A Critical Role for Tapasin in the Assembly and Function of Multimeric MHC Class I–TAP Complexes

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Newly assembled major histocompatibility complex (MHC) class I molecules, together with the endoplasmic reticulum chaperone calreticulin, interact with the transporter associated with antigen processing (TAP) through a molecule called tapasin. The molecular cloning of tapasin revealed it to be a transmembrane glycoprotein encoded by an MHC-linked gene. It is a member of the immunoglobulin superfamily with a probable cytoplasmic endoplasmic reticulum retention signal. Up to four MHC class I–tapasin complexes were found to bind to each TAP molecule. Expression of tapasin in a negative mutant human cell line (220) restored class I–TAP association and normal class I cell surface expression. Tapasin expression also corrected the defective recognition of virus-infected 220 cells by class I–restricted cytotoxic T cells, establishing a critical functional role for tapasin in MHC class I–restricted antigen processing.

Major histocompatibility complex molecules with specific bound peptides are the ligands for the antigen-specific receptors of T cells. Appropriate expression of MHC molecules with associated self peptides in the thymus and peripheral tissues is essential for the maintenance of T cell tolerance, and effective responses to infectious organisms require efficient assembly of both MHC class I and class II complexes with pathogen-derived peptides. Assembly of the MHC class I–peptide complex is initiated in the endoplasmic reticulum (ER) by the formation of MHC class I–β_2-microglobulin (β_2M) dimers and predominantly involves the chaperones calnexin and calreticulin (*1–5*). The peptides bound by MHC class I molecules are mainly generated by the proteasome-mediated cleavage of cytosolic proteins (*6*). Peptides are translocated into the ER by TAP, a specialized transporter that is a member of the adenosine triphosphate (ATP)–binding cassette family of transporters. It is composed of two homologous MHC-encoded subunits, TAP.1 and TAP.2 (*7*). Before peptide binding, calreticulin-associated class I molecules bind to TAP, an interaction mediated by tapasin (*4*). In the mutant cell line 220, which lacks tapasin expression, this interaction does not occur, and MHC class I assembly and subsequent cell surface expression is impaired (*4*, *8*, *9*). Somatic cell genetic evidence has suggested that either a gene regulating the expression of tapasin or the tapasin gene itself resides on the short arm of human chromosome 6 (*8*, *10*).

Translocation by TAP of an allele-specific, class I–binding peptide initiates dissociation of that class I molecule from the TAP complex (*1*, *2*). To investigate how translocation is coupled to peptide loading, we purified and characterized the human TAP complex, identified the different components, and determined their stoichiometry. We purified the class I–TAP complex from digitonin-solubilized membranes from the human Epstein-Barr virus (EBV)–transformed B cell line L001 using an affinity column containing monoclonal antibody (mAb) (148.3) to TAP1 (anti-TAP.1) (*11*). The L001 cell line was used because it expresses relatively large amounts of TAP and because its class I molecules associate stably with TAP. The material eluted at pH 3.5 is shown in Fig. 1A. On the basis of previous data, bands were tentatively identified as TAP.2 (72 kD), TAP.1 (70 kD), calreticulin (60 kD), tapasin (48 kD), human lymphocyte antigen (HLA) class I heavy chain (44 kD), and β_2M (12 kD). We also detected a band at 90 kD, which is the apparent size of calnexin on SDS–polyacrylamide gel electrophoresis (SDS-PAGE). To confirm the identity of the components, we analyzed similar material purified from the cell line Swei. Calnexin, TAP.2, calreticulin, tapasin, and HLA class I heavy chain were positively identified by immunoprecipitation after biotinylation of the purified material (*12*) (Fig. 1B).

