Tapasin Enhances Assembly of Transporters Associated with Antigen Processing-dependent and -independent Peptides with HLA-A2 and HLA-B27 Expressed in Insect Cells*

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Assembly of HLA class I-peptide complexes is assisted by multiple proteins that associate with HLA molecules in loading complexes. These include the housekeeping chaperones calnexin and calreticulin and two essential proteins, the transporters associated with antigen processing (TAP) for peptide supply, and the protein tapasin which is thought to act as a specialized chaperone. We dissected functional effects of processing cofactors by co-expressing in insect cells various combinations of the human proteins HLA-A2, HLA-B27, β₂m, 2-microglobulin, TAP, calnexin, and tapasin. Stability at 37 °C and surface expression of class I dimers correlated closely in baculovirus-infected Sf9 cells, suggesting that these cells retain empty dimers in the endoplasmic reticulum. Both HLA molecules form substantial quantities of stable complexes with insect cell-produced peptide pools. These pools are TAP-selected cytosolic peptides for HLA-B27 but endoplasmic reticulum-derived, i.e. TAP-independent peptides for HLA-A2. This discrepancy may be due to peptide selection by human TAP which is much better adapted to the HLA-B27 than to the HLA-A2 ligand preferences. HLA class I assembly with peptides from TAP-dependent and -independent pools was enhanced strongly by tapasin. Thus, tapasin acts as a chaperone and/or peptide editor that facilitates assembly of peptides with HLA class I molecules independently of their interaction with TAP and/or retention in the endoplasmic reticulum. HLA class I (HCI) molecules present short peptides mainly derived by cytosolic degradation of cellular proteins to cytotoxic T cells. Assembly of these peptides with newly synthesized HCI molecules in the endoplasmic reticulum (ER) is assisted and controlled by a multitude of proteins (1). HCI heavy chains associate initially with the ER chaperone calnexin which, although not essential for HCI assembly (2), has been reported to facilitate folding of heavy chains and prevent their aggregation (3). Upon binding of β₂m, HCI heavy chains dissociate from calnexin. Empty HCI/β₂m dimers are found in complexes including the soluble ER chaperone calreticulin (4), the putative chaperone tapasin (4–6), and Erp57, described previously as thiol-dependent oxidoreductase (7–9). These complexes associate then with the heterodimeric TAP1/TAP2 transporter which delivers cytosolic peptides into the ER. Once a peptide has bound to HCI molecules, these are released from assembly complexes, leave the ER, and transit to the cell surface.

Since most protein interactions in HCI loading complexes appear to be formed simultaneously, probably in a cooperative manner (10), the functions of individual proteins, and the precise nature of the formed contacts are only partially understood (11). So far only peptide supply by TAP (12) and HCI interaction with tapasin (5) have been shown to be essential for HCI assembly with peptides. Tapasin mediates association of empty HCI dimers with TAP complexes (5, 13). This role of an intermediary involves binding of a tapasin moiety within the carboxyl-terminal 128 residues to TAP and interaction of the 50 amino-terminal tapasin residues with HCI molecules (10, 14). Reconstitution of normal peptide assembly in the tapasin-deficient cell line .220.B8 by a soluble tapasin molecule unable to interact with TAP association suggests that tapasin may also act as a chaperone facilitating peptide assembly with HCI molecules (15).

HCI molecules display great polymorphic variation that determines their peptide ligand preferences but may also affect their associations with other proteins. Both parameters may affect the efficiency and mode of HCI assembly with peptide. Peptide preferences of individual HCI molecules may be more or less well adapted to the products of other components of the antigen processing machinery, for example peptides generated by proteasome or pumped by TAP. Variable interaction with processing cofactors in the ER may also affect HCI peptide loading.

Some evidence for HCI polymorphism-related variation in assembly of HCI molecules has been reported. The speed of assembly in complexes and progression to the cell surface has been reported to vary significantly among HCI alleles (16). Absence of TAP or tapasin affects HCI molecules to a different degree. HLA-A2, the most frequent HCI allele in caucasian populations, is affected the least by both deficiencies (17, 18), presumably because of its capacity of binding signal sequence-derived peptides which is shared with few other HCI alleles (1). More recently, HLA-B27 has been shown to depend less than two other HLA-B alleles on tapasin for peptide assembly (19). Moreover, HCI molecules have been found to display considerable variation with respect to the strength of TAP interaction (20) and may vary with respect to their dependence on the
proteasome for generation of antigenic peptides (21). However, the molecular mechanism of these differences has so far not been elucidated.

One candidate mechanism affecting efficiency of peptide presentation by HCl molecules is peptide supply by the TAP complex. Studies on rodent transporters have demonstrated that strongly incompatible ligand preferences of TAP and MHC class I molecules result in poor peptide supply to the latter (22, 23). Although the human transporter is clearly less selective than mouse TAP and rat TAP1-TAP2(9) complexes (24), we have found that ligand preferences of individual HCl molecules vary greatly with respect to their adaptation to human TAP preferences (25, 26). While HLA-A2 preferences are more difficult to observe at physiological expression levels. By using this system, we addressed the following issues: (i) what are the effects of tapasin, calnexin, and calreticulin on assembly of TAP-dependent and independent peptides with HCl molecules; (ii) are there allelic variations in these effects; and (iii) which interactions between HCl molecules, TAP, calnexin, and calreticulin can be observed in insect cells?

