Identification of an Alternate Splice Form of Tapasin in Human Melanoma

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Abstract

Assembly of MHC class I molecules with peptide in the endoplasmic reticulum requires the assistance of tapasin. Alternative splicing, which is known to regulate many genes, has been reported for tapasin only in the context of mutations. Here, we report on an alternate splice form of tapasin (tpsnΔEx3) derived from a human melanoma cell line that does not appear to be due to mutations. Excision of exon 3 results in deletion of amino acids 70 to 156 within the beta barrel region, but the membrane proximal Ig domain, the transmembrane domain and cytoplasmic tail of tapasin are intact. Introduction of tpsnΔEx3 into a tapasin deficient cell line does not restore MHC class I expression at the cell surface. Similar to a previously described tapasin mutant (tpsnΔN50), tpsnΔEx3 interacts with TAP. Therefore we utilized these altered forms of tapasin to test the importance of MHC class I interaction with TAP. In the presence of wild type tapasin, transfection of tpsnΔN50, but not tpsnΔEx3, reduced MHC class I expression at the cell surface likely due its ability to compete MHC class I molecules from TAP. Together these findings suggest that tumor cells may contain alternate splice forms of tapasin which may regulate MHC class I antigen presentation.

Keywords

antigen presentation; antigen processing

Introduction

MHC class I molecules consist of three components which must form a stable complex in the endoplasmic reticulum (ER) before transport to the cell surface; the highly polymorphic heavy chain, β_2-microglobulin, (β_2m) and a small peptide 8-10 amino acids in length. This trimeric complex is recognized by a specific clonotypic T cell receptor on the surface of a CD8^{+} T lymphocyte. Recognition of an immunogenic peptide associated with MHC class I molecules leads to the activation of a CD8^{+} T cell and subsequent killing of the target cell. Although *in vitro* studies suggest that a few hundred or possibly even one MHC-peptide complex is

sufficient for activation of a T cell [1,2], many reports have demonstrated that detectable, but reduced, MHC class I expression is associated with poor recognition by T cells [3-7]. Thus, T cell recognition is limited by the availability of surface MHC class I molecules bound to specific peptides.

Nascent MHC class I heavy chains first interact with calnexin upon their translocation into the ER [8]. Dissociation of heavy chains from calnexin is thought to precede their association with a preformed complex of TAP and tapasin [9-11]. These molecules, together with β_2m, the chaperone calreticulin, the thiol-oxidoreductase ERp57 and B cell receptor associated protein 31 (BAP31) make up the 'peptide loading complex' which brings several proteins together to assist in the assembly of MHC class I molecules with their peptide ligands [10,12-17]. Peptides, required for the stabilization of the immature heavy chain: β_2m dimers, are derived from the cytosol by proteasome activity and trimming peptidases [18]. The peptides are transported into the ER by the TAP1 and TAP2 heterodimer [19,20] where they are further trimmed by ERAAP [21,22] to optimize their size for binding to the cleft of the MHC class I molecule. The loading of the processed peptides into the peptide binding site of MHC class I molecules is enhanced by the coordinated effort of tapasin and ERp57.

Tapasin was first identified as a TAP associated protein that was required to detect MHC class I molecules associated with TAP [10,12,13]; an interaction whose importance in MHC class I assembly and export was suggested earlier [23-25]. This bridging function of tapasin is thought to be important for efficient MHC class I assembly with peptide as the absence of tapasin results in poor MHC class I export from the ER and hence low expression at the surface [13,25-27]. Although some MHC allelic products, such as HLA-B27, can achieve considerable surface expression in the absence of a TAP-MHC class I interaction (i.e. in the absence of tapasin), the MHC I-peptide complexes folded in the absence of tapasin are less stable arguing that the TAP-MHC class I interaction is important for optimal assembly [28]. Furthermore, single mutations in the transmembrane domain of tapasin diminishes interaction with TAP and compromises the conformation of surface MHC class I molecules [29,30]. Together these data argue that the bridging function is important for efficient assembly, export and surface expression. However, soluble tapasin which does not detectably associate with TAP, but promotes MHC class I expression challenged the idea that tapasin acted simply as a bridge between TAP and MHC class I molecules [31]. While more recent studies have demonstrated that the tapasin promotes peptide loading by enhancing the binding of peptides with slow off-rates [33,34], likely through the action of a tapasin-ERp57 disulfide intermediate [32,35], the importance of TAP-MHC class I proximity remains unclear.

Much of our information on how tapasin functions has been derived from transfection studies in the human B-LCL 721.220, tapasin deficient mice or more recently a tapasin deficient melanoma cell line [10,13,26,27,36]. Although the complete lack of tapasin protein drastically affects MHC class I expression, lower levels of tapasin is associated with lower MHC class I expression [37]. Therefore, the regulation of tapasin is an important aspect of efficient MHC class I expression and subsequent CTL and/or NK responses. Tapasin is upregulated by cytokines and extracellular signals such as IFN, IFNβ, TNF, IL-4 and ligation of TLRs [38, 39]. Gene regulation also may occur by alternate splicing [40] however, alternate splicing of tapasin has only been reported in the context of genomic mutations [41,42].

Here we report on an alternate splice form of tapasin (tpsnΔEx3) arising in a human melanoma cell line. This alternate splice form of tapasin lacks exon 3 but does not appear to be due to genomic mutation surrounding exon 3. TpsnΔEx3, like tpsnΔN50, interacts with TAP, but does not support MHC class I assembly and surface expression. Therefore, we tested if tapasinΔEx3 could act as a dominant negative by competing with wild type tapasin for TAP occupancy.

Materials and Methods

Cell lines

The human melanoma cell lines M1195 and M553 (HLA-A28, A2-haplotype loss, HLA-B*5001 and HLA-B*5701) and HeLaM cells have been described previously [11,36,43]. All cell lines used in this report were grown in RPMI-1640 medium (Life Technologies, Grand Island, NY) supplemented with 10% bovine calf serum (Hyclone Laboratories, Logan, UT) and 10mM HEPES.