The approximate stoichiometry of the TAP complex purified from L001 cells and that purified from β_2M-negative Daudi cells was calculated by excising bands from SDS-PAGE gels and subjecting each band to quantitative amino acid analysis. Two preparations from L001 cells were analyzed, one eluted from the affinity column at pH 3.5 and the other by competitive elution with the specific TAP.1 COOH-terminal peptide to which mAb 148.3 was raised (Table 1). This method eliminates the possibility that TAP.1-associated molecules might dis-

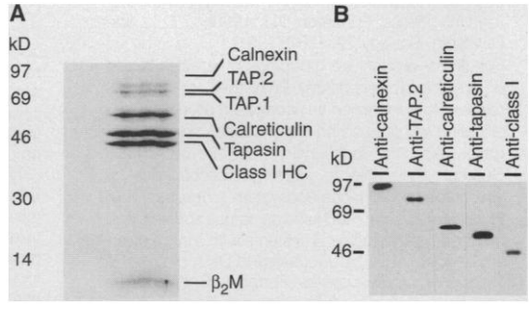

Fig. 1. Components of the MHC class I–TAP complex. (**A**) The TAP complex was affinity-purified from the cell line L001 by elution at pH 3.5 from an anti-TAP.1 (*13*) affinity column, ethanol precipitated, subjected to SDS-PAGE, and stained with Coomassie blue. Molecular size markers in kilodaltons are on the left. The components of the complex are identified on the right. Class I HC, HLA class I heavy chain. (**B**) The TAP complex similarly purified from the cell line Swei, but eluted with the specific COOH-terminal peptide of TAP.1, was biotinylated and denatured (*12*), and individual components immunoprecipitated with rabbit antisera to tapasin (*4*), calnexin (*28*), or calreticulin (Affinity Bioreagents, Neshantic Station, New Jersey), or the mAbs 435.3 (anti-TAP.2) (*29*) or 3B.10.7 (anti–class I heavy chain) (*30*). After SDS-PAGE and electrophoretic transfer to an Immobilon-P membrane, the components were detected by horseradish peroxidase (HRP)–conjugated avidin and a chemiluminescent substrate.

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sociate while leaving residual TAP.1 on the column. The ratio of TAP.1 to TAP.2 is about 1:1 as anticipated on the basis of previous characterization (*13*). In L001 cells the ratio of tapasin to HLA class I heavy chain is also about 1:1. This argues for a single class I binding site on the tapasin molecule. The ratio of tapasin to TAP dimer is considerably greater than 1:1 in both Daudi and L001 cells, and the data indicate that up to four tapasin–class I complexes are associated with a single TAP dimer, assuming that the purified material reflects a single complex of defined composition. The tapasin-TAP association is weak and detergent-dependent (*4*), necessitating the use of digitonin throughout the purification. Thus, the amount of tapasin relative to TAP after purification with an antibody to TAP is unlikely to be erroneously high. MHC class I molecules associate with TAP.1 in single-chain TAP.1–expressing cell lines (*1*, *14*), and we found that tapasin similarly associates with TAP.1 (*15*). Thus, the tapasin–class I complexes may all be bound to TAP.1, or a subset may be TAP.2-associated. Calreticulin association with class I molecules is maintained in the class I–TAP complex, although in one of the L001 preparations it was somewhat substoichiometric relative to class I and tapasin. This may reflect dissociation of calreticulin from the complexes after solubilization or variable association in vivo. Substoichiometric amounts of calnexin copurify with the complex, and preliminary evidence suggests that calnexin may be associated with a small number of assembling TAP complexes (*15*).

To determine the structure of tapasin, we separated the TAP-tapasin complexes from Daudi cells by SDS-PAGE, transferred them electrophoretically to Immobilon-P membrane, and excised the tapasin band. Edman degradation generated an unambiguous sequence from cycle 2 to cycle 31. We also identified residues 2 to 7 of a purified tryptic peptide. Degenerate polymerase chain reaction (PCR) primers were devised on the basis of the sequences and used to amplify fragments corresponding to the 5′ end of the gene. We completed the sequencing using a modification of the vectorette, or "bubble," PCR approach (*16*) adapted for use with cDNA.

Table 1. Composition of the purified TAP complex.

