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Impact of beta 2-microglobulin on tapasin expression and covalent association

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Abstract

Cellular immunity is dependent on major histocompatibility complex (MHC) class I molecules enabling cytotoxic T cell recognition of malignant and infected cells. Loading of antigenic peptides onto MHC class I is assisted by a peptide-loading protein complex including tapasin. We found that tapasin expression is enhanced by beta 2-microglobulin via both transcriptional and post-transcriptional mechanisms. In addition, using conditions which preserve the tapasin-ERp57 disulfide-bonded conjugate, we demonstrated that beta 2-microglobulin increases tapasin-containing protein complexes, and reduces the level of MHC class I/ERp57 complexes lacking tapasin. Overall, our results provide a new perspective on the regulation of tapasin expression and association.

Keywords

antigen presentation; beta 2-microglobulin; D^b; ERp57; major histocompatibility complex class I; peptide-loading complex; tapasin

1. Introduction

MHC class I molecules are cell-surface receptors for cytotoxic T lymphocytes, allowing them to target and lyse abnormal cells. The binding of peptides, including those derived from viruses and tumor-associated proteins, to heterodimers of MHC class I heavy chains and beta 2-microglobulin (β_2m) light chains completes the assembly of MHC class I molecules. Peptide binding to MHC class I molecules is assisted by a group of proteins known as the peptide-loading complex. Members of the MHC class I peptide-loading

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complex include the transporter associated with antigen processing (TAP), the lectin chaperone calreticulin, the thiol oxidoreductase ERp57, and tapasin. Tapasin plays crucial roles in MHC class I molecule assembly and in shaping the peptide repertoire that is ultimately presented to cytotoxic T lymphocytes [1]. The importance of tapasin for effective cytotoxic T cell-mediated immunity is evidenced by the down-regulation of tapasin in various cancers [2,3]. Tapasin is also targeted by some viruses in order to evade immune detection [4,5]. Recent reports have provided some perspective on how the expression of tapasin itself is regulated. Characterization of the tapasin promoter has shown binding motifs for NF-κB, GATA, E2F1, p300, AP1, SP1, and IRF-1/2 [6,7]. The transcription of tapasin is induced by the cytokines interferon (IFN)-α, IFN-γ and TNF-α [2,8]. Here, we present our findings that tapasin protein levels are lower in cells lacking β_2m, and that tapasin protein expression is enhanced by the presence of β_2m. Furthermore, tapasin mRNA levels are greater in β_2m-expressing cells, as compared to β_2m-negative cells, but the difference in mRNA levels is not sufficient to account entirely for the difference in tapasin protein expression. Thus, the mechanisms underlying the effect of β_2m on tapasin expression involve both transcriptional and post-transcriptional processes. Furthermore, we observed that β_2m increases the level of peptide-loading complexes containing tapasin. In total, our findings demonstrate a new role for β_2m in influencing tapasin expression.

2. Materials and Methods

2.1 Cell lines, immunoprecipitations, and western blotting

To determine the effect of β_2m on tapasin expression, we used several cell lines differing in β_2m expression. The R1.1 cell line was derived from a C58 (H-2^k haplotype) mouse thymoma [9]. The R1E cell line was generated from R1.1 cells and has a homozygous mutation of the β_2m gene [10]. R1E cells stably transfected with D^b alone (R1E-D^b) or also transfected with mouse β_2m (R1E-D^b-β_2m) were generated by Dr. R. A. Flavell and co-workers [11]. Daudi is a human Burkitt's lymphoma cell line that lacks β_2m expression [12], which we used in our studies in comparison with a β_2m transfectant (Daudi-β_2m). Immunoprecipitations and western blots were done on cell lysates, using previously described procedures [13]. Protein bands were quantified using a Molecular Imager ChemiDoc XRS system with Quantity One 1-D Analysis Software (Bio-Rad).

2.2 Quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) analysis

For analysis of tapasin mRNA transcripts, the following primers were used: 5'-ACA CTG CGA GAT GAG CCG CT-3' (forward) and 5' -GGT CAG CAC CAC TGT TGC CA-3' (reverse). As a control, the level of mouse glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA was determined using the following primers obtained from PrimerBank (http://pga.mgh.hardvard.edu/primerbank/) (PrimerBank ID 6679937a1): 5'-AGG TCG GTG TGA ACG GAT TTG-3' (forward) and 5'-TGT AGA CCA TGT AGT TGA GGT CA-3' (reverse).