**EXPERIMENTAL PROCEDURES**

**VIRUSES—**Baculoviruses expressing wild type human TAP1.0101 and TAP2.0101 proteins have been described previously (27). For expression of wild type TAP1-TAP2 complexes, a dual promoter virus containing human TAP1 and TAP2 cDNAs (a gift from Dr. R. Tampe(1), MPI Martinsried) was used. Mutant TAP1 and TAP2 proteins were generated by replacement of the Walker A sequence motifs by a peptide linker; single and double mutant TAP subunits form dimeric complexes that do not translocate peptides. Correct sequences of all cDNAs to be expressed in baculovirus were verified by PCR-based complete sequencing of plasmid inserts using an Applied Biosystems Inc. automated sequencer. cDNAs were sequenced after cloning of PCR products or restriction fragments into pCRll (Invitrogen, Carlsbad, CA), pTAG (R&D Systems, Abingdon, UK), or pBluescript SK(8) (Stratagene, La Jolla, CA) vectors. Human β2m cDNA was PCR-amplified in 22 cycles from plasmid HS4 provided by Dr. S. Kvit, Stockholm, Sweden, using primers with internal BamHl sites. Then the cDNA was cloned into pCRll and finally subcloned into the BamHl sites of pVL1393 (Invitrogen) and of the dual promoter vector pAcUW51 (PharMingen, San Diego, CA) already containing HLA-A2 or B27 inserts.

An HLA-A(2)0201 cDNA cloned into a M13 phage vector was obtained from Drs. P. Parham and J. Gumperz (Stanford). Insert was amplified from phage-derived double-stranded DNA in a 22-cycle PCR using primers with an internal BglII (5’)- or EcoRI (3’) site, respectively, and cloned into pCRll. A2 cDNA was then cloned as BglII fragment into pVL1392 (Invitrogen) and pAcUW51. An HLA-B27(9)05 cDNA cloned into pUHD was obtained from Dr. K. Fruh, San Diego. Insert was amplified in an 18-cycle PCR using primers with a BamHl (5’) or EcoRI (3’) site, respectively, cloned into pCRll, translated into pAcUW51 by Amersham Pharmacia Biotech) and then into pBluescript SK(8) for sequencing. Sequencing revealed that the amplified as well as the original pUHD-cloned cDNA differed from the published consensus sequence by a single nucleotide replacement resulting in an aspartic acid for asparagine substitution at codon 151. To correct the sequence, the B27 insert was transferred as BamHl/EcoRV fragment into BglII/Ecorl-digested pAcUW51, and site-directed mutagenesis using the QuikChange(8) kit (Stratagene) was performed to obtain the correct B27 sequence.

A human calmodulin cDNA cloned in pCRF-PAC was obtained from Drs. P. Cresswell and T. Mak, Yale University, and subcloned into pBluescript SK(8) containing six histidine codons followed by a stop codon. Calnexin stop codon and 3′-untranslated sequence were then removed by loop out mutagenesis so that the last calnexin codon was joined in frame to the first histidine codon. To shorten and modify the 5′-untranslated calnexin sequence, a 425-base pair KpnI/HindIII fragment was replaced by an equivalent PCR-amplified fragment with a 5′ BamHl site. Finally, the complete calnexin cDNA was subcloned into pVL1393. A human calreticulin cDNA cloned into pT7Z18 was obtained from Dr. J. D. Capra, Dallas. Insert was PCR-amplified using primers with an internal BglII (5’) or EcoRI (3’) site, respectively, and cloned into pT7oB after subcloning as BglII/EcoRI fragment into pVL1392 and pAcUW51.

A human tapasin cDNA with a 3′ polyhistidine extension was generated by PCR amplification of reverse-transcribed (Copy Kit(5), Invitrogen) cDNA from the cell line U937 using a high fidelity mixture of thermostable DNA polymerases (Advantage HF(1), CLONTECH). The tapasin cDNA was sequenced in pCRll before subcloning into pVL1393. Two TAP-independent peptides were expressed in the baculovirus system as monigens transferred from a vaccinia virus expression plasmid. Briefly, the vaccinia vector pSC11s was first modified by insertion of complementary oligonucleotides encoding the signal peptide of the adenovirus E3/19K protein. Then additional oligonucleotides for an HLA-A2-restricted epitope (FLPSDFPFSPS(28)), or an HLA-B27-binding peptide (RYYQGSTEL(29)), respectively, were inserted 3′ of the signal peptide encoding sequence, and the complete sequences were transferred into pVL1393. Both epitopes have high binding affinity for their restricting HLA molecules, and vaccinia-expressed sig/core 18-27 gives rise to high levels of TAP-independent target cell lysis by A2-restricted CTLs(3).

All recombinant viruses were produced by co-transfection of Spodoptera frugiperda (Sf9) cells with 100 ng of baculovirus DNA (BaculoGold(5), PharMingen) and 3 μg of pVL1392, pVL1390, or pAcUW51-cloned cDNAs as described (30). Control baculoviruses used in this study express human 65-kDa glutamic acid decarboxylase (GAD65) or the intracellular portion of the tyrosine phosphatase IA-2 (IA-2ic), two autoantigens targeted in type 1 diabetes (31).

**Antibodies—**Monoclonal antibody (mAb) AF8 specific for human calnexin (32) was provided by Dr. M. Brenner (Harvard Medical School, Boston), and mAb BBM.1, used for human calreticulin, was given by Dr. G. Moldenhauer, Heidelberg, Germany. Hybridomas producing mAb W6/32 (recognizing HCl/β2m dimers), BB7.2 (specific for HLA-A2), and BB27.1 (specific for HLA-B27) were obtained from American Type Culture Collection (Manassas, VA). Hybridoma HC10 with specificity for free HCl heavy chains (33) was obtained from Dr. H. Ploegh (Harvard). mAb 148.3 recognizing the carboxyl terminus of human TAP1 and mAb 429.3 (used for Western blots) and 435.3 (used for immunoprecipitations) with specificity for the carboxy-terminal domain of human TAP2 have been described previously (27). mAb were purified from ascites obtained from hybridoma-inoculated Balb/c mice by affinity purification on protein G-Sepharose columns (Amersham Pharmacia). Rabbit sera specific for human calnexin and calreticulin were purchased from StressGen Biotechnologies Corp. (Victoria, Canada), and a rabbit serum with specificity for human β2m was obtained from Dako (Glostrup, Denmark).