Cell line	Daudi*		L001*		L001†	
	Nanomoles	Ratio	Nanomoles	Ratio	Nanomoles	Ratio
Calnexin	ND‡	–	0.0086	0.74	0.0089	0.86
TAP.1	0.0126	1.17	0.0120	1.03	ND‡	–
TAP.2	0.0108	1	0.0116	1	0.0104	1
Calreticulin	0§	–	0.0229	1.97	0.0362	3.48
Tapasin	0.0395	3.66	0.0489	4.22	0.0369	3.55
HLA class I HC	0§	–	0.0469	4.04	0.0406	3.90

*Elution at pH 3.5. †Elution with specific peptide. ‡ND, not done. §No visible band.

Tapasin appears to be a type I membrane protein (Fig. 2A). The mature protein has 428 amino acids with a single N-linked glycosylation site at position 233. The COOH-terminal sequence has lysine residues at positions -3, -4, and -5, consistent with known cytoplasmic ER retention signals that have lysine residues at -3 and -4 or -3 and -5 (*17*). The hydrophobic region likely to constitute the transmembrane domain (underlined in Fig. 2A) contains a basic residue, Lys^{408}, which may be involved in intramembrane interactions with other proteins, for example, TAP. Such interactions occur between charged residues in the transmembrane domains of T cell receptor α and β chains and CD3 components (*18*). Alternatively, Lys^{408} could be the COOH-terminal border of a 15-residue transmembrane domain beginning at Ser^{393}. Hydrophobic sequences of comparable length can function as transmembrane sequences in recombinant membrane proteins (*19*). Of the 392 lumenal residues of the mature protein, 50 are prolines. They are distributed throughout the sequence with no evidence of periodicity.

The tapasin polypeptide sequence was analyzed by Smith-Waterman database searching and by threading onto known protein structures (*20*), and by comparison with known immuno-globulin (Ig) sequences (*21*). All three methods indicated that tapasin is a member of the Ig superfamily. A sequence incorporating two cysteine residues (Cys^{295} and Cys^{362}) exhibited significant homology to members of the Ig C1-SET constant region superfamily (Fig. 2B). Many of the key amino acids characteristic of the C1-SET are present, but there are 67 amino acids between the two cysteines in tapasin, in contrast to the 55 to 60 residues in most C domains (*21*). The increased span may be due to an extended loop at residues 317 to 323, corresponding to an insertion between the C and D β strands of the C1-SET structure. V-SET folds contain a large expansion at this position. An additional region with weak Ig C1-SET homology runs from residues 51 to 153, but the second Cys residue has not been conserved (*15*). The 118 residues (154 to 271) between these two putative Ig domains do not