Antibodies

The monoclonal antibody (mAb) W6/32 recognizes β2-microglobulin associated HLA-A, -B and -C molecules. The mAb SFR8.B6 recognizes the HLA-Bw6 epitope and is highly dependent upon the presence of residues Arg82 and Gly83 [44]. As we have previously shown in the M553 transfected with wild type tapasin cells, the SFR8.B6 supratypic antibody likely reacts with the HLA-B*5001 (Arg82, Gly83) product but not with HLA-B*5701 (Leu82, Arg83). The mAb TT4-A20 recognizes the HLA-Bw4 epitope, which is highly dependent upon residues 79–83 that are conserved in HLA-B*5701 expressed by M553 cells [36]. The following mAbs and antisera have been described previously: R.Ring4c antiserum (C-terminal peptide of TAP1) [23], mAbs NOB-2 (TAP2) and TO-3 (tapasin) [45,46]; R.gp48C antiserum (C-terminal peptide of tapasin [47]; mAb 3B10.7 (heavy chain) [48], mAbs TP25.99 (heavy chain) and CR11.351 (HLA-Aw68) [49]. The rabbit polyclonal calreticulin antibody was purchased from Stressgen (Vancouver, Canada). (San Diego, CA). The mAb specific for human tubulin was purchased from Sigma-Aldrich (St. Louis, MO). The mAb PaSta1 recognize a lumenal epitope within tapasin [32].

RT-PCR, cloning and genomic PCR

Total mRNA from M1195 cells was isolated using TRIZOL (Invitrogen, Carlsbad, CA) as per the manufacturer's recommendations. Two micrograms of mRNA were reverse transcribed to generate the first cDNA strand by using Superscript II (Invitrogen, Carlsbad, CA). cDNA was then used as template in a PCR reaction using the Accuprime enzyme (Invitrogen, Carlsbad, CA) to amplify tapasin cDNA using the primers 1F (5′-AGCGCCATGAAGTCCCTGTCTCTGCTC-3′) and 1R (5′-GTGCCCTCACTCTGCTTTCTTCTTTGA-3′). Amplicons were then gel purified and cloned into pDrive (Qiagen, Valencia, CA) before sequencing. The tapasinΔEx3 sequence obtained from pDrive has been submitted to genbank (**EU693375**). The cassette encoding wild type tapasin or tpsnΔEx3 cDNA was then excised and subcloned into pCDNA3.1(−) puro which was derived from pCDNA3.1(−)neo (Invitrogen, Carlsbad, CA) by replacement of the neomycin gene with the puromycin resistance gene. For analysis of genomic tapasin, genomic DNA was isolated from M1195 cells using TRIZOL according to manufacturers recommendations. PCR was performed using the primers FP_Ex3Genomic-225 (5′ AGTGTACACGGTGAGTCTCTAG 3′) and RP_Ex3Genomic+340 (5′ CATCACCTGACAAGTCTCTCAA 3′). The 826 bp product was cloned into pGEM-T-Easy (Promega Madison, WI) and single colonies were isolated and plasmid DNA purified for sequencing using the T7 and SP6 primers.

Transfection and cloning

For stable transfectants, tapasin expression constructs were transfected into M553, M553 (R240)tpsn or HeLaM cells using Lipofectamine (Invitrogen, Carlsbad, CA) or Effectene (Qiagen, Valencia, CA) according to the manufacturer's recommendations. Selection of clones containing the plasmid pCDNA3.1(−) PURO encoding a wild type tapasin cDNA or the tpsnΔEx cDNA was performed by limiting dilution and supplementing the media with

puromycin (Sigma-Aldrich St Louis, MO) at a final concentration of 1 microgram per mL. For transient transfection experiments, 100 ng of the pmaxGFP plasmid which encodes an enhanced GFP protein driven by the CMV promoter (Amaxa, Gaithersburg, MD) and 2 micrograms of the tpsnΔEx3 cDNA in pCDNA3.1(−)PURO or empty pCDNA3.1(−)puro plasmid were transfected into 1 million cells using an Amaxa Nucleoporator II instrument (solution V, program T-030) according to manufacturers recommendations.

Western blotting

Cells were lysed using 1% TritonX-100 in 10mM Tris, 150mM NaCl, pH7.4 supplemented with protease inhibitors PMSF (0.5mM) and N-ethylmaleimide (5mM) and incubated at 4°C for 30 minutes. Protein concentration of post nuclear supernatants was determined by the Bradford assay (Bio Rad, Hercules, CA). After SDS-PAGE and transfer of proteins to Immobilon-P membrane (Millipore, Bedford, MA), membranes were incubated in 3% Bovine Serum Albumin (BSA). Proteins of interest were detected using the indicated primary antibodies overnight at 4°C followed by three washes in PBS/0.02% TWEEN 20. Secondary antibodies coupled to alkaline phosphatase were then added in 3% Bovine Serum Albumin and incubated at room temperature for 1 hour. After three washes in PBS/0.02% TWEEN 20, VistraECF substrate (Amersham Pharmacia, Piscataway, NJ) was applied and fluorescence was measured using a STORM860 instrument (Molecular Dynamics, Palo Alto, CA). Fluorescence intensity was acquired and analyzed using ImageQuant 5.2 software by integration of area under the histogram analysis.

Radiolabeling and Immunoprecipitation

Cells were washed in PBS and starved for 30 minutes in the absence of cysteine and methionine prior to labeling with ^{35}S-methionine for 2.5 hours at 37°C. For immunoprecipitation cells were lysed in either 1% Digitonin (Calbiochem, Gibbstown, NJ) or 1% TritonX-100 in 10mM Tris, 150mM NaCl, (pH7.4) 0.5mM PMSF and 5mM NEM. After pelleting nuclei, post nuclear supernatants were diluted in 1% TritonX-100. The resultant supernatants were subjected to immunoprecipitation using the antibodies indicated and protein G Sepharose. After washing, the bound proteins were eluted with reducing Laemmli sample buffer, separated by SDS-PAGE and subjected to autoradiography. For immunoprecipitation of TAP, cells were lysed in digitonin containing lysis buffer indicated above (to preserve interactions between TAP and MHC class I molecules) without the addition of the 1% TritonX-100 dilution. After immunoprecipitation, bound proteins were eluted by boiling in 1% SDS, 10mM Tris-Cl, 150mM NaCl. The resulting supernatants were diluted 10 fold in 1% TritonX-100, 10mM Tris-Cl, 150mM NaCl (pH7.4) and subjected to a second round of immunoprecipitation.

