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A Mutant Cell with a Novel Defect in MHC Class I Quality Control

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Cytotoxic T lymphocyte survey tissues for the presence of foreign Ags, such as those of viral or tumor origin, presented on MHC class I molecules on the surface of cells. MHC class I molecules are trimolecular complexes, consisting of a polymorphic glycoprotein, the H chain, which is noncovalently associated with β2-microglobulin (β2m), and a small peptide, generally 8–10 residues long, which is usually the product of proteolysis of cellular and viral proteins in the cytosol (reviewed in Refs. 1 and 2). H chain and β2m are cotranslationally translocated into the endoplasmic reticulum (ER), where they fold, form intrachain disulfide bonds, and associate with each other. Before binding peptide, this complex is relatively thermolabile and is retained within the ER. Peptides are transported into the lumen of the ER by the peptide transporter TAP, and if a peptide has the appropriate sequence motif, it can bind to the H chain/β2m complex. Upon binding peptide, the association between H chain/β2m becomes more stable, and the trimolecular complex is released from the ER and transported to the cell surface.

Each stage of MHC class I maturation is associated with ER chaperones (reviewed in Refs. 3 and 4). GRP78 (BiP) interacts with H chain shortly after its synthesis (5, 6). Protein disulfide isomerase (PDI) may help form some of the H chain intrachain disulfide bonds. Calnexin (CNX) interacts with newly synthesized H chain. CNX dissociates from human MHC when H chain interacts with β2m but remains associated with mouse H chain/β2m complexes (6, 7). Calreticulin (CRT) and ERp57 both associate with H chain/β2m complexes (8–12). Tapasin brings the peptide-receptive H chain/β2m complex into physical proximity with TAP-transported peptides (13–15) and otherwise facilitates peptide binding to the H chain/β2m dimer (16–18). When peptide is bound to the MHC complex, the chaperones all dissociate from MHC, and the mature H chain/β2m/peptide complex is allowed to exit the ER to the cell surface.

In general, these chaperones are believed to perform quality control, monitoring protein folding and either directing folding toward the proper conformation or targeting misfolded protein to degradation. Thus, various chaperones have been shown to facilitate folding and assembly (19) and retain peptide-empty MHC class I in the ER (16, 18). However, the details of these functions are not well understood as yet.

The identity and functions of several proteins associated with Ag processing, such as TAP and tapasin, have been elucidated by studying mutant cell lines with defects in cell surface MHC class I expression. To further investigate the MHC class I pathway, we generated a series of mutant cell lines with defects in surface MHC class I expression and in this study describe one of these mutants that has a defect in ER quality control.

Materials and Methods

Abs and cell lines

COS7 cells were stably transfected with the mouse MHC class I H chain allele H-2Kb in the plasmid pcDNA1Neo (Invitrogen Life Technologies) to generate COS-Kb cells. COS-Kb and its mutant derivative 4S8.12 were grown in RPMI 1640 medium (Invitrogen Life Technologies) supplemented with 10% FCS and G418 (1 mg/ml) in the presence of 5% CO2. W6/32 (20), BB7.5 (21), and PA2.6 (22) are mAbs that recognize HLA-A, -B, and -C only when not associated with β2m. HC10 (23) recognizes HLA-B and -C only when not associated with β2m. Anti-GRP78 antisera (which also recognizes gp96/GRP94) and anti-CNX, anti-PDI, anti-CRT, anti-TAP1, and anti-HSP70 rabbit sera were purchased from StressGen Bio-technologies. Anti-ERp57 rabbit serum was kindly provided by S. High (University of Manchester, Manchester, U.K.). JD12 (24) recognizes HLA-A alleles associated with β2m and was provided by D. Scheinberg.
(Memorial Sloan-Kettering Cancer Center, New York, NY). 4E (25) recognizes HLA-B alleles associated with β₂m and was a gift from Dr. S. Yang (Memorial Sloan-Kettering Cancer Center). Anti-human-β₂m rabbit serum was purchased from DakoCytomation. Preliminary experiments indicated that all of these Abs cross-reacted with the COS7 (African Green monkey) proteins. GAP-A3 (26) recognizes HLA-A3 associated with β₂m. 28.14.85 (27) recognizes H-2D<sup>b</sup>. 19.8.503 (28) recognizes mouse β₂m and does not cross-react with human or African Green monkey β₂m. 25.D1.16 (29) is specific for the immunodominant epitope from chicken OVA, SINFEKL, in association with H-2<sup>K<sub>b</sub></sup>. H36.5-4.2 recognizes influenza hemagglutinin in its mature conformation at the cell surface (30). H-2K<sub>b</sub> and H-2D<sub>b</sub> were subcloned into pTracerCMV (Invitrogen Life Technologies). Cells were lysed in 1% CHAPS in PBS with protease inhibitors (Roche). Cells were lysed in 1% CHAPS in PBS with protease inhibitors (Roche) and incubated on ice for 15 min. Following this, the sample was centrifuged at 14,000 g for 10 min at 4°C. The supernatant was collected and stored at −70°C. The protein A-Sepharose complexes were incubated in 1% Triton X-100 in PBS for 1 h at 4°C. Immunoblotting and immunoprecipitations were performed as described previously (31). For coprecipitation of MHC class I complexes with TAP, individual clones produced with Pfx and subcloned into plasmid vectors were sequenced at the University of Massachusetts Medical Center Nucleic Acids Core Facility.