**Metabolic Labeling and Immunoprecipitation—**5 × 10(5) Sf9 cells adhering to 6-cm inner diameter tissue culture dishes were infected with 2 × 10(6) pfu of HCl/β2m double insert virus supernatants together with 4 × 10(6) pfu of each of two other viral supernatants for 1 h. Infectious supernatants were then replaced by 5 ml of complete TMN-FH (Roche Molecular Biochemicals or Sigma). To assess steady state levels of HCl molecules, cells were labeled after 24 h infection by incubation for 40 min in 0.6 ml of Grace’s medium without methionine supplemented with 0.33 mCi of [35S]methionine (NEN Life Science Products). Then 0.9 ml of methionine-deficient Grace’s medium with 10% fetal calf serum dialyzed against PBS was added, and labeling was continued overnight. Labeled cells were recovered by rinsing in ice-cold PBS, washed once in PBS with 1 mM PMPS and lysed by incubating 1 h at 4°C.
in 1 ml of a buffer containing 150 mM NaCl, 40 mM Tris, pH 7.4, 1% Nonidet P-40 (Fierce), and a mixture of protease inhibitors: 0.2 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride-HCl (ICN, Costa Mesa, CA), 1 μg/ml apritin (ICN), 1 mM EDTA, 1 mM benzamidine (Calbiochem), 5 μM leupeptin (Alexis, Laufelfingen, Switzerland), and 10 μM pepstatin (Alexis). Lysates were clarified by centrifugation at 20,000 × g for 10 min and precleared by incubation for 2 h at 4 °C with 40 μl of protein G-Sepharose beads (Gamma-Bind Plus®, Amersham Pharmacia Biotech) or Sepharose 4B beads coupled to a control mAb. After removal of beads by centrifugation for 1 min at 1,000 × g, cleared lysates were split in equal parts (for HLA-A2) or at a ratio of 1:2 (for HLA-B27) for immunoprecipitation by mAb HC10 and W6/32 (10 μg each), respectively. 2.5 h later, 12.5 μl of protein G-Sepharose was added for a further 30 min to recover immunoprecipitated material. Then beads were washed with a series of Tris/NaCl buffers containing 0.1% Nonidet P-40 with increasing pH (2 × pH 7.4, 1 × pH 8.0, 1 × pH 9.0, 1 × pH 9.0 with 250 mM NaCl added), washed once in 50 mM Tris, pH 6.8, with 0.01% Nonidet P-40, and finally boiled in 20 μl reducing SDS-PAGE sample buffer.

In experiments on HCl stability at 37 °C, precleared lysates were split in two equal parts that were incubated for 1 h at 34 or 37 °C, respectively. Then HCl molecules were immunoprecipitated by incubation for 45 min with 10 μg of mAb HC10 or W6/32 pre-absorbed onto 8 μl of protein G-Sepharose. Beads were washed as described above. Precipitated proteins were separated in 10% SDS-PAGE gels. Gels were fixed in 50% trichloroacetic acid, destained in 50% methanol, 10% acetic acid, enhanced in 1 m sodium salicylate, pH 6.0, and finally autoradiographed for 24 h to 1 week. Autoradiograms were scanned and analyzed by densitometry using NIH Image 1.62 software.

Co-precipitation Experiments—To analyze protein interactions, 5 × 10⁶ plastic-adherent Sf9 cells were infected for 1 h with 1.5 × 10⁶ pfu in a total volume of 5 ml of TMN-FH. Infectious supernatant was then replaced by 500 μl of a buffer containing 150 mM NaCl, 40 mM Tris, pH 7.4, 1% Nonidet P-40 with increasing pH (2× pH 7.4, 1× pH 8.0, 1× pH 9.0, 1× pH 9.0 with 250 mM NaCl added) containing 1% digitonin (Sigma) and protease inhibitors on steady state levels of HCl dimers, insect cells were infected with one or several baculoviruses as indicated by lysing in a Nonidet P-40 buffer, and the indicated amount of total lysate protein was separated in SDS-PAGE gels and blotted onto PVDF membranes. Reombinant or control Jeshothm proteins were then stained with serum R425 specific for HCl heavy chains (upper left panel), a rabbit serum specific for β₂m (top right), mAb AF8 recognizing calnexin (bottom left panel), a rabbit serum specific for calreticulin (bottom center), or mAb 148.3 recognizing human TAP1 (bottom right). Viruses used for infections harbored cDNAs for the following human proteins: HLA-A2 (A), HLA-B27 (B), HLA-A2 + β₂m (AB), calnexin (C), TAP1 + TAP2 (T), HLA-B27 + β₂m (Bb), β₂m (β), and calreticulin (CR).