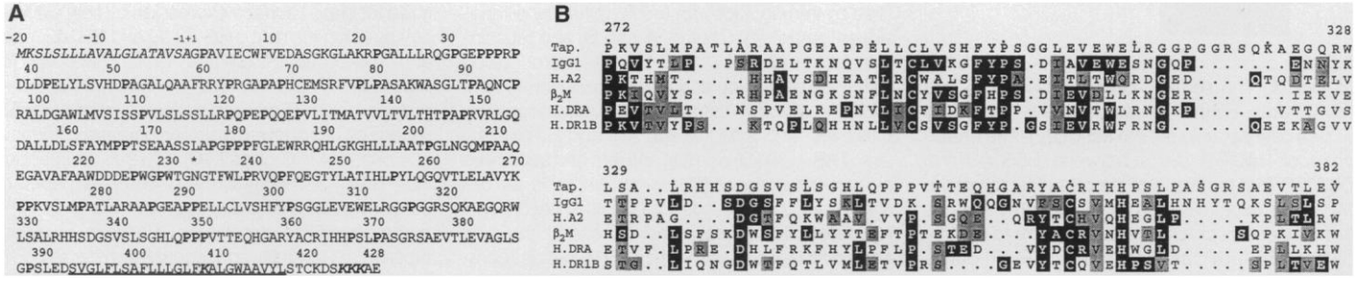

Fig. 2. (**A**) Deduced amino acid sequence (*31*) of tapasin. The signal sequence (residues –20 to –1) is italicized, and proline residues are in bold type. Lysine residues 408, within the putative transmembrane region (underlined), and 424, 425, and 426, constituting a putative ER retention signal, are bold and italicized. The asterisk (Asn^{233}) indicates the putative site of N-linked glycosylation. The cDNA sequence is available through GenBank (accession number: BankIt 122101 AF009510). (**B**) Tapasin is a member of the Ig superfamily. Comparison of residues 272 to 382 of tapasin (Tap.) to some other members of the Ig superfamily that contain a C1-SET domain. Numbering is from the NH_2-terminus of the mature protein. Residues identical to those in tapasin are shaded in black; similar residues are shaded in gray. Gaps are indicated by a dot. H., HLA. These have been introduced to maximize homology with the Ig-C1 fold.

show any obvious Ig homology.

To map the gene encoding tapasin, we used a PCR probe that gave a positive signal on two human chromosome fragment–rodent somatic cell hybrids whose only common human chromosome band includes the MHC (Fig. 3A). This is consistent with the location of the tapasin coding gene rather than a regulatory gene being on chromosome arm 6p (*8*, *10*). To refine the map position, we used a tapasin cDNA probe (base pairs 1 through 796) to screen gridded libraries of PAC clones (United Kingdom Human Genome Mapping Project resource center). This yielded a single clone, PAC 36A2, which we labeled with biotin and used for fluorescence in situ hybridization (FISH) analysis on 40 metaphase spreads from phytohemagglutin-stimulated normal human lymphocytes (*22*). A consistent signal was observed at band 6p21.3 on both copies of chromosome 6 (Fig. 3B). This chromosomal band includes the MHC. The result indicates that the tapasin gene is within $\sim 10^6$ bp of the MHC.

The mutant cell line 220 is defective in MHC class I assembly and lacks tapasin expression (*4*, *8*, *9*). To determine whether introducing tapasin could restore a normal phenotype, we transfected tapasin cDNA into 220 cells expressing HLA-A1 or HLA-B8 (*8*). Immunoprecipitation with anti-TAP or anti-β_2M showed that tapasin restored the association of HLA-B8 with TAP in 220-B8 cells (Fig. 4). Restoration of a functional TAP–tapasin–class I complex also resulted in an ~10-fold increase in class I surface expression (Fig. 5). The restored surface expression is similar to that seen in normal HLA-B8– or HLA-A1–positive B cell lines (*15*). To address the functional importance of tapasin, we infected 220-B8, 220-A1, and their tapasin-expressing derivatives with either influenza virus A/PR8/(HIN1) or a vaccinia virus recombinant expressing cytomegalovirus (CMV) pp65 protein and tested for their susceptibility to lysis by HLA-B8–restricted, influenza nucleoprotein–specific, or HLA-A1–restricted CMV pp65-specific, cytotoxic T cell lines (CTLs), respectively. Tapasin expression restored CTL sensitivity to virus-infected 220-B8 and 220-A1 cells (Fig. 5, C and D), indicating that tapasin plays an essential role in MHC class I function.

MHC class I molecules are more selective than TAP, in that they bind restricted sets of peptides, whereas TAP is highly promiscuous in the peptides it translocates (*23*). Thus, having multiple class I molecules associated with a single TAP dimer may increase the probability that an individual translocated peptide binds successfully. Certain HLA class I alleles have been found to associate poorly with TAP (*24*), which may reflect a weak interaction with tapasin. However, absence of association in detergent solution may not reflect the situation in vivo where the components of the complex remain membrane-bound. The extent to which the antigen processing and presentation function of these weakly associated class I alleles is affected by the absence of tapasin remains to be determined.