Results

4S8.12 cells have reduced endogenous MHC class I surface expression

We chose to mutagenize COS7 cells because these cells epistemically replicate plasmids containing the SV40 origin of replication, leading to high expression of transfected genes, and because they react broadly with the many available reagents directed against components of the human Ag processing system. These characteristics make expression cloning of mutant genes practical in COS7 cells. To study presentation of defined Ags, we stably transfected COS7 cells with the well-characterized mouse MHC allele H-2<sup>K<sub>b</sub></sup> to produce COS-K<sub>b</sub> cells. COS<sup>−</sup>K<sub>b</sub> cells were mutagenized with ethyl methanesulfonate and repeatedly selected for low surface expression of MHC class I molecules, using a panel of anti-MHC class I mAbs. To sequence CR, CRT, and 2m constructs. A plasmid expressing human ERp57 was provided by S. High (University of Manchester, Manchester, UK) and HLA-B-like molecules associated with β₂m (mAb 4E-reactive) (Fig. 1B) and HLA-A-like molecules associated with β₂m (reactive with the mAb JDI2) (Fig. 1C) were both markedly lower than the parent cell line’s levels. The decreased surface expression of endogenous MHC class I did not reflect a general defect in glycoprotein expression because transiently transfected influenza hemagglutinin was expressed at equal levels on the surface of 4S8.12 and wild-type COS<sup>−</sup>K<sub>b</sub> cells (Fig. 1D); however, the defect was not limited to the endogenous monkey MHC class I because transiently transfected human MHC class I alleles (e.g., HLA-A*0302; Fig. 1E) were also expressed at lower levels on 4S8.12 cells than on COS-K<sup>−</sup>.

The 4S8.12 population showed biphasic levels of endogenous surface MHC class I, with a minority of cells (~5–20% under normal culture conditions) expressing nearly wild-type levels of MHC class I (Fig. 1A). This was not because of contamination...
with wild-type cells because repeated subcloning by limiting dilution invariably yielded biphasic populations (at least 30 subclones), rather than either uniformly high and low populations (data not shown). Furthermore, when nearly pure populations of either low- or high-MHC class I-expressing 4S8.12 cells were selected using anti-MHC class I Ab-coated magnetic beads, each population became biphasic again within 24 h of culture (Fig. 1F). This suggests that individual cells in the population rapidly switch between a normal MHC class I phenotype and a mutant (low surface MHC class I) phenotype. The trigger for this switch is unknown but may be related to environmental conditions. It is not related to the phase of cell cycle (data not shown).