Expression of HLA-A2 and B27 and Human Tapasin, Calnexin, and Calreticulin in Baculovirus-infected Insect Cells—We expressed full-length cDNA clones coding for human calnexin, calreticulin, tapasin, β₂m, HLA-A*0201, and HLA-B*2705, and two leader sequence-coupled peptides with high affinity for HLA-A2 or B27, respectively, in recombinant baculoviruses under the control of the polyhedrin promoter. Calnexin and tapasin were expressed as fusion proteins joined to six carboxy-terminal histidine codons. For expression of HLA class I heavy chain/β₂m dimers and of TAP1-TAP2 complexes, we also produced viruses expressing two proteins under the control of the polyhedrin and the p10 promoter, respectively.

To verify expression of proteins with correct molecular weights, we blotted Nonidet P-40 lysates of insect cells infected for the 3 days with one or several viruses onto PVDF membranes and quantified expressed proteins using specific mAb or sera and an ECL protocol (Fig. 1A). As controls, lysates from the human B cell line Jeshothm expressing HLA-A2 and HLA-B27 were analyzed. As shown in Fig. 1, all reombinant proteins had molecular weights that were similar or identical to their physiological counterparts expressed in the human B cell. This includes HLA-A2 and B27 heavy chains, suggesting that the insect cell-expressed HCl molecules are glycosylated to a similar extent as in human cells. Recombinant calnexin migrated slightly more slowly than its physiological equivalent due to the carboxy-terminal polyhistidine extension. Recombinant tapasin could be purified as a 49-kDa protein based on the interaction of its polyhistidine extension with Ni²⁺-nitrilotriacetic acid resins (not shown).

Reombinant proteins represented a higher percentage of total Nonidet P-40-solubilized cellular protein than their physiological counterparts in the human B cell line. The two TAP subunits and the chaperones calnexin and calreticulin showed the highest relative levels of expression; equal protein amounts of B cells contained on average 10-fold (between 5- and 30-fold in several experiments) less of these proteins than insect cells infected with the relevant viruses. HCl heavy chains and β₂m were expressed at relatively more moderate levels; in several independent experiments, the ratio of expression in insect cells to B cells (normalized for equal protein amounts) was between 1 and 3. Tapasin expression levels were not compared with those in human B cells.

Effect of Chaperones and TAP on Assembly in Dimers of HLA-A2 and HLA-B27—To study the effect of antigen processing cofactors on steady state levels of HCl dimers, insect cells were infected with various combinations of three viruses, driving expression of up to five human proteins. 24 h after infection, cells were labeled metabolically for 12 h, followed by immunoprecipitation of unfolded free heavy chains or folded HCl dimers with an excess of mAb HC10 or W6/32, respectively. Precipitated HCl molecules were separated by SDS-PAGE and quantified by densitometry. For each condition,
for dimer precipitation to facilitate gel visualization (densitometry. For HLA-A2 precipitations lysates were split in equal parts (A), whereas for HLA-B27 precipitations two-thirds of lysates was used for dimer precipitation to facilitate gel visualization (B). Virus 1 was HLA-A2/β2m in A and HLA-B27/β2m in B. Viruses 2 and 3 are indicated above A and were as follows: CON1, GAD65; CON2, IA-2ic; CALN, calnexin; CRET, calreticulin; TAP, TAP1/TAP2; PEP, TAP-independent peptide; TAPA, tapasin. For each combination of expressed proteins, HCI molecules precipitated by HC10 (specific for free heavy chains; left lane) and W6/32 (specific for dimers; right lane) are shown. Autoradiographs were scanned, and scans corresponding to HC10/W6/32 pairs for separate conditions were re-assembled for the figure. All scans are derived from a single experiment and the same exposure time. Histograms below gel scans indicate the precipitated amount of free heavy chains (open columns) and dimers (filled columns) relative to that obtained from control infections (HCI/CON1/CON2), with the amounts obtained for the latter condition set at 100. Numbers indicate the percentage of HCI molecules precipitated by W6/32 relative to the sum of HCI molecules precipitated by HC10 plus W6/32 for the same condition.

Fig. 2 shows three parameters as follows: (i) the percentage of HCI molecules recovered by dimer-specific mAb W6/32 relative to the sum of HCI molecules precipitated by HC10 and W6/32 (given as numbers); (ii) the amounts of recovered free heavy chains and (iii) of dimers relative to control infections, in which cells expressed HCI/β2m together with two control proteins (expressed as a bar graph). Fig. 2 is representative of three independent experiments. Similar results were obtained in four experiments in which the amounts of dimers and free heavy chains were quantified by Western blot staining with a serum (R425) recognizing denatured heavy chains (not shown). In control experiments on Jesthom B cells, 10–20 times less HCI molecules were recovered by HC10 than by W6/32 (not shown). Only HCI10-reactive heavy chains could be recovered from insect cells lacking β2m expression (not shown).

Both the percentage assembling in dimers in the absence of processing cofactors and the effect of cofactors differed substantially between HLA-A2 and B27. In the case of HLA-A2 (Fig. 2A), 35% of heavy chains formed dimers with β2m in the absence of any human cofactor. Surprisingly, this percentage was not affected by co-expression of chaperones or peptide sources including a TAP-independent peptide with high A2 binding affinity. However, although not affecting the percentage of A2 molecules forming dimers, calnexin and calreticulin co-expression resulted in modest (30–50%) simultaneous increases in the amount of free heavy chains and dimers relative to control infections. Dimer formation of HLA-A2 was limited to less than 40% of HLA-A2 molecules under all conditions. Less than 10% of B27 molecules formed dimers in the absence of cofactors (Fig. 2B). Co-expression of peptide sources was sufficient to increase this percentage and the amount of HLA-B27 dimers relative to control infections without affecting the amount of free heavy chains. A TAP-independent peptide increased the percentage of dimers much more efficiently (factor 3.9) than TAP alone (factor 1.6). Co-expression of chaperones did not enhance the effect of the TAP-independent peptide with high B27 affinity. However, tapasin, which had only a small effect when co-expressed alone or with calreticulin, and a modest effect together with calnexin, enhanced the TAP effect 2-fold so that a similar percentage of dimers as in the presence of the TAP-independent peptide was formed. Thus, formation of B27 dimers required co-expression of peptide sources and, in the case of TAP, was enhanced by co-expression of tapasin.