It remains unclear precisely how tapasin, TAP, and calreticulin combine to facilitate MHC class I peptide loading. Cells that

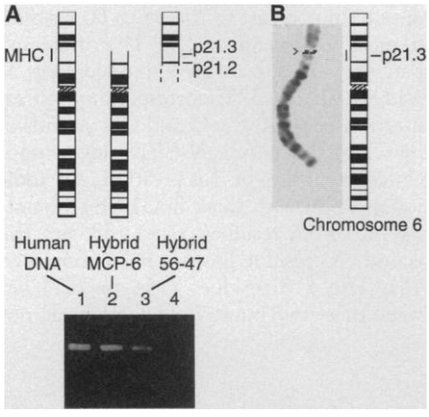

Fig. 3. Location of the tapasin gene in the MHC region. (**A**) The somatic cell hybrids MCP-6 and 56-47 both contain chromosome 6 fragments whose overlap includes the MHC (*32*). The breakpoint on MCP-6 maps between D6S105 and D6S276, just telomeric of the class I region of the MHC (*15*). In cell 56-47, the chromosome extends from the end of chromosome 6, p arm, to centromeric of HLA-DP, as determined by PCR, but its break point has been determined cytogenetically. The PCR primers were TTTAGATCCGGCAGTGAC and TCCTGAGTGTAGAGAAGGAAG, yielding a product of 157 bp. Lane 4 contains mouse genomic DNA. (**B**) The fluorescent signal of labeled PAC 36A2 DNA containing the tapasin gene is shown on the G-banded chromosome and corresponding idiogram, at band 6p21.3.

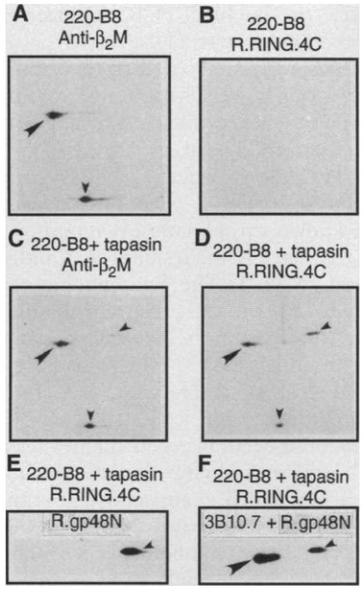

Fig. 4. Tapasin in 220-B8 restores assembly of the TAP–class I complex. 220-B8 cells were transfected with tapasin cDNA subcloned in the pMCFR-PAC vector (*33*) and positive clones identified by immunoblot with the R.gp48N (anti-tapasin) antiserum (*4*). Control (**A** and **B**) and tapasin-transfected (**C** and **D**) 220-B8 cells were labeled with [^{35}S]methionine and [^{35}S]cysteine for 20 min, and 1% digitonin extracts were immunoprecipitated with anti-β_2M [(A) and (C)] and R.RING.4C (anti-TAP.1) (*4*) [(B) and (D)] antisera and separated by two-dimensional (2D) electrophoresis as described (*4*). Arrowheads to the left of each panel represent class I heavy chain, those to the right represent tapasin, and vertical arrowheads represent β_2M. To confirm the identity of the bands, digitonin extracts from tapasin-transfected 220-B8 cells were immunoprecipitated with R.RING.4C, separated by 2D electrophoresis, and following transfer to Immobilon-P membranes probed with R.gp48N (**E**). Simultaneous detection of class I heavy chains with mAb 3B10.7 (**F**) confirmed the relative position of the tapasin spot.

