and H210, K211, R213 (TN7) on TAPBPR contributing to the interaction. The C-terminal domain of TAPBPR also appeared to contribute to the interaction with MHC class I. No association was observed between TAPBPR and MHC class I using the TC2 or TC3 mutant TAPBPR molecules in which residue R335 or Q336/S337 was altered (Fig. 2A). In contrast, MHC class I could bind to TAPBPR in which nearby residue A339 (TC4) or S343/I344 (TC5) was mutated (Fig. 2A). The t\(_{1/2}\) of the five TAPBPR mutants that showed a reduction in binding to MHC class I was determined by pulse-chase analysis to determine whether the loss of association was a consequence of TAPBPR instability. Pulse-chase analysis revealed that the TAPBPR mutants TN5, TN7, and TC3 had a similar t\(_{1/2}\) to WT TAPBPR when expressed in HeLa cells (Fig. 2B). A difference was observed in radiolabeling efficiency for TN6. However, the labeled TAPBPR TN6 protein also appeared stable over time. The TC2 mutant was the only mutant that appeared to be less stable than WT TAPBPR (Fig. 2B). To further examine protein stability of the TAPBPR

Table 1. Panel of TAPBPR mutant molecules

<table>
<thead>
<tr>
<th>Name</th>
<th>Residues Mutated</th>
<th>Primers Used for Site-Directed Mutagenesis</th>
<th>Predicted TAPBPR Domain</th>
</tr>
</thead>
<tbody>
<tr>
<td>TN4</td>
<td>K22E, D23R</td>
<td>5'-GTCCTAGACTGTTTCCTTGGAGGACGCCGTTCAGGCTCTG-3'</td>
<td>N-terminal</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5'-CTATGCAGCCAGAGGTGTCAGTCGAGCCGCTCTG-3'</td>
<td>N-terminal</td>
</tr>
<tr>
<td>TN5</td>
<td>I261K</td>
<td>5'-GTCCTAGACTGTTTCCTTGGAGGACGCCGTTCAGGCTCTG-3'</td>
<td>N-terminal</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5'-CTATGCAGCCAGAGGTGTCAGTCGAGCCGCTCTG-3'</td>
<td>N-terminal</td>
</tr>
<tr>
<td>TN6</td>
<td>E205K, R207E, Q209S, Q272</td>
<td>5'-GTCCTAGACTGTTTCCTTGGAGGACGCCGTTCAGGCTCTG-3'</td>
<td>N-terminal</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5'-CTATGCAGCCAGAGGTGTCAGTCGAGCCGCTCTG-3'</td>
<td>N-terminal</td>
</tr>
<tr>
<td>TN7</td>
<td>H210S, K211E, R213</td>
<td>5'-GTCCTAGACTGTTTCCTTGGAGGACGCCGTTCAGGCTCTG-3'</td>
<td>N-terminal</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5'-CTATGCAGCCAGAGGTGTCAGTCGAGCCGCTCTG-3'</td>
<td>N-terminal</td>
</tr>
<tr>
<td>TC2</td>
<td>R213E</td>
<td>5'-GTCCTAGACTGTTTCCTTGGAGGACGCCGTTCAGGCTCTG-3'</td>
<td>C-terminal</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5'-CTATGCAGCCAGAGGTGTCAGTCGAGCCGCTCTG-3'</td>
<td>C-terminal</td>
</tr>
<tr>
<td>TC3</td>
<td>Q336D, S337D</td>
<td>5'-GTCCTAGACTGTTTCCTTGGAGGACGCCGTTCAGGCTCTG-3'</td>
<td>C-terminal</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5'-CTATGCAGCCAGAGGTGTCAGTCGAGCCGCTCTG-3'</td>
<td>C-terminal</td>
</tr>
<tr>
<td>TC4</td>
<td>A339D</td>
<td>5'-GTCCTAGACTGTTTCCTTGGAGGACGCCGTTCAGGCTCTG-3'</td>
<td>C-terminal</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5'-CTATGCAGCCAGAGGTGTCAGTCGAGCCGCTCTG-3'</td>
<td>C-terminal</td>
</tr>
<tr>
<td>TC5</td>
<td>S343R, I344T</td>
<td>5'-GTCCTAGACTGTTTCCTTGGAGGACGCCGTTCAGGCTCTG-3'</td>
<td>C-terminal</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5'-CTATGCAGCCAGAGGTGTCAGTCGAGCCGCTCTG-3'</td>
<td>C-terminal</td>
</tr>
</tbody>
</table>