Note that the percentage of dimers did not exceed one-third of B27 molecules in all settings.

It has been proposed that TAP may also act as a chaperone for HCI molecules, for example by retaining them in the ER or stabilizing them until peptides bind. To determine whether this was the case in the insect cell system, we tested the effect on dimer formation of TAP proteins with mutated Walker A sequences (not shown). These mutant TAP proteins assemble normally in complexes but cannot transport peptide.2 Co-expression of mutant TAP dimers had no effect on HLA-B27 dimer formation or cell surface expression (see below). This demonstrates that active peptide transport by TAP is required for its effect on HLA-B27 dimer formation and argues against an important role of TAP complexes as chaperones for HLA-B27 dimers.

Effect of Chaperones and TAP on Cell Surface Expression of HLA-A2 and B27—In vertebrate cells, newly formed MHC dimers are retained in the ER until they acquire peptide ligands with sufficient affinity (34). Therefore, the amount of MHC dimers acquiring mature N-linked glycans in the Golgi compartments and then expressed on the cell surface reflects the functional performance of the machinery for generation, delivery, and assembly of peptides in the cytosol and ER. However, murine MHC class I molecules can reach the surface of vertebrate cells when these are incubated at 26 °C (35, 36), whereas most human MHC class I molecules cannot (37, 38). Empty murine and human MHC class I molecules have also been reported to be expressed on the cell surface of Drosophila cells lacking antigen processing cofactors, presumably because insect cells are cultured at 27 °C (39). We therefore analyzed the level of HLA-A2 and B27 dimers on the surface of SF9 cells in the absence or presence of processing cofactors. HCl surface expression was analyzed under the conditions also applied in intracellular dimer formation, i.e. 36 h after triple infections by 2-fold higher infectious doses of cofactor viruses relative to HCl viruses.

Insect cells expressing HLA-A2/β2m plus two irrelevant proteins expressed significant amounts of W6/32-reactive dimers on the surface, whereas only small amounts of surface B27 dimers were expressed under these conditions (mean fluorescence 4.4 with isotype control, 9.8 with mAb W6/32; Figs. 3 and 4). Co-expression of TAP or the TAP-independent peptide

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**Fig. 2.** Formation of HLA-A2/β2m and HLA-B27/β2m dimers in triple-infected insect cells. SF9 insect cells were infected with a combination of three viral supernatants and labeled metabolically for 12 h starting 24 h after infection. Then cells were lysed, lysates were split, free heavy chains and HCI dimers were immunoprecipitated with mAb HC10 and W6/32, respectively, and quantified by SDS-PAGE and densitometry. For HLA-A2 precipitations lysates were split in equal parts (A), whereas for HLA-B27 precipitations two-thirds of lysates was used for dimer precipitation to facilitate gel visualization (B).
had negligible effects on expression of A2 dimers but increased B27 surface expression dramatically (A and C). Additional expression of tapasin increased most substantially surface expression of HLA-A2 in the absence of a peptide source and of HLA-B27 in cells co-expressing TAP (B and D). Equivalent results were obtained with additional conformation-specific mAb recognizing A2 (BB7.2) or B27 (B27M1) dimers; the latter mAb has been reported to recognize a subset of peptide-filled B27 molecules (40) whose assembly with B27 may be highly tapasin-dependent (19).

A quantitative view of the effects of processing cofactors on HCl surface expression is provided in Fig. 4. HLA-A2 surface expression was modestly increased by calnexin and the TAP-independent peptide, more by calreticulin, and most significantly by tapasin. TAP co-expression alone or in combination with other factors had no effect. Expression of the TAP-independent peptide was synergistic with all three chaperones, i.e. tapasin, calreticulin, and calnexin. Thus, different from the percentage of intracellular dimers, cell surface expression of HLA-A2/β2m was affected by processing cofactors, especially availability of chaperones. Moderate effects of calnexin and calreticulin may be due to increased total cellular amounts of HLA-A2 in their presence (Fig. 2A). In contrast, since tapasin did not affect the total amount of cellular HLA-A2, its stronger effect was likely to be related to more efficient peptide assembly and thereby stabilization and export of A2 dimers in its presence. Importantly, in these experiments, tapasin exerted its effect on a pool of TAP-independent peptides.

Fig. 4 also illustrates the striking effect of peptide sources on surface expression of HLA-B27 dimers (7-fold increase with TAP and 10-fold with the TAP-independent peptide). Chaperones enhanced these effects but in a distinct fashion according to the peptide source. Formation of cell surface-expressed dimers in the presence of the TAP-independent peptide was most strongly (but still modestly) enhanced by calreticulin, whereas formation of exported dimers with TAP-supplied peptides was strongly increased by tapasin (factor 1.7), little by calreticulin, and not at all by calnexin.