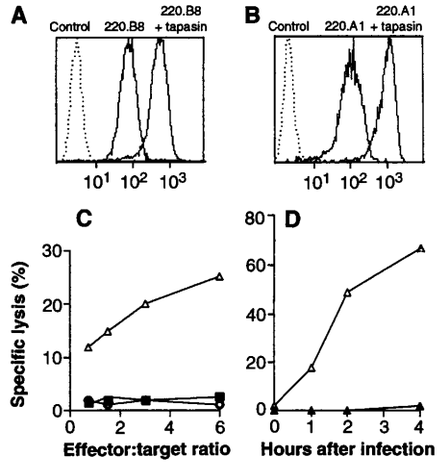

Fig. 5. Tapasin transfection restores MHC class I surface expression and CTL lysis of 220-B8 and 220-A1 cells. Control and tapasin-transfected 220-B8 (**A**) and 220-A1 cells (**B**) (*34*) were analyzed for class I surface expression by flow cytometry by using the mAbs w6/32 (A) and GS142.1 (B) and fluorescein-conjugated rabbit antibody to mouse IgG. The control was a nonspecific isotype-matched mAb. For each series of transfectants, several clones showed a similar increase in surface amounts of MHC class I. (**C**) Influenza A–specific, HLA-B8/NP380-88 restricted CTLs were generated from peripheral blood mononuclear cells (*35*) and assayed in a standard ^{51}Cr release assay on tapasin- (△ and ■) and control-transfected (○), influenza A–infected (△ and ○) 220-B8 target cells. (**D**) Tapasin- (△) and control-transfected (▲) 220-A1 cells were infected with recombinant vaccinia-CMVpp65 for the indicated periods of time, and cytotoxicity was assayed with the CMVpp65-specific, HLA-A1–restricted CTL clone DLS13B7 (*36*) at a saturating effector to target ratio in a standard 5-hour ^{51}Cr release assay.

lack TAP but express tapasin form calreticulin–class I–tapasin complexes in the ER (*4*), and HLA-A2 molecules in such cells are quite efficiently assembled and transported, binding a subset of signal sequence–derived peptides (*25*). Thus TAP, although it is the primary source of peptides, is not absolutely required for peptide loading of class I molecules. HLA-B8 molecules expressed in 220 cells are degraded more rapidly than when expressed in TAP-negative 174 cells (*10*). Therefore, one role of tapasin may be to stabilize class I molecules in the absence of associated peptides, similar to the way HLA-DM association stabilizes empty MHC class II molecules (*26*). Tapasin could also function like the invariant chain does in the class II system, preventing premature nonspecific interactions with unfolded proteins in the ER (*27*). The interaction with TAP may simply provide a means to ensure proximity to the source of translocated peptides, with no additional mechanistic role in peptide loading. These possibilities remain to be evaluated.