The C-terminal domain of TAPBPR also appeared to contribute to the interaction with MHC class I. No association was observed between TAPBPR and MHC class I using the TC2 or TC3 mutant TAPBPR molecules in which residue R335 or Q336/S337 was altered (Fig. 2A). In contrast, MHC class I could bind to TAPBPR in which nearby residue A339 (TC4) or S343/I344 (TC5) was mutated (Fig. 2A). The t\(_{1/2}\) of the five TAPBPR mutants that showed a reduction in binding to MHC class I was determined by pulse-chase analysis to determine whether the loss of association was a consequence of TAPBPR instability. Pulse-chase analysis revealed that the TAPBPR mutants TN5, TN7, and TC3 had a similar t\(_{1/2}\) to WT TAPBPR when expressed in HeLa cells (Fig. 2B). A difference was observed in radiolabeling efficiency for TN6. However, the labeled TAPBPR TN6 protein also appeared stable over time. The TC2 mutant was the only mutant that appeared to be less stable than WT TAPBPR (Fig. 2B). To further examine protein stability of the TAPBPR
Overexpression of TAPBPR in HeLa cells results in down-regulation of MHC class I expression on the cell surface (27). This phenotypic effect was used as an assay to determine the effect of the TAPBPR mutants on MHC class I surface expression. WT TAPBPR resulted in a significant downregulation of HLA-A68 expression (Fig. 2C–E). The other TAPBPR mutants that bind to MHC class I (TN4, TN6, TC2, TC3) did not downregulate HLA-A68 expression (Fig. 2C–E). The other TAPBPR mutants that bind to MHC class I (TN4, TN7, TC4, TC5) downregulated MHC class I surface expression (Fig. 2E). However, they were not quite as efficient as WT TAPBPR (Fig. 2E). Surprisingly, the TN4 mutant, which appeared to show a strong association with MHC class I, as measured by coimmunoprecipitation with TAPBPR (Fig. 2A), resulted in only a 50% reduction in HLA-A68 surface expression compared with the 90% reduction observed with WT TAPBPR. Therefore, this mutant may have a more complex effect on TAPBPR function than originally suggested.