Flow cytometric analysis

Cells were washed and incubated with saturating concentrations of W6/32, SFR8.B6 and TT4-A20 or control antibodies for 30 minutes at 4°C. After washing, half a million cells were resuspended in saturating concentrations (10 microgram/ml) of fluorescein isothiocyanate or phycoerythrin conjugated, goat anti-mouse immunoglobulin G antibodies. When cells transfected with GFP were used for flow cytometric analysis, the cells were stained with W6/32-PE directly conjugated antibody (BD biosciences, San Jose, CA) and analyzed without fixation. Otherwise, after removing the excess primary and secondary antibody by washing, cells were fixed in 2% paraformaldehyde and cells were analyzed on a FACScan instrument (Becton Dickinson, San Jose, CA). Data were analyzed with WINMDI software (Joe Trotter, The Scripps Research Institute, La Jolla, CA).

Results

M1195 cells encode a tapasin variant lacking the third exon in the absence of genomic mutations

While characterizing antigen presentation genes in human melanoma cell lines we discovered an alternate splice form of tapasin lacking exon 3 in M1195 (tpsnΔEx3, Figure 1A). Tapasin cDNA was amplified by PCR using primers designed to anneal at the 5′ and 3′ ends of the coding region. Sequencing of independent colonies revealed a full length wild type tapasin gene encoding the R240 tapasin allele ((R240)tpsn) and tpsnΔEx3. To determine if a genomic mutation in the splice sites surrounding exon 3 may be the cause of tpsnΔEx3 we sequenced genomic DNA from M1195 cells (Figure 1B). Exon3 including 200bp 5′ and 200bp 3′ of the exon was amplified by PCR. In an attempt to isolate and sequence both genomic copies of tapasin, eight independent PCR reactions and 24 independent colonies from those eight PCR reactions were sequenced. All 32 sequences revealed identical wild type splice site sequences (Figure 1B). All sequences contained an intact exon 3 suggesting that deletion of exon 3 in the genomic DNA is unlikely. Furthermore, these wild type sequences indicate intact splice acceptor and donor sites on either end of exon3.

TpsnΔEx3 encodes a protein that can be expressed at appreciable levels within cells

The tpsnΔEx3 sequence predicts an in frame truncated protein lacking 86 amino acids (from position 70 to 156) and an expected molecular weight of 38kD instead of the wild types 48kD. To test whether the alternate splice lacking exon 3 could be translated and expressed at appreciable levels, the tpsnΔEx3 cDNA isolated from M1195 was transfected into M553 cells where endogenous tapasin protein is undetectable [36]. To verify the expression of the truncated protein product we examined newly synthesized tpsnΔEx3 protein (Figure 2A) and steady state levels (Figure 2B). Control, tapasin or heavy chain specific antibodies were used to immunoprecipitate (IP) the respective proteins from ^{35}S-methionine labeled M553, M553 (R240)tpsn or M553tpsnΔEx3 cell lysates (Figure 2A). While class I heavy chains are detectable in all three cell types (lanes 3, 6 and 9), tapasin IP from M553 lysates confirmed the lack of detectable endogenous tapasin protein (compare control antibody, lane 1, and tapasin antibody, lane 2). Tapasin IP from M553(R240)tpsn cell lysates revealed a specific band corresponding to wild type tapasin (lane 5) that was not present in the M553 parental cell line (lane 2). In M553tpsnΔEx3 cells, the tapasin specific IP (lane 8) revealed a band of lower molecular weight (as predicted) than that of the transfected wild type tapasin (lane 5) and specific to the tapasin antibody (compare control and tapasin specific lanes 7 and 8 respectively). The presence of a detectable radiolabeled tpsnΔEx3 after ^{35}S-methionine labeling argues that tpsnΔEx3 protein is synthesized. The tpsnΔEx3 protein from M553tpsnΔEx3 cells was also successfully detected by western blot (Figure 2B) suggesting that tpsnΔEx3 is not rapidly degraded following synthesis.

TpsnΔEx3 does not restore MHC class I surface expression in M553 cells

We have previously reported that levels of either HLA-B allele, (HLA-B*5701 and – B*5001), on M553 cells are low to undetectable because this cell line does not express tapasin protein, and that this defect is restored by transfecting a wild type tapasin cDNA [36]. To determine if tpsnΔEx3 was sufficient to promote efficient class I expression at the cell surface (in the absence of wild type tapasin) tpsnΔEx3 cDNA was over-expressed in M553 cells and compared to wild type tapasin transfectants. As expected, transfection of wild type tapasin restores MHC class I expression at the cell surface (Figure 2C, MFI for M553 parental and (R240)tpsn transfected were 25 and 228 for HLA-B*5701, and 4 and 17 for HLA-B*5001, respectively). Surface MHC class I levels on MtpsnΔEx3 cells remained unaltered when compared to the parental M553 cells (Figure 2C, MFI values for M553 parental vs M553tpsnΔEx3 were 25 vs

22 for HLA-B*5701 and 4 vs 5 for HLA-B*5001), arguing that tpsnΔEx3 is not capable of restoring the tapasin deficiency of M553 cells.

TpsnΔEx3 interacts with TAP

The transmembrane domain of tapasin is required for its interaction with TAP [29,31] and this domain is present in tpsnΔEx3. We therefore hypothesized that tpsnΔEx3 would interact with the TAP heterodimer. M553, M553(R240)tpsn and M553tpsnΔEx3 cells were metabolically labeled as in Figure 2A, and an equivalent number of cells were lysed in digitonin containing buffer to preserve the interactions within the peptide loading complex. TAP2 IPs were eluted and re-precipitated using either a control or tapasin specific antibody (Figure 3). Parental M553 cells were used to distinguish between the specific interaction mediated by the transfected tapasin and the interactions that occur in the absence of tapasin (Figure 2, lanes 1 and 2). As previously shown, in M553(R240)tpsn cells, there is a specific band corresponding to the transfected wild type tapasin (compare tapasin IP, lane 4, to lane 3 control IP). In M553tpsnΔEx3 cells, a specific band in lane 6 corresponds to tpsnΔEx3, evidenced by a lower molecular weight band than the wild type tapasin (lane 4). Similar results were obtained using TAP1 specific antibody for the first immunoprecipitation (data not shown). Together, these results suggest that tpsnΔEx3 interacts with TAP despite the lack of exon 3.