MHC class I complexes expressed on the surface of Drosophila (39) and, as recently reported (41), also Aedes insect cells can be devoid of peptide. This can be revealed by incubation of the cells at 37 °C for 1 h (41) which leads to disappearance of unstable empty molecules. To determine whether HLA-A2 and B27 molecules expressed on Sf9 cells are peptide-filled, we incubated cells 36 h after infection for 60 min at 37 °C followed by staining of cell surface HC1 dimers by mAb W6/32 (not shown). These incubations resulted in a dramatic reduction in the number of viable insect cells, presumably due to the cumulative cell damage from viral infection and heat shock. However, cells surviving after 37 °C incubations expressed about 70–80% of surface HCl dimers of cells incubated at 27 °C, regardless of the combination of human proteins expressed. This suggested that under all conditions the vast majority of HCl dimers reaching the surface of infected Sf9 cells were peptide-filled.
Effect of Chaperones and TAP on Stability of HLA-A2 and HLA-B27 Dimers—Similar to empty cell surface dimers, empty detergent-solubilized HCl/β2m dimers dissociate at 37 °C, a property that can be used experimentally to distinguish empty and peptide-filled dimers (42). We used this method to analyze peptide filling of HLA-A2 and B27 dimers that were metabolically labeled for 12 h at the end of a 36-h infection period (Fig. 5). Equal aliquots of Nonidet P-40 lysates were incubated for 1 h at 4 or 37 °C before HCI dimers or free heavy chains were recovered in a rapid immunoprecipitation to avoid stabilization of empty dimers by mAb during the precipitation (36). Dimers precipitated from human B cells were completely stable under these conditions (not shown). For each condition, Fig. 5 indicates the percentage of stable dimers (numbers), and the amount of dimers recovered at 4 or 37 °C relative to the amounts recovered in cells expressing HCl/β2m dimers together with two control proteins (bar graph).

Less than 20% of cellular HLA-A2 dimers were stable at 37 °C in cells devoid of processing cofactors (Fig. 5A). This proportion was increased slightly by calreticulin and the TAP-independent peptide and doubled by tapasin. TAP had no effect on A2 dimer stability (not shown). Simultaneous expression of calreticulin and the TAP-independent peptide was synergistic and also doubled the proportion of stable dimers. Thus, stability at 37 °C of A2 dimers paralleled closely their expression at the cell surface; chaperones, especially tapasin, enhanced A2 assembly with peptides derived from an endogenous TAP-independent pool, and assembly of the defined TAP-independent peptide with high A2 affinity appeared to be facilitated mainly by calreticulin.

Similar to cell surface expression, stability of B27 dimers was strikingly enhanced by co-expression of peptide sources (Fig. 5B). The defined TAP-independent peptide alone increased the percentage of stable molecules almost 10-fold, resulting in a more than 50-fold increase in the amount of stable dimers relative to control cells. Expression of additional cofactors did not enhance stable dimer formation with the TAP-independent peptide. Expression of TAP alone also increased the proportion of stable dimers more than 5-fold and thereby their total amount 10-fold relative to control cells. Tapasin, which alone or in combination with calreticulin had little effect on stability, increased the percentage and total amount of dimers formed in the presence of TAP substantially. Thus, also in the case of HLA-B27, results for dimer stability were closely related to those for cell surface expression.

We also studied stability of metabolically labeled free heavy chains recognized by mAb HC10 (Fig. 5, A and B, right lanes). In the absence of processing cofactors, the majority of heavy chains was lost during 37 °C incubations, probably due to aggregation. Co-expression of housekeeping chaperones calnexin (for A2 and B27) and calreticulin (B27 only), but also of the TAP-independent peptide alone (B27), increased the amount of heavy chains stable at 37 °C (Fig. 5, and not shown). Small but significant amounts of HC10-reactive A2 and B27 heavy chains could also be detected on the surface of live insect cells (not shown). Surface expression of free heavy chains was also increased in the presence of calnexin, calreticulin (A2 and B27), and the TAP-independent peptide (B27).

Interactions of TAP and Chaperones with HCI Molecules—Taking advantage of high recombinant protein levels and the possibility of freely combining proteins for expression in the insect cell system, we also analyzed interactions between HCI molecules and processing cofactors (with the exception of tapasin). Before performing experiments on lysates from co-infected cells, we asked whether the various proteins could associate with cell lysis. We lysed insect cells infected with single viruses and expressing high levels of HCI heavy and light chains, TAP1-TAP2 complexes, calnexin, or calreticulin in digitonin, mixed lysates containing HCI molecules with a lysate containing another protein of interest, and incubated 16 h before recovering free HCI heavy chains with mAb HC10. As shown in Fig. 6A, prolonged incubation of large amounts of digitoninsolubilized calreticulin or TAP with A2 or B27 heavy chains did not result in detectable formation of complexes between any two proteins; only when calnexin and B27 heavy chains were mixed, a small quantity of the chaperone associated with the free heavy chain.

After having demonstrated that at least TAP and calreticulin
interaction with HCl proteins required protein co-expression in the same cell, we studied TAP interaction with HCl molecules (Fig. 6B). We found a highly significant association of HC10-reactive free A2 and B27 heavy chains with TAP complexes (left lane); in cells expressing HCl heavy chains only together with individual TAP subunits, large amounts of TAP1 can be co-precipitated with HCl heavy chains, whereas a much smaller amount of TAP2 associates with heavy chains. This association can be detected in cells expressing HCl heavy chains only or heavy chain/β2m together and is also observed when calnexin and/or calreticulin are co-expressed (not shown); the most substantial association is observed in cells expressing the TAP1 subunit and an HCl heavy chain only.