**Mutation of T134 on MHC class I inhibits TAPBPR binding**

The central region of the N-terminal domain of tapasin is predicted to bind to residues 128–136 of the MHC class I H chain. Mutation of these residues on MHC class I inhibits tapasin binding (12–15, 20). Given the importance of the central region of the N-terminal domain of TAPBPR in associating with MHC class I, we asked whether amino acids in the loop under the α2-1 domain of MHC class I were also required to associate with TAPBPR. Using an N-terminally tagged GFP HLA-A2 construct, we created a panel of mutant HLA-A2 molecules (Table II) that were expressed in HeLa cells. The GFP tag was used to differentiate between the endogenous HLA alleles in HeLa cells and the transduced HLA-A2, which runs at ~70 kDa. We reported previously that this N-terminal GFP tag did not significantly alter export rates or surface expression of HLA-A2 compared with untagged HLA-A2 (31). To induce TAPBPR expression, the HeLa cells were treated with IFN-γ (27). In TAPBPR immunoprecipitation experiments, an association was observed between TAPBPR and the GFP-A2 WT molecule (Fig. 3A). In contrast, no association was observed between TAPBPR and GFP-A2–T134K in which a threonine at position 134 was mutated to a lysine. In agreement with previously reported findings (13, 14), mutation of T134K reduced the expression of conformation HLA-A2 detected by BB7.2 to ~30% of WT-A2 in IFN-γ-induced HeLa cells (Fig. 3B). Other single-point mutations (D122R, E128R, W133T) in the loop under the α2-1 of MHC class I similarly affected the ability of TAPBPR to associate with HLA-A2 (Supplemental Fig. 3). The lysine residue at position 144, which points outward in the α2-1 helix, was also important for the association between TAPBPR and MHC class I H chain (Supplemental Fig. 3), whereas the lysine residue at position 146, which points in toward the peptide-binding groove, was not critical in the association between TAPBPR and MHC class I (Fig. 3A). As controls, immunoprecipitation experiments were performed in parallel for tapasin in the IFN-γ-treated HeLa panel. As expected, GFP-A2 WT bound to tapasin, but GFP-A2–T134K failed to associate (Fig. 3A). These findings were confirmed in reciprocal immunoprecipitation
experiments in which GFP-A2 was immunoprecipitated, followed by blotting for tapasin and TAPBPR (Fig. 3C).

Mutation of residues in MHC class I α3 domain inhibit TAPBPR binding

The C-terminal domain of tapasin is predicted to bind to residues 222–229 in the α3 domain of the MHC class I H chain (20, 22–25). To determine whether these residues on MHC class I were required for association with TAPBPR, two GFP-A2 constructs were made in which amino acids in this region were mutated (Table II). The GFP-A2 α3-1 mutant, in which four residues in the α3 domain were mutated (E222K, D223R, D227R, E229K), no longer bound to TAPBPR, demonstrating that this region is crucial for MHC class I to associate with TAPBPR (Fig. 3A, 3C). The GFP-A2 α3-2 mutant, in which only residues D227 and E229 were mutated, also did not bind to TAPBPR, refining the binding site further to involve these two amino acids. As expected, mutation to this region of the α3 domain also inhibited binding of tapasin to the MHC class I H chain (Fig. 3A, 3C). Again, cell surface expression of conformational HLA-A2 detected with BB7.2 was severely reduced in these mutants, which were no longer able to bind to tapasin or TAPBPR (Fig. 3B).

Mutant MHC class I molecules associate with β2m

To further verify the importance of T134 and residues in the α3 domain of MHC class I in the association with TAPBPR, untagged WT A2, T134K, or α3-2 A2 was expressed in the classical MHC class I+ cell line 721.221. In TAPBPR immunoprecipitation experiments, only untagged WT-A2 was found to associate with TAPBPR (Fig. 3D). Neither untagged T143K nor untagged α3-2 was able to associate with TAPBPR (Fig. 3D). These results using untagged HLA-A2 molecules expressed in 721.221 confirm the finding observed with the GFP-tagged HLA-A2 molecules expressed in HeLa cells.

Because β2m is crucial for the association of both TAPBPR and tapasin with the MHC class I H chain, the ability of the T134K and α3-2 mutant HLA-A2 molecules to associate with β2m was determined. Like WT A2, both T134K and α3-2 mutant HLA-A2 were found to associate with β2m when expressed in 721.221 (Fig. 3D). Therefore, the loss of interaction of the mutant HLA-A2 molecules with TAPBPR is not an indirect consequence of failure to associate with β2m.

The association of MHC class I with TAPBPR is increased in the absence of tapasin

Because TAPBPR and tapasin bind in a similar orientation to the same face of MHC class I, it is possible that they compete with each other for MHC class I binding. To investigate whether tapasin competes with TAPBPR for MHC class I, the association of MHC class I with TAPBPR was compared in WT and tapasin-deficient KBM-7 cells. Immunoprecipitation of total cellular TAPBPR revealed a significant increase in the association of MHC class I with TAPBPR in tapasin-deficient cells compared with WT cells (Fig. 4A). This increased association was quantified further in radiolabeled cells, which revealed an approximate 2-fold increase (1.97 ± 0.065) in the association of MHC class I with TAPBPR in the absence of tapasin (Fig. 4B). Therefore, tapasin can compete with TAPBPR for MHC class I binding.