RNA was purified from cells using RNAzol-RT (Molecular Research Center), and after purification 500 ng of RNA was used to generate cDNA using the AccuScript High Fidelity 1st Strand cDNA Synthesis Kit (Stratagene). For each qRT-PCR reaction, 1μl of the cDNA reaction was combined with 10μM forward primer, 10μM reverse primer, 12.5μl RT^2 SYBR Green qPCR Master Mix (SuperArray Bioscience), and 10.5μl water. The qRT-PCR reactions were analyzed on a Cepheid SmartCycler using Cepheid software version 2.0c. The following thermal cycling program was used: 95°C, 900 s, then 39 cycles of 95°C, 30 s; 55°C, 30 s, and 72°C, 30 s. After completing the thermal cycling program, the following melting curve program was run: 60 to 95°C at 0.2°C per s.

The cycle threshold values were converted into relative expression levels using standard curves generated for the mouse tapasin and mouse GAPDH primers. The relative expression levels obtained from four tapasin and four glyceraldehyde 3-phosphate dehydrogenase (GAPDH) qRT-PCR analyses of a cDNA preparation were averaged. Next, the relative expression of tapasin was normalized to the relative expression of GAPDH for each line. The normalized relative expression of mouse tapasin in R1E cells was set as the control and used to calculate the change in mouse tapasin mRNA expression in the R1.1, R1E-D^b, and R1E-D^b-β_2m cells. According to the results of an F-test, the two-sample equal variance Student's *t*-test was used to determine the significance of the difference in mouse tapasin mRNA expression in R1E versus R1.1 cells. Results with R1E were compared to results with either R1E-D^b or R1E-D^b-β_2m by the two-sample unequal variance Student's t-test.

3. Results

3.1 Expression of β_2m augments murine tapasin protein levels

In our analysis of the R1.1, R1E, R1E-D^b, and R1E-D^b-β_2m cell lines, we noted that β_2m, alone or in conjunction with D^b, appeared to increase the amount of tapasin protein (Fig. 1a→e). The increase was particularly noticeable for free tapasin. Additionally, β_2m enhanced the expression of D^b heavy chain (Fig. 1a), consistent with previous reports [14]. Tapasin was also observed in ~145 kD disulfide-bonded complexes, similar in molecular mass to tapasin-containing complexes previously reported by our laboratory [13]. A second complex of ~110-120 kD, representing a conjugate between ERp57 and D^b, was found in D^b and ERp57 western blots of R1E-D^b and R1E-D^b-β_2m (Fig. 1a,c). This complex was also apparent in immunoprecipitates of D^b probed by anti-ERp57 (data not shown). In the presence of β_2m, the balance shifted toward the larger (~145 kD) complexes that included tapasin, whereas in the absence of β_2m the balance tilted toward the ERp57/D^b dimer (Fig. 1a,c). Studies by others have also detected ERp57-MHC class I disulfide-bonded dimers, and implicated ERp57 in the reduction of sub-optimally folded MHC class I prior to its degradation [15,16,17]. In support of this hypothesis, our findings show that the level of the ERp57-D^b conjugate (not including tapasin) is enhanced by the absence of β_2m, likely due to hampered D^b folding.

By densitometric quantification of western blots, tapasin protein was increased ~4× in R1.1 cells (endogenously expressing β_2m) and in cells that express transfected D^b and β_2m (R1E-D^b-β_2m), as compared to β_2m-negative R1E (Fig. 1e). Expression of transfected D^b (without β_2m) enhanced tapasin protein expression to ~2.5× that of R1E (Fig. 1e). In contrast, calreticulin expression was not increased by D^b and β_2m transfection into R1E cells (data not shown). We also compared tapasin expression in Daudi and Daudi-β_2m, and (as shown in Fig. 1f) tapasin was increased 1.4× by the presence of human β_2m.

3.2 Expression of β_2m or the MHC class I heavy chain augments murine tapasin mRNA levels

Variation in tapasin protein levels could be due to differences in tapasin protein stability and/or in regulation of tapasin gene expression. In order to examine the extent to which β_2m and/or D^b impacts tapasin expression at the mRNA level, we performed qRT-PCR on the R1.1, R1E, R1E-D^b, and R1E-D^b-β_2m cell lines. This analysis revealed that tapasin mRNA levels were enhanced ~2-3× in cells which express β_2m, D^b, or both β_2m and D^b (Fig. 1g). Thus, in the R1.1, R1E-D^b, and R1E-D^b-β_2m cell lines, the ~2.5-4× increase in tapasin protein expression may be attributable in part to the concomitant increase in tapasin gene transcription. These data suggest that a mechanism exists to regulate the level of tapasin expression in relation to the level of MHC class I-β_2m heterodimers present in the cell. In the R1.1 and R1E-D^b-β_2m cell lines, tapasin mRNA was enhanced ~2.4×, whereas the level

of tapasin protein was increased even more (~4×). Therefore, in the R1.1 and R1E-D^b-β_2m cell lines, the elevation in tapasin transcription cannot fully account for the nearly 4× enrichment of tapasin at the protein level.