**Assembly of endogenous H chain/β₂m complexes is defective in 4S8.12 cells**

Endogenous (monkey) MHC class I is expressed on the surface of 4S8.12 at markedly lower levels than in wild-type cells (Fig. 1A). This could have many causes: reduced MHC class I synthesis, failure to assemble β₂m and H chain molecules in the ER, reduced peptide supply or peptide binding, increased degradation of H chain or β₂m, or because mature (peptide-containing) molecules are abnormally retained in the ER. We analyzed maturation of MHC class I in wild-type and mutant cells, using the mAbs W6/32 (which recognizes MHC class I only when it is assembled with β₂m, regardless of the presence or absence of peptide) and HC10, which recognizes only H chain that is not assembled with β₂m. Synthesis of MHC class I H chain and β₂m was similar in parent and mutant cells (Fig. 2A, compare HC10 pulse bands). However, assembly of H chain and β₂m to produce W6/32-reactive complexes was reduced markedly in the mutant cells (Fig. 2A). The levels of free H chain dropped relatively rapidly in 4S8.12 cells, although free H chain was not being converted into β₂m-associated, W6/32-reactive H chain. This probably represents proteolytic degradation of misfolded or unassembled H chain, as part of the normal ER quality control system. (It is formally possible that H chain aggregates, takes on a conformation that is not recognized by available mAbs, or is otherwise sequestered from detection. However, we have not been able to find any evidence for such a hidden pool of H chain.) The small amount of assembled MHC class I in 4S8.12 probably represents the small subpopulation of cells in the overall 4S8.12 population that at any time have near-normal surface MHC class I levels.

There were two possible explanations for the reduction in H chain/β₂m complexes in immunoprecipitates from 4S8.12. H chain and β₂m could have failed to assemble altogether. Alternatively, H chain and β₂m might have assembled but failed to associate with the peptide. Because peptide-empty complexes are less stable than peptide-loaded complexes, the empty complexes could have dissociated during the immunoprecipitation procedure. To differentiate between these possibilities, we needed to confirm that, under our immunoprecipitation protocol, we were able to detect peptide-empty (unstable) complexes. Therefore, we performed immunoprecipitations in COS-K⁺ cells expressing the herpes simplex virus protein ICP47 (Fig. 2A), which binds to the TAP peptide transporter and blocks peptide transport into the ER (35, 36), using the adenovirus construct AdICP47. Surface expression of MHC class I is dependent generally on peptide supply to the ER, and ICP47 drastically reduced MHC class I surface expression in the parent COS-K⁺ cells (Fig. 2B). The reduction in surface expression of MHC class I in COS-K⁺ cells expressing ICP47 was comparable to that in COS-K⁺ cells treated with the ER-to-Golgi transport blocker brefeldin A (data not shown), demonstrating that peptide supply to nascent MHC class I complexes was almost completely blocked. Even under these conditions, when the H chain/β₂m complexes must have been peptide-empty and relatively unstable, we were able to immunoprecipitate W6/32-reactive complexes (Fig. 2A, COS-K⁺ + ICP47), even though some of the complexes dissociated during the 37°C incubation of the 45-min chase.

Additionally, we confirmed that W6/32 could recognize peptide-empty MHC class I by examining the thermostability of the complexes this Ab recognized (Fig. 2, C and D). Cell lysates were either kept on ice and immunoprecipitated immediately or incubated at 40°C for 1 h before immunoprecipitation. Under these conditions, MHC class I complexes could be immunoprecipitated from COS-K⁺ cells by W6/32 even after a 15-min pulse. However, these complexes were predominately thermostable because incubation at 40°C for 1 h abrogated recognition by W6/32. The complexes became progressively more thermostable during the chase period, although at 15 min of chase and even after 45 min of chase.
a significant proportion of the complexes failed to immunoprecipitate after 40°C incubation. The increasing stability of the complex presumably corresponds to acquisition of peptide as the complexes mature, and therefore, the dissociating (thermolabile) fraction represents peptide-empty complexes. Lysates from 4S8.12 cells, immunoprecipitated in parallel with those from the parent COS-Kb cells (but without the 40°C incubation), again showed few or no W6/32-reactive complexes. Thus, although W6/32 immunoprecipitated peptide-empty complexes immediately after the pulse, this Ab failed to recognize complexes from 4S8.12 cells.

These experiments confirmed that, with our immunoprecipitation conditions, we could detect assembly of MHC class I complexes even when the complexes were not stabilized by association with high-affinity peptide. Therefore, the inability to detect any W6/32-reactive complexes in 4S8.12 cells, in contrast to those from the parent COS-Kb cells (but without the 40°C incubation), again showed few or no W6/32-reactive complexes. Thus, although W6/32 immunoprecipitated peptide-empty complexes immediately after the pulse, this Ab failed to recognize complexes from 4S8.12 cells.