The association of MHC class I with tapasin does not increase in the absence of TAPBPR

Next, we determined whether TAPBPR can compete with tapasin for MHC class I binding. If TAPBPR competes with tapasin for MHC class I binding, then the association of MHC class I with tapasin should be regulated negatively by TAPBPR expression (i.e., depletion of TAPBPR should increase the association of MHC I with tapasin, whereas overexpression of TAPBPR should decrease the association of MHC I with tapasin). However, tapasin immunoprecipitation experiments did not reveal an increase in MHC class I binding to tapasin upon TAPBPR depletion in KBM-7 (Fig. 4A) or HeLa (Fig. 4C) cells. In contrast, densitometry in radiolabeled cells revealed that the association between tapasin and MHC class I was reduced slightly (by 6.5 ± 0.02%) in the absence of TAPBPR (Fig. 4B). Supporting this finding, a slight decrease in the association between tapasin and MHC class I was observed in the absence of TAPBPR in steady-state immunoprecipitation experiments in HeLa cells (Fig. 4C). Finally, the inability of TAPBPR to compete with tapasin for MHC class I was confirmed in HeLa cells overexpressing TAPBPR, in which a slight, but consistent, increase in association was observed between tapasin and MHC class I compared with WT HeLa cells (Fig. 4C). Taken together, these results suggest that TAPBPR does not compete with tapasin for MHC class I.

Discussion

In this study, we show that TAPBPR binds in a similar orientation to the same face of MHC class I as does tapasin. Therefore, a single MHC class I molecule cannot bind tapasin and TAPBPR at the same time. Thus, TAPBPR and tapasin binding to MHC class I are mutually exclusive. In agreement with this, we showed previously that there was no association between tapasin and TAPBPR in...
immunoprecipitation experiments. Furthermore, tapasin bound to MHC class I in the absence of TAPBPR, and TAPBPR bound to MHC class I in the absence of tapasin (27).

FIGURE 3. Residues in the α2 and α3 domain of MHC class I are crucial for associating with TAPBPR. (A) TAPBPR or tapasin was isolated by immunoprecipitation using PeTe4 and Pasta1, respectively, from IFN-γ-treated HeLa cells stably expressing a panel of GFP-tagged HLA-A2 mutant molecules lysed in 1% digitonin–TBS. Western blot analysis was performed for TAPBPR (R021), tapasin (Rgp48N), or GFP-A2 (using anti-GFP) on lysates and immunoprecipitates, as indicated. The last lane on the TAPBPR and tapasin immunoprecipitation gels is a lysate from GFP-A2 WT cells. (B) Cytofluorometric analysis of HLA-A2 on HeLa cells stably transduced with selected GFP-A2 mutant molecules. All cell lines were treated with IFN-γ for 48 h before staining with the HLA-A2 conformational-specific Ab BB7.2. HeLa cells (gray dashed line), GFP-A2 WT (filled graph), GFP-A2–T134K (black line), and GFP-A2 α3-2 (gray line). (C) GFP-A2 was isolated by immunoprecipitation using a GFP-specific Ab from IFN-γ-treated HeLa cells stably expressing the panel of GFP-tagged HLA-A2 mutant molecules. Western blot analysis was performed for TAPBPR, tapasin, or GFP-A2 (using an anti-GFP Ab), as indicated. The last lane on the gel is a lysate from GFP-A2 WT cells. All experiments were repeated independently at least three times. (D) Untagged HLA-A2 WT, A2-T134K, or A2 α3-2 was expressed in the classical MHC class I cell line 721.221. After solubilization in 1% digitonin–TBS, immunoprecipitation was performed for TAPBPR (using mAb PeTe4) or β2m from precleared postnuclear lysates. Western blot analysis was performed for HLA-A2 using HCA2. Nontransfected 721.221 cells were included as a negative control (–).