TpsnΔN50, but not TpsnΔEx3 diminishes MHC class I surface expression in the presence of wild type tapasin

Previous studies have demonstrated that deletion of the N-terminal 300 amino acids (tpsnΔN300) or as little as the N-terminal 50 residues from tapasin renders it incapable of interacting with MHC class I molecules, yet able to interact with, and stabilize, TAP [47]. Therefore, if the tapasin bridging function is important, then tapasin mutants that interact with TAP but not with MHC class I molecules may compete for TAP binding resulting in fewer MHC class I molecules associated with TAP. We therefore tested if tpsnΔEx3 or tpsnΔN50 could compete for TAP and diminish MHC class I assembly and expression at the cell surface.

TpsnΔN50 cDNA was transfected into HeLaM cells, which have an intact peptide loading complex, and transfectants were selected for puromycin resistance. Of 11 isolated clones, five expressed abundant tpsnΔN50 at steady state (at a much greater level than the barely detectable endogenous tapasin in HeLaM cells), while six did not express detectable tpsnΔN50 (Figure 4A). Flow cytometric analysis of the HLA-A and HLA-B alleles revealed that tpsnΔN50 expression correlates with lower levels of MHC class I at the cell surface (Figure 4B). Two clones were chosen for further examination of interactions with TAP; clone F7, which expresses high levels of tpsnΔN50 and a clone that does not express tpsnΔN50, clone E7. In clone F7, the tpsnΔN50 interacts robustly with TAP (Figure 4C, marked by *) and the level of endogenous wild type tapasin associated with TAP1 was diminished by approximately fifty percent when compared to clone E7 (Figure 4C). Confirmation of the reduced ability of endogenous tapasin to function as a bridge between the TAP and MHC class I molecules is shown by the decreased level (~50%) of associated heavy chain and β_2m (Figure 4C, HC panel and β_2m panel). Together these data argue that tpsnΔN50 can act as a dominant negative by reducing MHC class I association with TAP and reducing MHC class I surface assembly and surface expression.

Next we tested if tpsnΔEx3 could also act as a dominant negative. Stable transfection of tpsnΔEx3 into HeLaM cells also led to high expression relative to the endogenous wild type tapasin (Figure 5A). This ratio of mutant to wild type tapasin is comparable to that observed in HeLaM tpsnΔN50 cells (Figure 5A vs 4A). Flow cytometric analysis of staining with CR11-351 (recognizing HLA-Aw68 on HeLaM cells) or W6/32 (anti-HLA-A, -B, -C) showed no significant differences between HeLaM and HeLaMtpsnΔEx3; (Figure 4B the MFI for

HeLaM and HeLaMtpsnΔEx3 were 266 and 235 for CR11-351 and 171 and 191 for W6/32, respectively). Therefore it appears that tpsnΔEx3 does not act as a dominant negative.

Everett and Edidin recently reported that transient (rather than stable) transfection of a tapasin truncation mutant reduced MHC class I expression at the cell surface [50]. Therefore, we attempted a similar transient transfection approach of M553(R240)tpsn cells with tpsnΔEx3 or tpsnΔN50 along with an EGFP encoding plasmid. Populations of cells successfully co-transfected were gated based on GFP fluorescence. Then, MHC class I levels were analyzed in cells transfected, gated on R3 (GFP positive) and untransfected, gated on R2 (GFP negative) using W6/32 directly conjugated to PE (FL-2). Examination of the mean fluorescent intensity (MFI) on FL-2 revealed no significant difference in the surface expression of MHC class I in M553(R240)tpsn cells transfected with tpsnΔN50 or tpsnΔEx3 when compared to those transfected with empty vector and GFP (Figure 6A). The lack of effect in the transient transfection may be due to the lower ratio of transfected tpsnΔN50 to wild type tapasin in the M553 cells compared to HeLaM cells (compare Figure 6B to Figure 4A). Transient transfection of YFP-ΔN300 into M553(R240)tpsn cells reduced surface MHC class I expression by approximately 20% suggesting that the transient transfection approach to demonstrate dominant negative tapasin activity is feasible (Figure 7). Collectively these data argue that tpsnΔN50 and YFP-tpsnΔN300 can have dominant negative activity when expressed at high enough levels. However, similar over-expression of tpsnΔEx3 (in HeLaM cells) does not result in dominant negative activity.

Discussion

In the present study we have demonstrated the presence of a novel splice form of tapasin in a human melanoma cell line. This splice form, which lacks exon 3, encodes a protein that can be synthesized within cells and associate with TAP but does not promote MHC class I assembly and surface expression when expressed in a tapasin deficient cell line. Over-expression of tpsnΔEx3 in the presence of wild type tapasin (in HeLaM or M553(R240)tpsn cells) does not appear to diminish MHC class I assembly and surface expression. However, a deletion mutant of tapasin that lacks the N-terminal fifty amino acids does appear to compete with wild type tapasin and reduce MHC class I association with TAP and subsequent assembly and surface expression.

Two previous reports of tapasin alternate splice forms have demonstrated that their occurrence was due to genomic mutations. First, Copeman and colleagues identified the tapasin defect in the B-LCL .220; a cell line that was selected for low class I expression after γ-irradiation [41]. In .220 cells, a single nucleotide substitution abolishes the 5′ splice site of the second intron leading to the generation of a truncated tapasin protein that lacked a portion of the signal sequence and the N-terminal fifty amino acids. The protein product of this alternate splice form is barely detectable in .220 cells presumably due to the incomplete signal sequence that is predicted to impair its translocation into the ER. Second, Elliott and colleagues analyzed an alternate splice form of tapasin from the existing cDNA database [42]. This splice variant of tapasin failed to exclude introns 4 through 6 due to a mutation in the intron-exon boundary. However, when this mutated splice form of tapasin was transfected into .220 cells, it was processed to remove the 4th and the 6th intron but the 5th intron remained as part of the translated product, introducing an early stop codon generating a soluble tapasin protein. This truncated tapasin protein, although unable to interact with TAP (as the transmembrane domain was absent), was able to restore surface presentation of HLA-B5 expression of the .220 cells. However, the stability of the HLA-B5 allele was much lower when .220 cells were transfected with the alternate splice form of tapasin than with wild type tapasin suggesting suboptimal assembly with peptide [42]. We have identified a unique previously undescribed tapasin alternate splice form in which the third exon is deleted. Exon 3 'skipping' is likely not caused

by genomic mutations because sequencing of exon 3 and the surrounding nucleotides reveals intact splice acceptor and donor sites (Figure 1B).