4. Discussion

A plausible explanation for the increase in tapasin protein, above what can be accounted for by increased tapasin transcription, is that D^b-β_2m heterodimers may stabilize the tapasin protein. Thus, just as tapasin stabilizes MHC class I molecules with open peptide-binding grooves [18], MHC class I heavy chain-β_2m heterodimers may reciprocally stabilize tapasin. Tapasin has a long half-life (~24 hours) [19], which precluded our ability to determine the influence of β_2m and D^b on tapasin protein stability by the use of cycloheximide to inhibit protein synthesis (data not shown). The expression of human tapasin was also enhanced in the presence of β_2m in transfected Daudi cells, although the extent of tapasin upregulation was less dramatic than observed in the R1E-D^b-β_2m cell line. Future studies in our laboratory will address whether the mechanisms controlling tapasin expression differ depending on MHC allotypes present in the cell, as well as how these mechanisms differ among various cell lines and tissues.

Relevant to our data, calreticulin has also been shown to impact mouse tapasin protein levels. In a recent report, Del Cid et al. showed that transfection of calreticulin into calreticulin-deficient mouse cells augmented the steady-state level of tapasin protein [20]. These authors suggested that calreticulin may aid in the folding of nascent tapasin molecules. In light of our findings, it is possible that calreticulin may also contribute to the regulation of tapasin expression indirectly by supporting MHC class I heavy chain and/or β_2m stability.

Overall, these studies will assist in uncovering mechanisms that control the stable expression of MHC class I assembly proteins. The MHC class I peptide-loading complex is often disrupted or disregulated in infected and cancerous cells. Thus, understanding the pathways that normally coordinate peptide-loading complex protein expression will advance the development of strategies to restore antigen presentation in the context of infection and cancer.

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Highlights

- Beta 2-microglobulin enhances tapasin expression by a post-transcriptional mechanism.
- Tapasin mRNA levels are also augmented by beta 2-microglobulin.
- Beta 2-microglobulin increases the presence of tapasin-containing protein complexes.