FIGURE 4. The MHC I:TAPBPR association increases in the absence of tapasin, but the MHC I:tapasin association does not increase in the absence of TAPBPR. (A) Total cellular levels of TAPBPR and tapasin were isolated by immunoprecipitation with PeTe4 and Pasta1, respectively, from IFN-γ-treated WT, tapasin gene trap knockout, or KBM-7 cells depleted of TAPBPR by stable transduction with TAPBPR-specific shRNA lysed in 1% digitonin–TBS. Western blot analysis was performed for TAPBPR (R021), tapasin (Rgp48N), MHC class I (HC10 and HCA2), or calnexin (rabbit anti-calnexin). (B) IFN-γ-treated WT, tapasin gene trap knockout, or TAPBPR-depleted KBM-7 cells were radiolabeled with [35S]cysteine/methionine for 30 min, followed by immunoprecipitation of TAPBPR (using mAb PeTe4) or tapasin (using Pasta1). Signal intensity of the MHC class I H chain bands was determined by densitometry. To calculate the fold change in MHC class I association with tapasin or TAPBPR, the following calculation was used: MHC class I H chain signal intensity for test cell line/MHC class I H chain signal intensity for WT cell line. This was normalized to the fold change in signal intensity of immunoprecipitated protein (i.e., tapasin or TAPBPR). (C) Total cellular levels of TAPBPR and tapasin were isolated by immunoprecipitation with PeTe4 and Pasta1, respectively, from IFN-γ-treated HeLa-S, HeLa-S shTAPBPR, HeLa-M, or HeLa-M overexpressing WT TAPBPR cells lysed in 1% digitonin. Western blot analysis was performed for TAPBPR (R021), tapasin (Rgp48N), MHC class I (HC10 and HCA2), and calnexin (rabbit anti-calnexin). All experiments were repeated independently at least three times.

We previously demonstrated that overexpression of TAPBPR severely reduces surface levels of peptide-loaded MHC class I and increases MHC class I free H chain expression (27). Because overexpression of TAPBPR resembles the phenotype of a tapasin-deficient cell, superficially TAPBPR appears to oppose the function of tapasin in the MHC class I Ag-presentation system. This is highly reminiscent of the opposing effect of HLA-DO on the function of HLA-DM in the MHC class II Ag-presentation system (36–38), raising the possibility that TAPBPR is the equivalent of...
HLA-DO for the MHC class I system. It was shown recently that HLA-DO is a MHC class II mimic and binds tightly to HLA-DM, directly suppressing HLA-DM from interacting with MHC class II (39). Because there is no direct association of TAPBPR with tapasin, mechanistically TAPBPR does not appear to work in the same manner as HLA-DO. However, with TAPBPR and tapasin being oriented on MHC class I in a similar manner, an opposing role of TAPBPR could be envisaged by it competing directly with tapasin for MHC class I binding. However, this also does not appear to be the case because TAPBPR expression does not decrease the interaction between MHC class I and tapasin. Surprisingly, TAPBPR expression slightly increases the tapasin:MHC class I association. Thus, TAPBPR does not compete with tapasin for MHC class I binding but actually may cooperate with tapasin. However, tapasin competes with TAPBPR for MHC class I. Therefore, how can we explain the fact that MHC class I surface expression in HeLa cells overexpressing TAPBPR resembles tapasin deficiency? In both situations, the ratio of tapasin/TAPBPR is altered in favor of TAPBPR, implicating an increased association of MHC class I with TAPBPR in some of the phenotypic effects observed in tapasin-deficient cells.