Deletion of exon 3 (amino acid residues 70-156) eliminates the central beta sheet (S4, S5, S6 and S7) of the N-terminal three-tiered beta sheet sandwich. This structure of tapasin is known to be essential for interaction with ERp57 since Cys95 forms a disulfide intermediate with ERp57 (Cys57 on ERp57). The phenotype of tpsnΔEx3 is more severe than C95A mutation [32]. Mutation of Arg72 within this region leads to diminished peptide loading by the tapasin-ERp57 conjugate [35] supporting the idea that residues beyond Cys95 are critical for efficient assembly by the tapasin-ERp57 conjugate. Therefore, it is not surprising that tpsnΔEx3 does not support efficient MHC class I export and surface expression. However, its ability to interact with TAP prompted us to test whether tpsnΔEx3 may compete with wild type tapasin for TAP occupancy which would result in fewer MHC class I molecules associated with TAP. Although our studies demonstrate that tpsnΔEx3 does not reduce surface expression, we show that tpsnΔN50 can reduce MHC class I association with TAP and surface expression significantly. Everett and Edidin recently showed that a mutant tapasin lacking 300 amino acids at the N-terminus fused with yellow fluorescent protein (YFP) diminishes MHC class I expression possibly due to competition for TAP accessibility [50]. In our studies, we demonstrate a correlation between reduced MHC class I association with TAP and reduced surface expression of MHC class I molecules. It should be noted that we observe this dominant negative activity when the mutant tapasin is expressed at much higher levels than the wild type tapasin (in HeLaM cells) but could not reproduce this phenotype in M553(R240)tpsn cells likely due to the high expression of wild type tapasin.

Together these data provide evidence for the importance of the MHC class I – TAP interaction and also for the presence of alternate splice forms of tapasin which may represent a potential mechanism of regulation. Initial attempts to determine the prevalence of tapasinΔExon3 in cell lines that express wild type tapasin have been unsuccessful. Whether this is due to the low abundance of tapasinΔEx3 transcripts relative to wild type tapasin transcripts is unclear. Future studies into identifying the prevalence of tapasinΔEx3 and/or the signals that regulate the generation of tapasinΔEx3 may uncover novel aspects of regulation of antigen presentation in normal and tumor cells.
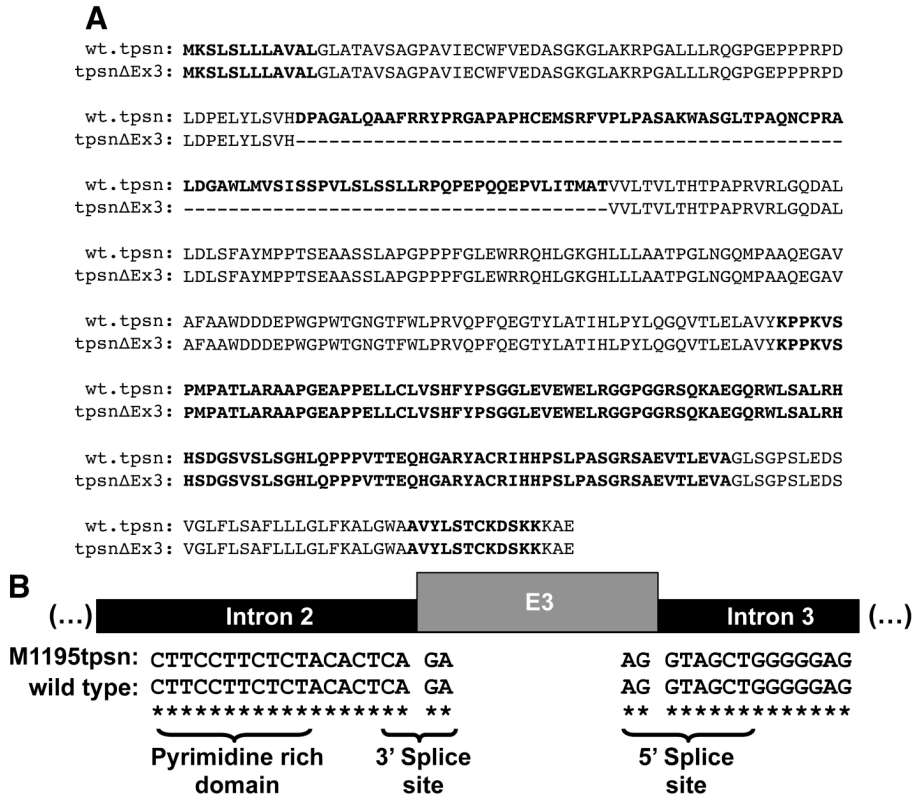
Acknowledgments

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A

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wt.tpsn:   MKSLSLLLAVALGLATAVSAGPAVIECWFVEDASGKGLAKRPGALLLRQGPGEPPPRPD
tpsnΔEx3:  MKSLSLLLAVALGLATAVSAGPAVIECWFVEDASGKGLAKRPGALLLRQGPGEPPPRPD

wt.tpsn:   LDPELYLSVHDPAGALQAAFRRYPRGAPAPHCEMSRFVPLPASAKWASGLTPAQNCPRA
tpsnΔEx3:  LDPELYLSVH-------------------------------------------------

wt.tpsn:   LDGAWLMVSISSPVLSLSSLLRPQPEPQQEPVLITMATVVLTVLTHTPAPRVRLGQDAL
tpsnΔEx3:  -------------------------------------VVLTVLTHTPAPRVRLGQDAL

wt.tpsn:   LDLSFAYMPPTSEAASSLAPGPPPFGLEWRRQHLGKGHLLLAATPGLNGQMPAAQEGAV
tpsnΔEx3:  LDLSFAYMPPTSEAASSLAPGPPPFGLEWRRQHLGKGHLLLAATPGLNGQMPAAQEGAV

wt.tpsn:   AFAAWDDDEPWGPWTGNGTFWLPRVQPFQEGTYLATIHLPYLQGQVTLELAVYKPPKVS
tpsnΔEx3:  AFAAWDDDEPWGPWTGNGTFWLPRVQPFQEGTYLATIHLPYLQGQVTLELAVYKPPKVS

wt.tpsn:   PMPATLARAAPGEAPPELLCLVSHFYPSGGLEVEWELRGGPGGRSQKAEGQRWLSALRH
tpsnΔEx3:  PMPATLARAAPGEAPPELLCLVSHFYPSGGLEVEWELRGGPGGRSQKAEGQRWLSALRH