4S8.12 compared with parent COS-Kb cells. To further confirm that the reduction in assembly was not due to a primary mutation in endogenous β2m or MHC class I genes, we transfected the parent and mutant cells with a plasmid expressing human β2m covalently attached through a flexible linker to the N terminus of HLA-A*0302 (single-chain HLA-A3, “SC-A3”). In these transfectants, although both members of the complex were of wild-type sequence, assembly and surface expression of the HLA-A3/human β2m molecule (assessed with the conformation-sensitive Ab GAP-A3) was markedly lower in the mutant 4S8.12 than the parent

**FIGURE 2.** MHC class I assembly is impaired in 4S8.12 cells. A, COS-Kb cells or 4S8.12 cells or COS-Kb cells infected with AdICP47 (COS-Kb + ICP47) were labeled metabolically with [35S]methionine and cysteine for 15 min (pulse, P) and chased with nonradioactive medium for 15 or 45 min. At each time point, cells were lysed, and equal amounts of lysate were immunoprecipitated with Ab directed against HLA-A, -B, and -C only in association with β2m (mAb H6/32) or with anti-HLA-B and -C not associated with β2m (mAb HC10). B, COS-Kb cells infected with AdICP47 (thick trace) or with a control adenovirus (thin trace) for 48 h were stained with W6/32 and analyzed by flow cytometry. Shaded trace, Background staining with an irrelevant Ab. C, COS-Kb and 4S8.12 cells were radiolabeled and lysed as in A. The lysates were immunoprecipitated with HC10 (one-third of each lysate) or W6/32 (two-thirds of each lysate) as in A, except that half of each COS-Kb lysate was incubated at 40°C for 1 h before immunoprecipitation to ensure that heat-labile complexes dissociated. D, The autoradiograph from C was scanned, and densitometry was performed using NIH Image. Open symbols, unheated samples; closed symbols, 40°C incubation. Triangles, COS-Kb HC10; squares, COS-Kb W6/32; diamonds, 4S8.12 HC10; and circles, 4S8.12 W6/32.
COS-Kb cell line (Fig. 3A). Therefore, the mutation in these cells must affect an accessory factor that facilitates assembly of β2m with human and monkey MHC class I H chain.

Stable assembly of MHC class I H chain and β2m depends on association with the peptide, as noted above. We transfected 4S8.12 cells with the SC-A3 construct as previously described and delivered a HLA-A3-binding peptide RVCEKMALY (38) to the ER by fusing it to the adenovirus E3gp19K signal sequence (ss-R9Y). Expression of the SC-A3 construct was not increased by this peptide (Fig. 3A), although the same peptide construct markedly increased levels of HLA-A3 in a Hela cell line stably expressing ICP47 (Fig. 3B), confirming that the peptide is transported into the ER and can bind efficiently to HLA-A3. This further demonstrates that the assembly defect in 4S8.12 cells is not related to delivery of peptide to the ER and suggests that H chain and β2m fail to assemble into a peptide-recptive complex.

Surface mouse MHC class I expression is not reduced in 4S8.12 cells

In contrast to endogenous MHC class I, expression of the stably transfected mouse MHC class I H chain, H-2Kb, was not reduced in 4S8.12; indeed, H-2Kb expression was ∼5-fold higher on 4S8.12 than on the wild-type COS-Kb (Fig. 4A). As well, transiently transfected mouse MHC class I allele H-2Dβ was expressed at equal or slightly increased levels on 4S8.12 compared with COS-Kb cells (Fig. 4B).