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11. Membranes were prepared from frozen pellets of Swei (10^9), Daudi (10^{10}), or L001 cells (10^{10}) by extracting the cells twice in 0.15 M NaCl, 0.01M M tris, pH 7.4, containing 5 mM iodoacetamide and 50 μM phenylmethylsulfonyl fluoride, and in 10 mM tris-HCl, pH 7.4. The pooled supernatants were centrifuged at 100,000*g* for 1 hour at 4°C. The pelleted membranes were solubilized in 100 ml per 10^{10} cells of 1% digitonin in tris-buffered saline (TBS) and the lysate clarified at 100,000*g* for 1 hour at 4°C. The lysate was applied to tandem columns of mouse immunoglobulin G and mAb 148.3 conjugated to Biogel A15 M, equilibrated in 0.1% digitonin in TBS. After extensive washing in 0.1% digitonin the column with mAb 148.3 was eluted either with 0.1% digitonin in 0.1 M NaCl, 0.05 M sodium acetate, pH 3.5, or competitively eluted with 100 μM synthetic peptide corresponding to the TAP.1 COOH-terminus in 1% digitonin in TBS. In the latter case the column was incubated overnight at 4°C with the peptide before fractions were collected. For stoichiometry estimation, the TAP complexes were ethanol precipitated and subjected to SDS-PAGE and the gel stained with Coomassie blue (0.25%). The bands were excised and subjected to amino acid analysis by the Keck Foundation Biotechnology Resource Laboratory, Yale University. In combination with the known molecular weights of the proteins (exclusive of carbohydrate), these data were used to calculate the amount of each component. Glycine, cysteine, and tryptophan were excluded from the calculations of protein amounts. For NH_2-terminal sequencing, the proteins were electrophoretically transferred to Immobilon-P membrane (Millipore), the membrane stained with Coomassie blue, and the tapasin band excised. NH_2-terminal sequencing was performed by the Keck Foundation Biotechnology Research Foundation, Yale University.
12. TAP complexes isolated from Swei cells were dialyzed extensively against 10 mM bicine, 150 mM NaCl, pH 8.2, and biotinylated by adding NHS-LC-biotin (Pierce) to a final concentration of 200 μM and incubating for 30 min at 4°C. Biotinylation was terminated by adding glycine to a final concentration of 10 mM. The biotinylated proteins were denatured by adding SDS (2%) and dithiothreitol (2 mM) and heating the preparation to 100°C for 5 min. For immunoprecipitation, the denatured proteins were diluted 10-fold with 1% Triton X-100 in TBS containing 10 mM iodoacetamide, incubated for 30 min at 25°C, and then precipitated with the appropriate antibodies and protein A– or protein G–Sepharose (Pharmacia) before being subjected to SDS-PAGE. Biotinylated proteins were detected with avidin-HRP and a chemiluminescent substrate (ECL, Amersham).
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16. From the sequence of residues 2 through 31 obtained by NH_2-terminal amino acid sequencing, and from a sequence from a tryptic peptide that proved to be residues 77 through 83 (Fig. 2A), oligonucleotide primers were designed according to preferred human codon usage, but they were degenerate where necessary in the last six bases. RNA was extracted from Swei cells with TRIZOL (Gibco-BRL) and reverse transcriptase–PCR performed. Products were gel purified and cloned into pCR2.1 (Invitrogen). We generated the cycle sequencing template by PCR using resuspended colonies of clones as the template. Sequencing was performed by the Keck facility (Howard Hughes Medical Institute, Yale Medical School). The remaining sequence was obtained from a walking approach by vectorette PCR [P. M. Sharp *et al.*, *Nucleic Acids Res.* **16**, 8207 (1988)]. Polyadenylated RNA was purified with Oligotex (Qiagen), and the cDNA prepared (Gibco-BRL). Restriction digestions of 500 ng of cDNA were performed with Alu I, Hae III, Bsa AI, Hinc II, Msl I, Eco RV with Xmn I, and Pvu II with Stu I, and the vectorettes were ligated. Vectorette PCR was performed with a tapasin- and a vectorette-specific primer, with the above cDNA digestions as separate templates. PCR products were cloned and sequenced as above. For each tapasin-specific primer, a number of products from different cDNA digestions were sequenced in both directions. New tapasin-specific primers were designed to the sequence obtained. Alignment of the multiple overlapping sequences allowed the derivation of a consensus sequence. We verified this by amplifying the full tapasin coding region from a separate synthesis of intact Swei cDNA using an enzyme with proofreading capability (*Pfu* DNA polymerase; Stratagene). Products were cloned into pCR2.1 and the clones sequenced in both directions. These sequences corresponded to the consensus sequence from vectorette PCR. Details of PCR reactions are available upon request.
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31. Single-letter abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.
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33. Tapasin cDNA was amplified by PCR with the *Pfu* DNA polymerase (Stratagene) under standard PCR conditions. The forward primer was 5′-GGGGTACCGCCGCCATGAAGTCCCTGTCTCTGCTCCT-3′ and the reverse primer was 5′-CCATCGATTCACTCTGCTTTCTTCTTTGAATCCTTGCA-3′. A consensus Kozak sequence was introduced at the 5′ end to ensure optimal expression. The 1367-bp amplification product was cloned into the polylinker of the pCR2.1 cloning vector (Invitrogen), sequenced in both directions, and subcloned into the pMCFR-PAC vector by using the 5′ Kpn and 3′ Cla sites. Stable tapasin-transfected 220-B8 clones were generated by electroporation at 210 volts, 960 mF, and selected in medium containing puromycin (500 ng/ml).
34. We transfected 220-A1 cells with the tapasin cDNA in pREP8D by electroporation and selected transfectants with L-histidinol as described [D. Arnold *et al.*, *Nature* **360**, 171 (1992)]. Positive isolates expressing increased surface amounts of HLA-A1 were identified by indirect immunofluorescence with mAb GS142.1 [K. A. Nelson, J. G. Bodmer, A. Martin, C. Navarette, D. M. Strong, in *Immunobiology of HLA*, B. Dupont, Ed. (Springer-Verlag, New York, 1989), pp. 292–301].
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