wt.tpsn:   HSDGSVSLSGHLQPPPVTTEQHGARYACRIHHPSLPASGRSAEVTLEVAGLSGPSLEDS
tpsnΔEx3:  HSDGSVSLSGHLQPPPVTTEQHGARYACRIHHPSLPASGRSAEVTLEVAGLSGPSLEDS

wt.tpsn:   VGLFLSAFLLLGLFKALGWAAVYLSTCKDSKKKAE
tpsnΔEx3:  VGLFLSAFLLLGLFKALGWAAVYLSTCKDSKKKAE
```

B

Figure 1. TpsnΔEx3 is likely not due to genomic mutations
A. Sequence of wild type tapasin and a variant alternate splice form lacking exon 3. *B*. Exon 3 is not deleted from the genome and splice sites surrounding exon 3 are not mutated. Genomic sequence surrounding exon 3 in M1195 cells compared to wild type sequence (NT007592).
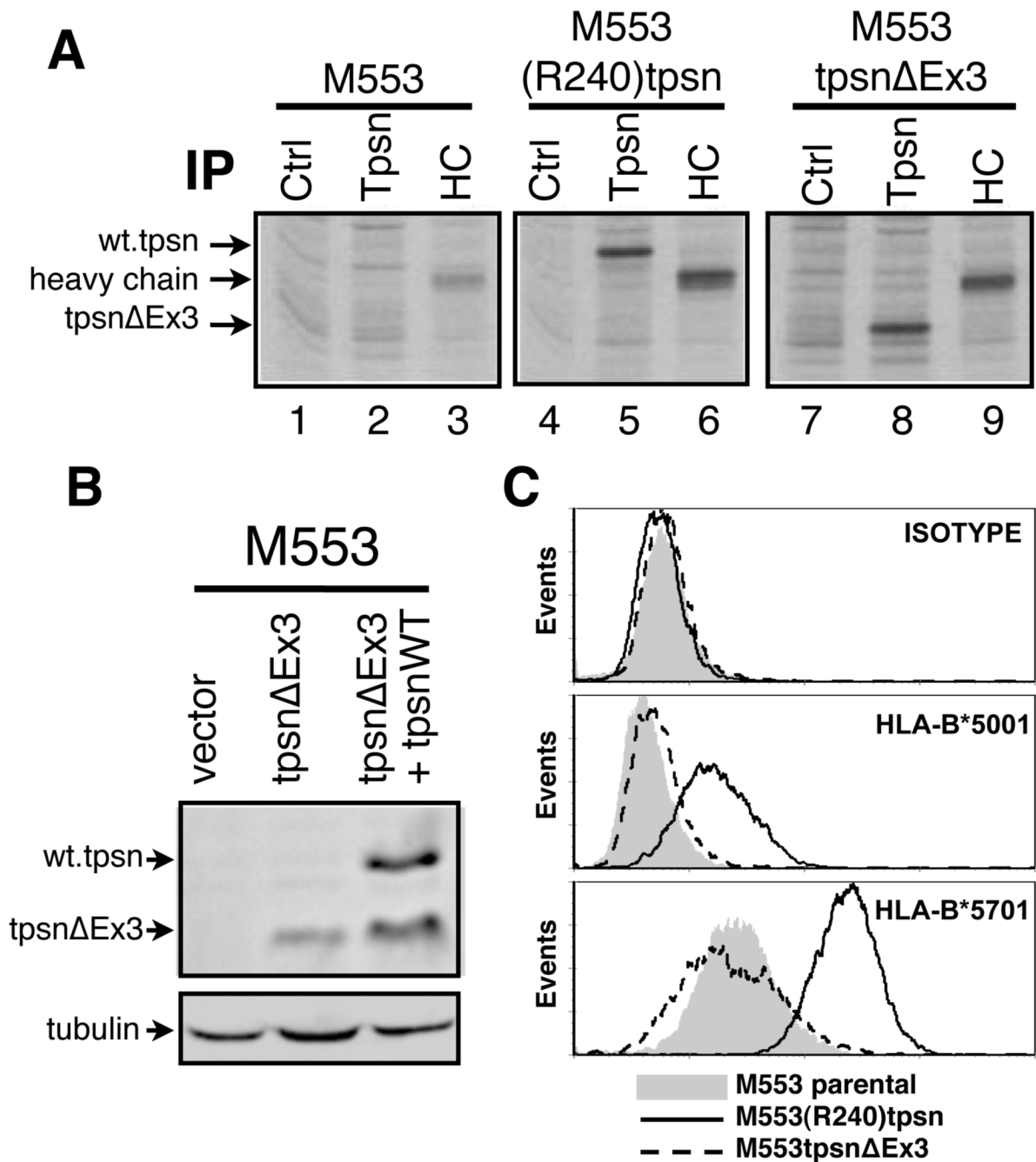
**Figure 2. TpsnΔEx3 expression does not restore tapasin deficiency**
A. Tapasin deficient M553 cells or the indicated transfectants were metabolically labeled, lysed in TritonX100 containing lysis buffer and immunoprecipitations (IP) performed with control (Ctrl) antibody (L243), tapasin (Tpsn) specific antibody TO-3 or heavy chain (HC) specific antibody TP25.99. *B*. Lysates from M553 cells transfected with empty plasmid (vector) or tpsnEx3 were assessed for for tapasin by western blot using TO-3 antibody. Control lane shows relative position of wild type tapasin and tpsnΔEx3. *C*. Untransfected M553 cells (filled histograms) or stable transfectants containing wild type R240tpsn (solid line) or tpsnΔEx3 (dotted line) were examined for MHC class I expression by TT4-A20 staining (HLA-B*5701),

SFR8.B6 (HLA-B*5001) staining, or isotype control staining. A representative figure of at least three independent experiments is shown.