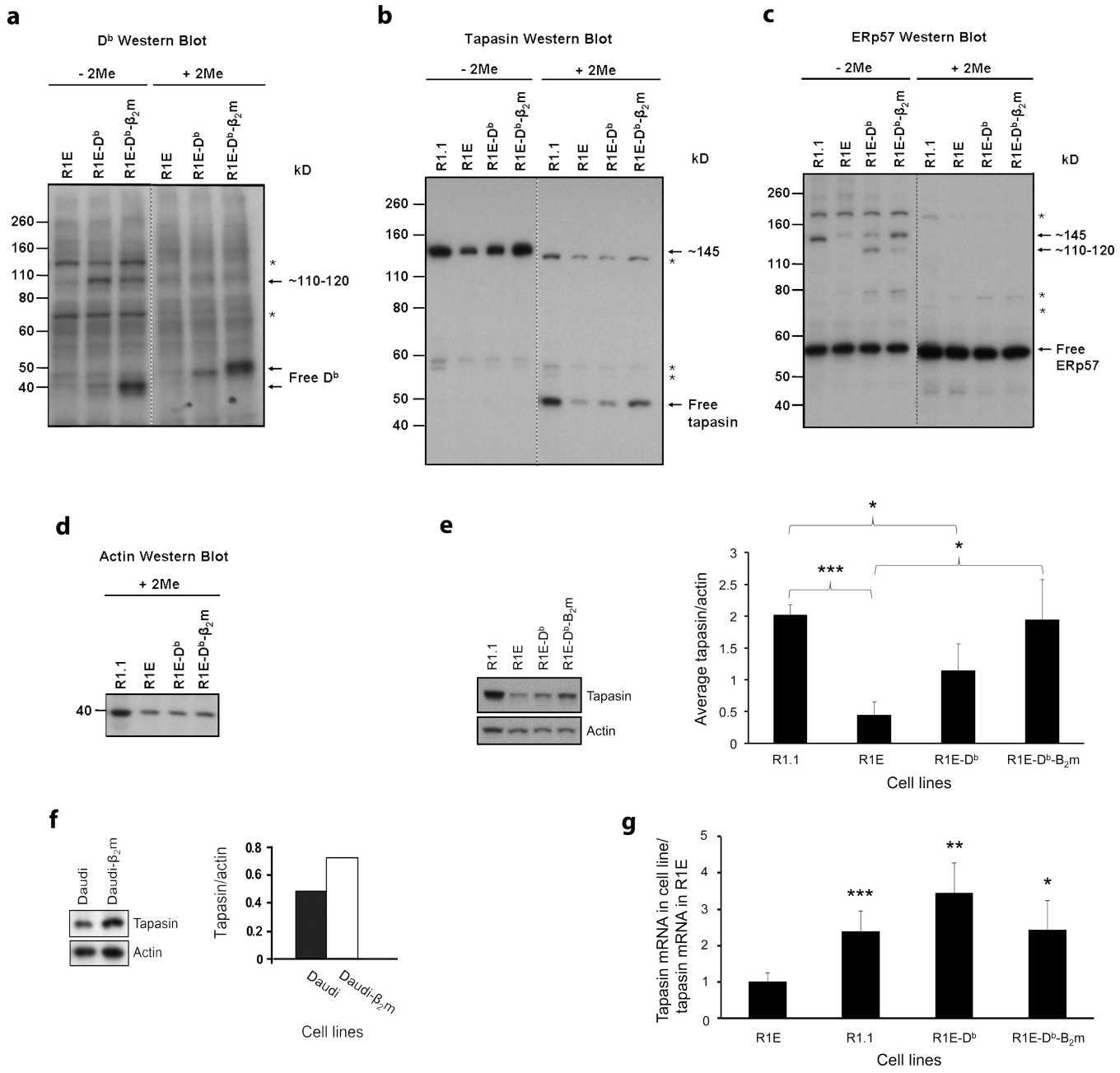

Fig. 1.

β_2m increases the level of mouse tapasin protein existing in free form or in disulfide-linked complexes. Lysates of equal numbers of cells from the indicated cell lines were prepared in methyl methanethiosulfonate to preserve disulfide bonds, and samples of the lysates were added to NuPAGE® lithium dodecyl sulfate (LDS) sample buffer (Invitrogen) +/- 2-mercaptoethanol (2-Me). The samples were then electrophoresed on 10% acrylamide Trisglycine sodium dodecyl sulfate (SDS) gels, transferred to membranes, and probed on western blots with rabbit anti-D^b serum (a), hamster anti-mouse tapasin monoclonal antibody (mAb) (b), rabbit anti-mouse ERp57 serum (c), or mouse anti-actin mAb (d). Dotted lines in (a), (b), and (c) indicate areas where lanes were removed. The approximate molecular masses of disulfide-linked complexes on the tapasin, ERp57, and D^b blots are

indicated with arrows. Additionally, free, non-disulfide-conjugated tapasin, ERp57, and D^b molecules are marked with arrows. The Tris-glycine SDS gels used for the tapasin, ERp57 and actin blots were purchased from Invitrogen, whereas the Tris-glycine SDS gel used for the D^b blot was from Bio-Rad. Asterisks indicate background bands. (e) Lysates of the indicated cell lines were prepared in iodoacetamide and mixed with NuPAGE® LDS sample buffer containing 2-Me. Actin was used as the loading control, with the loading of samples for the tapasin and actin blots set at the volumes that would yield similar intensities for actin bands. The samples were electrophoresed, transferred, and probed with a hamster anti-mouse tapasin mAb. The antibody was then stripped off, and the membrane was probed with a mouse anti-actin mAb. Densitometry was performed on the tapasin and actin bands of three replicate lysates from each cell line and used to calculate the average tapasin/actin ratio. Cell lines with significantly different tapasin/actin ratios as determined by the two-sample equal variance Student's *t*-test are indicated with asterisks (* $p < 0.05$, *** $p < 0.001$). (f) Electrophoresis, blotting and densitometry were performed as described above, except that a mouse anti-human tapasin mAb was used for the tapasin blot. (g) qRT-PCR was performed to determine the level of mouse tapasin and mouse GAPDH mRNA in the R1E, R1.1, R1E-D^b, and R1E-D^b-β_2m cells, and the data was analyzed as described in the Materials and Methods section. According to results obtained by an F-test, the two-sample equal variance Student's *t*-test was performed to determine the significance of the difference in the expression of mouse tapasin mRNA in R1.1 compared to R1E cells. For comparison of R1E to either R1E-D^b or R1E-D^b-β_2m, the two-sample unequal variance Student's t-test was performed (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).