In several experiments, we also searched for a potential association between TAP and β2m or HCl dimers. However, we have not found any association of W6/32-reactive A2 or B27 dimers with TAP complexes or individual subunits (not shown). We tried to detect association of β2m with TAP complexes or subunits, using antibodies to TAP, to β2m, and to free heavy chains (43) and cells infected with various virus combinations; in no case could we detect interaction of β2m or β2m-assembled HCl heavy chains with TAP (not shown). Reasoning that HCl dimer association may be difficult to detect due to rapid binding of TAP-delivered peptides to HCl molecules followed by HCl dissociation from TAP, we tried to co-precipitate β2m or HCl dimers with mutant TAP complexes unable to transport peptides (see above); again, β2m could not be co-precipitated with TAP (not shown). We conclude that in the absence of tapasin, insect cell-expressed free heavy chains associate efficiently with TAP complexes and especially with the TAP1 subunit, but HCl dimers cannot interact with TAP. There was no difference between HLA-A2 and B27 with regard to TAP association.

Next we studied calnexin association with HCl molecules. Calnexin could be co-precipitated with A2 heavy chains in cells expressing A2 heavy chains alone or in combination with β2m; co-expression of calreticulin or TAP did not affect significantly this association (Fig. 6C). In cells expressing β2m with HLA-A2, a small amount of the chaperone was associated with W6/32-reactive A2 dimers (Fig. 6C, right panel). As already suggested by the experiment shown in Fig. 2, expression of calnexin resulted in increased recovery of free A2 heavy chains regardless of the presence of β2m. In analogous experiments on cells expressing HLA-B27 and calnexin, identical results were obtained (not shown).

Finally, interactions of calreticulin with insect cell-expressed HCl molecules were analyzed (Fig. 6D). Different from calnexin, calreticulin could be precipitated with HLA-A2 and HLA-B27 dimers recognized by mAb W6/32 as well as allele-specific mAbs BB7.2 and B27M1. However, larger amounts of calreticulin were recovered with free heavy chains immunoprecipitated by mAb HC10. Free heavy chains associated with calreticulin were not derived from unstable empty HCl dimers dissociating during immunoprecipitation since calreticulin could also be co-precipitated very efficiently with A2 and B27 heavy chains expressed in the absence of β2m (not shown).

**DISCUSSION**

This is the first study of the functional interactions between human MHC class I molecules and TAP, tapasin, and other ER chaperones in non-vertebrate cells. Novel results obtained in this study bear on three issues as follows: (i) the effect of tapasin on the assembly of TAP-dependent -independent peptides with HCl molecules; (ii) distinct utilization of peptide sources by the two HCl molecules; and (iii) interactions between TAP and HCl molecules.

Insect cells have previously been used to express MHC class I molecules. Baculovirus-encoded H-2K\(^d\) and HLA-B27 have been reported to be expressed at relatively high levels and to assemble poorly (<5 or <10%, respectively) in dimers (44, 45). Jackson and co-workers (39) expressed three murine and four
human MHC class I molecules in stably transfected Drosophila cells, whereas a very recent study described expression of murine MHC class I molecules by Aedes insect cells infected by recombinant vaccinia viruses (41). These studies provided convincing evidence that Drosophila as well as Aedes cells synthesize and assemble MHC class I dimers (including HLA-B27) at a similar rate as vertebrate cells and express them on the surface as empty dimers that disappear almost completely upon incubation at 37 °C (39, 41). In striking contrast to these reports, our results suggest that baculovirus-infected Sf9 cells do not express empty HCI molecules on their surface. This conclusion is based on three findings as follows: (i) almost undetectable surface expression of HLA-B27 in the absence of peptide sources; (ii) small and invariant changes in HCI surface expression upon cell incubation at 37 °C; (iii) close quantitative correlation between stability at 37 °C of metabolically labeled HCI dimers and HCI dimer expression on the surface of cells incubated at 27 °C (Figs. 2 and 4). Thus, baculovirus-infected Sf9 cells surprisingly appear to possess a retention mechanism for empty HCI molecules; we do not know whether this mechanism is related to insect cell or viral proteins. Degradation of empty dimers is unlikely to account for this phenomenon since, at least in the case of HLA-A2, tapasin expressed doubled surface expression without affecting the total cellular amount of HCI dimers (Figs. 2 and 4). Digestion of Sf9-expressed HCI dimers by endoglycosidase H suggested that empty HCI dimers carried immature glycan moieties and were therefore retained in the ER (not shown).

Notably, even in the presence of a suitable peptide source and tapasin, no more than 30–40% of HCI molecules formed dimers. This may be due to incomplete insect cell processing of overexpressed heavy chains that migrate in SDS-PAGE as at least two molecular forms (not shown); only one of these may be able to assemble correctly. Alternatively, other factors, e.g., ERIp57 also found in class I loading complexes (7), may be required for complete restoration of class I antigen processing in Sf9 cells.

Our results confirm the crucial role of tapasin for peptide assembly with human as well as murine MHC class I molecules (10, 15, 19, 46–49). By coincidence, for this study we had selected the two HCI molecules that have been suggested to be less dependent on tapasin than other alleles (18, 19, 50). The significant effects observed underlie that tapasin is likely to act on all HCI molecules. Our study reports several novel findings with respect to tapasin. First, since the mutant human line .220 expresses low amounts of a truncated tapasin molecule (14), this is the first study of the effect of tapasin on human MHC class I molecules in an entirely tapasin-deficient cell. Our observation of enhanced peptide binding to HLA-A2 in the presence of tapasin but absence of TAP demonstrates conclusively that tapasin acts indeed independently of its role as an intermediary between TAP and HCI dimers, as suggested by Lehner and associates (15). Moreover, that same observation is the first evidence reported so far for a role of tapasin in HCI assembly with a TAP-independent peptide pool. Very recently, Schoenhals and associates (46) reported that assembly of a peptide epitope (expressed in the cytosol or in a TAP-independent form) with K\(^{\alpha}\) was promoted by tapasin. Since Drosophila transfectants used in that study do not retain empty MHC class I dimers in the ER, this effect may solely have been due to retention of empty K\(^{\alpha}\) molecules in the ER by tapasin. As infected Sf9 cells appear to possess an unidentified tapasin-unrelated mechanism of preventing surface export of empty HCI dimers, our study demonstrates that ER retention of such dimers by tapasin is at least not the sole mechanism by which it enhances peptide binding to insect cell-expressed MHC class I molecules.