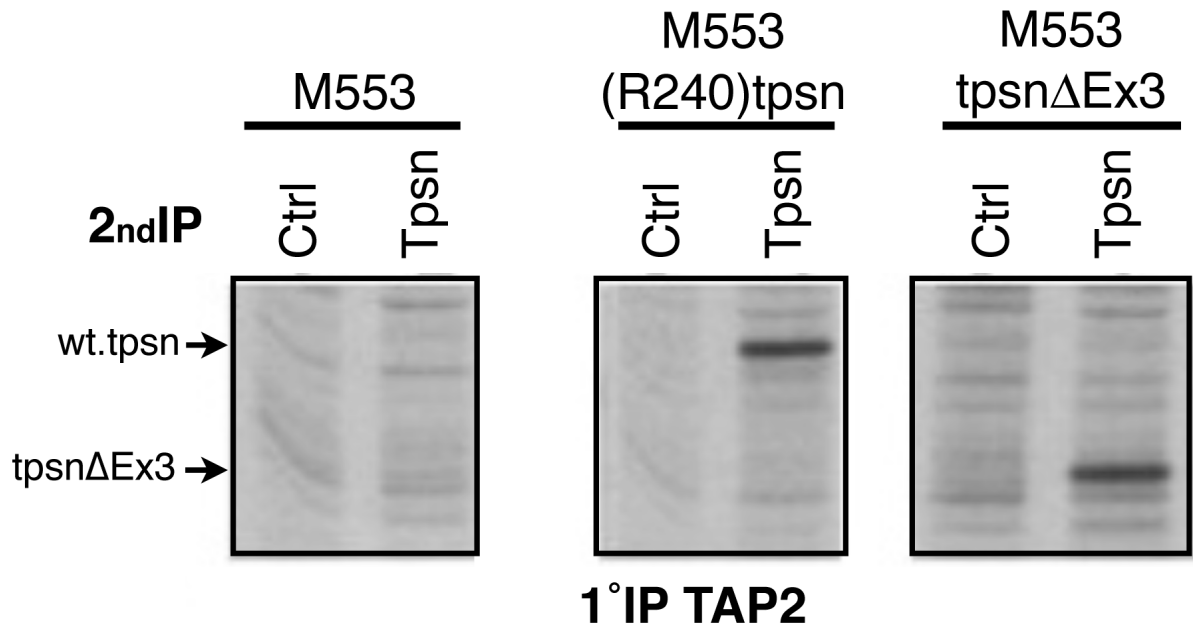
Figure 3. TpsnΔEx3 interacts with TAP
M553 cells were treated with IFN for 16-20 hours and metabolically labeled using ^{35}S methionine prior to lysis in digitonin containing lysis buffer. Cell lysates were subjected to immunoprecipitation (1°IP) using a TAP2 specific antibody. After elution, re-IP (2°IP) with a control antibody (Ctrl) or TO-3 (Tpsn) was performed and gels subject to autoradiography.
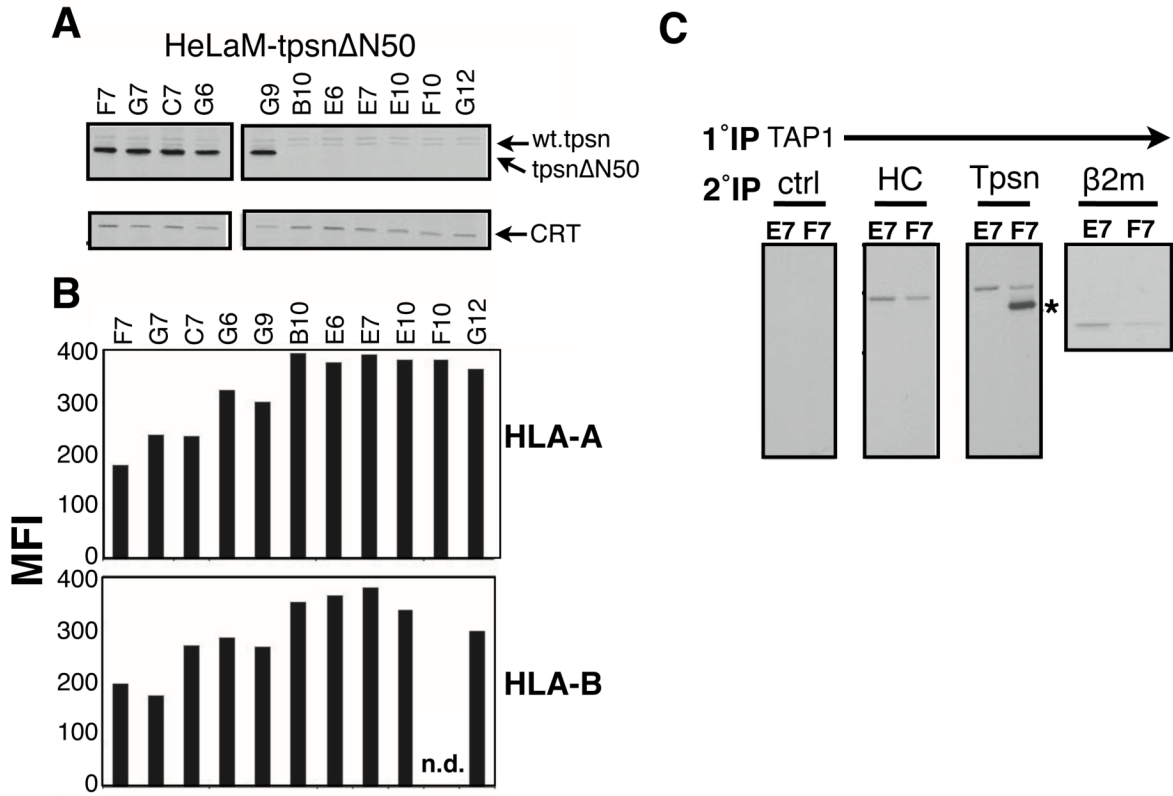
Figure 4. TpsnΔN50 downregulates surface MHC class I molecules by competing with wild type tapasin in HeLa cells

A. The indicated puromycin resistant transfectants of HeLaM cells were labeled with ^{35}S-methionine and immunoprecipitated for tpsnΔN50 (Rgp48C) or calreticulin. *B*. HLA-A (CR11.351) and HLA-B (SFR8.B6) expression on the surface of clones indicated. Mean Fluorescence Intensity (MFI) is plotted for each clone. *C*. Clones F7 (expressing tpsnN50) and E7 (not expressing tpsnN50), were lysed in digitonin and immunoprecipitated using a Ring4C (TAP1) specific antibody. After elution, re-immnoprecipitation was performed using control, heavy chain (HC, 3B10.7), tapasin (R.gp48C) or β2m specific antibodies. A representative figure of at least three independent experiments is shown.
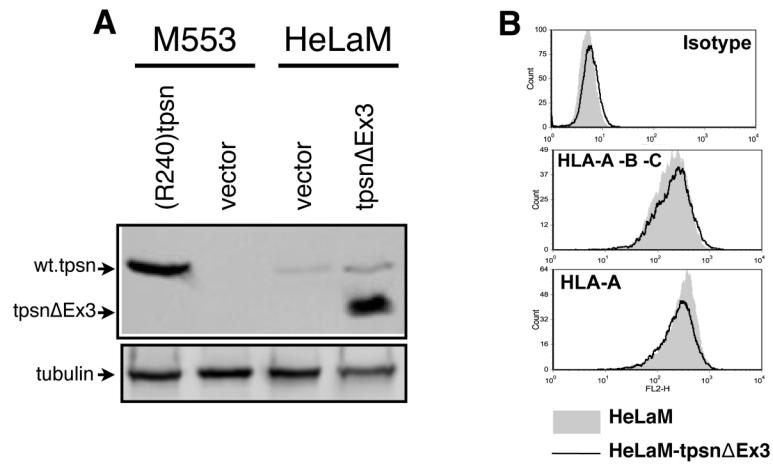
Figure 5. TpsnΔEx3 does not reduce MHC class I expression on HeLa cells