In light of the discovery of TAPBPR as an additional chaperone oriented on MHC class I in the same way as tapasin, the precise function of tapasin in the Ag-presentation pathway now needs to be re-evaluated. Much of the work examining tapasin function has understandably been performed using either tapasin-deficient cells (6, 8–45) or mutant MHC class I molecules that no longer associate with tapasin (13, 14, 16, 23, 25). However, in tapasin-deficient cells, it is now apparent that TAPBPR is present and still capable of binding to MHC class I. In fact, MHC class I molecules exhibit increased binding to TAPBPR in the absence of tapasin. Therefore, the findings observed in cells lacking tapasin are likely to be highly reflective of TAPBPR function. More alarming is the issue of experiments in which MHC class I mutants were used, such as T134K, because it is clear from our data that it is not only the functional effect of tapasin on MHC class I that is lost, but also that of TAPBPR.

Our discovery of the interaction between TAPBPR and MHC class I helps to explain some of the conflicting data generated with regard to the function of tapasin over the past 2 decades. There is no doubt about the critical role played by tapasin in the loading of peptide onto MHC class I given the severe reduction in MHC class I surface expression in the absence of tapasin (2, 8, 40, 45, 46). However, there have been some discrepancies with regard to the function of tapasin as a peptide editor, a process in which low-affinity peptides are replaced with those exhibiting higher affinity (3, 6, 8–10, 17, 43, 47–49). It is likely that some of the conflicting data generated are due to the influence of TAPBPR on MHC class I. For example, the T134K mutation has a more severe effect on surface expression of conformational HLA-A2 than does the absence of tapasin (T134K-A2 is expressed at only 20% of WT A2 in C1R cells, whereas HLA-A2 in 721.220 cells is expressed at 50–60% of HLA-A2 in 721.221 cells) (13, 14, 41). Such differences are likely to be due to a lack of binding of TAPBPR to HLA-A2→T134K compared with an increased association between MHC class I and TAPBPR in the experiments using 721.220 cells.

Our findings also raise the question of why are there two MHC class I-specific chaperones in the Ag-presentation pathway oriented on MHC class I in a similar manner. Are there two alternative pathways of peptide loading or a single pathway that involves sequential engagement of the two related proteins? Is one protein primarily involved in peptide loading and the other in peptide editing? Is one the ER-resident MHC class I chaperone, whereas the other performs a similar function outside the peptide loading complex? We favor the idea of a single pathway for MHC class I peptide loading in which tapasin and TAPBPR represent sequential steps in the same pathway. We speculate that MHC class I first associates with tapasin to be loaded with peptide and then interacts with TAPBPR. An initial interaction of MHC class I with tapasin instead of TAPBPR could be influenced by subtle alterations in the form of MHC class I, accessory proteins, or spatial separation. If MHC class I sequentially engages with the two related proteins in this manner, TAPBPR could monitor the stability of MHC class I as it dissociates from the peptide loading complex. This is consistent with the increased binding of TAPBPR to MHC class I in the absence of tapasin, a condition that produces suboptimally loaded MHC class I. In this way, TAPBPR acts as a second quality control checkpoint or post–peptide loading complex gatekeeper for MHC class I. Given the orientation of TAPBPR on MHC class I, it is conceivable that TAPBPR is capable of peptide editing. In support of an influence of TAPBPR in peptide selection, TAPBPR slows down the anterograde transport of MHC class I, a property that was shown to allow MHC class I to efficiently optimize its peptide cargo (16, 27). Furthermore, TAPBPR prolongs the contact between MHC class I and the peptide loading complex, an event that is highly likely to alter peptide selection by MHC class I (27). Another question under investigation is whether tapasin and TAPBPR exhibit differential preference for specific HLA gene products. Separation of the function of tapasin from that of TAPBPR is needed to further elucidate the molecular mechanism governing peptide selection by MHC class I molecules.

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Disclosures

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