Experimental reagents allowed us to assess the tapasin effect on HCI assembly of peptides from three sources as follows: a heterogeneous TAP-dependent peptide pool of cytosolic origin, a heterogeneous TAP-independent peptide pool with a probable ER-luminal origin, and two defined epitopes with high HCl binding affinity presumably generated in the ER by signal peptidase. Interestingly, tapasin enhanced HCI assembly with peptides from both heterogeneous peptide pools but had a much smaller effect on assembly of the high affinity TAP-independent epitopes. This discrepancy is compatible with the hypothesis of peptide “editing” by tapasin which has been proposed by several authors (15, 19, 46, 51). The latter result is in accordance with a recent study in Aedes cells in which human tapasin failed to promote assembly of a TAP-independent epitope with high affinity for H-2K\(^{\beta}\) (41); however, this result might also be due to species specificity in tapasin interaction with MHC class I molecules, a hypothesis supported by some (19, 37, 38) but not all (48) evidence.

Our study provides some insight into the surprisingly distinct peptides suitable for assembly with HLA-A2 and B27. Quantity and quality of peptides available in the ER of infected Sf9 cells in the absence of TAP appear to be sufficient for attaining maximum levels of stable A2 dimers in the system, provided tapasin is co-expressed to facilitate their assembly. In contrast, HLA-B27 depends almost entirely on TAP-supplied peptides; in the presence of these peptides, tapasin has the same effect on B27 as on A2 in the absence of TAP. Relatively efficient generation of stable HLA-A2 and B27 dimers in Sf9 cells co-expressing tapasin and (in the case of B27) TAP suggests that peptide generation both in the cytosol and the ER of infected insect cells supplies a sufficient number of high affinity ligands for both HCI molecules, or at least their precursors. Thus, not only are insect cell proteasomes capable of generating selected HCI ligands (46, 52), but also both their cytosolic and luminal protein degradation machineries provide a sufficient amount of ligands for two human MHC class I molecules.

HLA-A2 and B27 are known to depend to a different degree on TAP for peptide supply (37, 38, 53). The relative TAP independence of HLA-A2 is at least partly due to its capacity of binding signal peptide-derived peptides (54) which are also likely to represent the major TAP-independent peptide source in Sf9 cells. Nevertheless, in view of the reduction of A2 surface expression in TAP-deficient human cells by at least 50% (53), it was surprising that even co-expression of TAP with tapasin had no effect on the formation of stable HLA-A2 molecules. This phenomenon may be related to the ligand preferences of the HCI molecules. Although the HLA-B27 preferences are very well adapted to those of human TAP, HLA-A2 prefers ligands that are poorly adapted to TAP (25). Therefore, HLA-A2 ligands may frequently need to enter the ER as longer ligands that are poorly adapted to TAP (25). Possibly, by a mechanism absent from insect cells. In contrast, potential HLA-B27 ligands are much more likely to be translocated from the cytosol into the ER (25). Moreover, B27 is known to be capable of binding longer peptides and may therefore depend less on a specific trimming activity in the ER (56). It is also possible that A2 depends more than B27 on additional processing cofactors, some of which remain to be identified (7, 57, 58).

In any case, the two HCI molecules studied here may bind insect cell peptides derived from TAP-dependent (B27) or -independent (A2) sources, respectively, with exceptional efficiency since peptide filled H-2 K\(^{\beta}\) molecules have been reported to be generated at a much lower level in similarly reconstituted Drosophila cells (46).
The relatively small effects of calnexin co-expression in our system are compatible with a role in preventing aggregation and degradation of free heavy chains, thereby increasing the amount of HCI molecules available for assembly with β2m and peptide (59). Thus, we do not find evidence for a role of calnexin in folding and assembly of HCl dimers (60). Moreover, despite high expression levels and very efficient co-precipitation of calnexin with free heavy chains in our system, only very small quantities associated with dimers, suggesting that its described association with HCl dimers possesses very low efficiency (61).

Our findings on calreticulin associations are in conflict with some but not all previous reports. Calreticulin has been reported to associate not at all (4) or very poorly (62) with free heavy chains in cells lacking β2m. In contrast, we find that it associates with equally high efficiency with free heavy chains and (presumably empty (4)) dimers. Several studies suggest that preferential calreticulin binding to free dimers in human cells may be related to HCl conformation rather than glycan modification (3, 63), so that its binding to insect cell-expressed free heavy chains may reflect an altered conformation. However, two very recent studies in which the chaperone co-precipitated with HCl molecules recognized by mAb HC10 (64, 65) suggest that calreticulin can associate with free heavy chains in human cells. In support of this conclusion, we have been able to co-precipitate calreticulin with HC10-recognized heavy chains expressed by human cells cultured at 27 and 37 °C (not shown). In any case, the moderate but reproducible effect of calnexin and G. Moldenhauer for mAb. In support of this conclusion, we have been able to co-precipitate calreticulin with HC10-recognized heavy chains expressed by human cells cultured at 27 and 37 °C (not shown). In any case, the moderate but reproducible effect of calnexin and G. Moldenhauer for mAb.

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