A. Stable transfectants of HeLaM, or M553, cells containing empty vector or tpsnΔEx3 were analyzed for tapasin protein by Western blot using TO-3 antibody. *B*. The effect of stable tpsnΔEx3 expression on MHC class I expression on the surface of HeLaM cells was determined using W6/32 (HLA-A, -B, -C) or CR11-351 (HLA-Aw68).
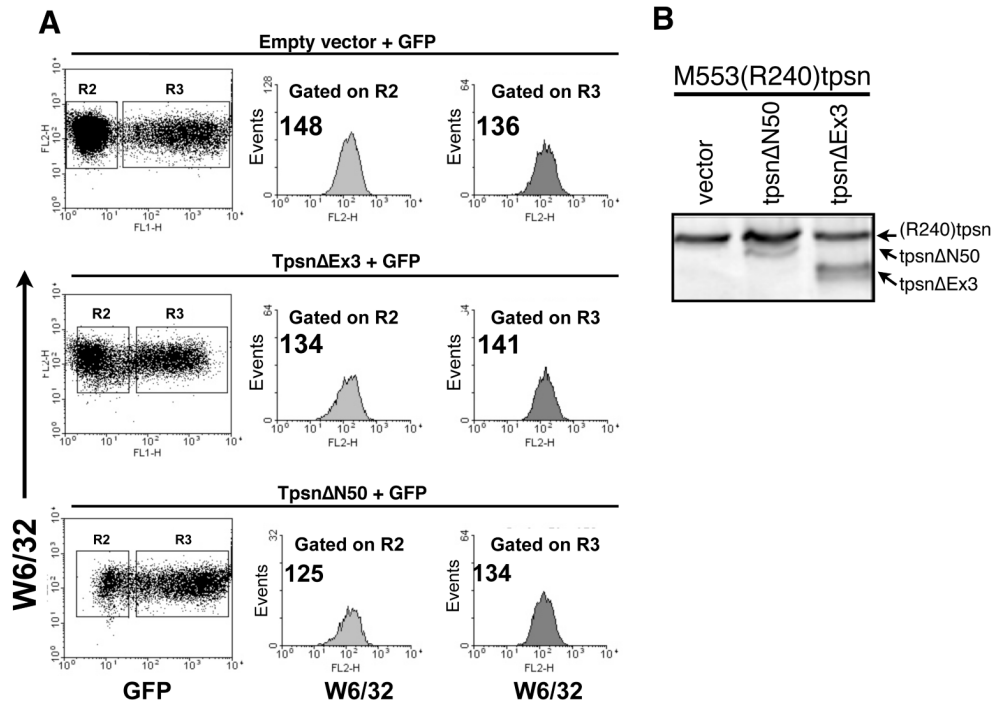
Figure 6. Transient expression of truncated tapasin molecules does not reduce MHC class I expression in M553(R240)Tpsn cells

A. Transient co-transfection of M553(R240)Tpsn cells with pmaxGFP and the indicated tapasin variants followed by W6/32 staining 24 hours post transfection. W6/32 staining on populations of cells gated on forward and side scatter (R1) and for the negative or positive expression of GFP (R2 vs. R3, respectively). *B*. The transient transfectants from A. were analyzed by western blot for the ratio of wild type (R240)tpsn to tpsnΔEx3 or tpsnΔN50 using TO-3 antibody.
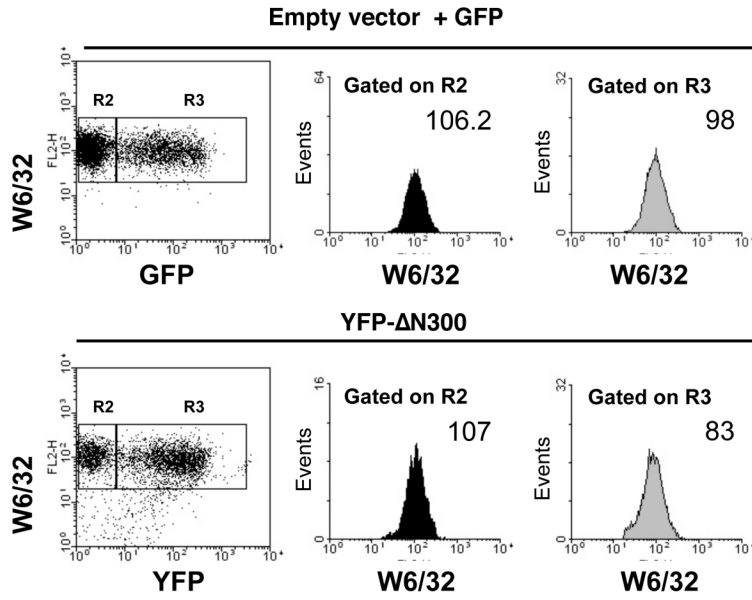
Figure 7. YFP-N300 downregulates MHC class I expression in M553(R240)tpsn cells
M553(R240)Tpsn cells were transiently transfected with YFP-N300 or co-transfected with empty vector and pmaxGFP. Twenty-four hours post transfection, MHC class I expression was analyzed by flow cytometry using a PE directly conjugated W6/32 (MHC class I) antibody. Mean fluorescent intensity was determined for W6/32 staining on populations of cells gated on forward and side scatter (R1) and for the negative or positive expression of GFP/YFP (R2, GFP/YFP negative) and R3, (GFP/YFP positive).