Because the endogenous monkey MHC class I fails to associate with β2m in 4S8.12 cells, there may be a large pool of free β2m available for association with H-2Kb. As well, mouse MHC class I binds to human β2m (and presumably also to African Green Monkey β2m, which is 90% identical with human β2m) with higher affinity than to mouse β2m (39, 40), which might affect surface levels of H-2Kb. To reduce the influence of the endogenous β2m, we transiently transfected COS-Kb or 4S8.12 cells with single-chain H-2Kb covalently linked to mouse β2m. Surface expression of this complex was measured by staining for mouse β2m to distinguish it from the stably transfected H-2Kb. The single-chain H-2Kb was expressed at the surface of both 4S8.12 and COS-Kb cells, although at lower levels than the heterodimer of stably transfected H-2Kb and monkey β2m, perhaps because in this transient transfection steady-state levels were not achieved or because association with monkey β2m permits higher expression. Importantly, the single-chain H-2Kb was expressed at similar or higher levels on 4S8.12 compared with COS-Kb cells (Fig. 4C), in contrast to the single-chain human HLA-A*0302, which was expressed at lower levels in the 4S8.12 than the COS-Kb cells (Fig. 1E). This shows that H-2Kb did not require an exceptionally high-affinity β2m partner to achieve wild-type levels of expression on 4S8.12 cells.

Sequentialing of cDNA from 4S8.12 cells confirmed that the primary sequence of the H-2Kb expressed in these cells was wild-type (data not shown). To test whether the H-2Kb expressed on mutant 4S8.12 cells was functional, we delivered a peptide, SIINFEKL, (the immunodominant H-2Kb-binding peptide from chicken OVA), to the ER of wild-type and mutant cells by transfecting cells with a plasmid expressing SIINFEKL preceded by a signal sequence and stained the cells with the mAb 25.D1.16, which recognizes SIINFEKL only in combination with H-2Kb (29). 4S8.12 presented at least as much or slightly more H-2Kb-SIINFEKL as did the wild-type COS-Kb (Fig. 4D).

Because 4S8.12 expressing ER-localized SIINFEKL generated similar levels of surface H-2Kb-SIINFEKL as COS-Kb, we were able to test the overall Ag presentation pathway of 4S8.12 cells. We transfected cells with full-length OVA and measured presentation of SIINFEKL on H-2Kb. Previous studies have established that efficient presentation of SIINFEKL from full-length OVA requires at the least functional proteasome (41), TAP (42–44), and tapasin (45, 46). 4S8.12 consistently generated somewhat higher levels of H-2Kb-SIINFEKL complexes from full-length OVA compared with COS-Kb cells (Fig. 4E), demonstrating that these known components of Ag presentation are intact in 4S8.12 cells.

Chaperone levels are normal in 4S8.12 cells

Therefore, 4S8.12 cells showed a phenotype in which primate but not mouse H chain and β2m failed to normally assemble. This suggested an abnormality in chaperone function because chaperones facilitate assembly of multiprotein complexes. Several chaperones, including GRP78, CNX, CRT, and ERp57, are known to associate with MHC class I molecules during their maturation in the ER. As well, tapasin associates transiently with ER-localized MHC class I and facilitates peptide loading. We used semiquantitative Western blots to test the presence and amount of several ER-localized chaperones. Levels of CNX, gp96, GRP78, CRT, ERp57, and PDI were very similar in 4S8.12 and COS-Kb cells (Fig. 5A). We were unable to detect tapasin directly in either the wild-type or mutant cells, either because this protein is expressed normally at very low levels or because the available Abs do not efficiently recognize African Green Monkey tapasin. However,
precipitation of TAP from mutant and wild-type cells coprecipitated MHC class I equivalently, as indicated by the presence of β2m (Fig. 5B). (We examined β2m, rather than H chain, because unlike human and mouse H chain, it is expressed at similar levels on the surface of COS-Kb and 4S8.12 cells. The β2m detected in 4S8.12 cells is presumably mainly associated with H-2K* rather than endogenous monkey H chain because little β2m in these cells is associated with the latter. However, because tapasin is required for the association of H-2K* with TAP, this does not affect the conclusions.) Because tapasin is required to link HC to TAP (13), this coprecipitation demonstrates that tapasin is functionally present in the mutant cells. In addition, we cloned and sequenced tapasin from COS-Kb and 4S8.12 cells; the sequences were identical (GenBank accession no. AY570382). We also sequenced CRT (GenBank accession no. AY901963) and ERp57 (GenBank accession no. AY901964) from wild-type and mutant cells and confirmed that the sequences in 4S8.12 were the same as wild type. Finally, transient transfections of 4S8.12 cells with CNX, CRT, GRP78, ERp57, or tapasin did not restore surface MHC class I expression (Fig. 5, C–G), confirming that these known chaperones are not only present at normal levels but are functional in 4S8.12 cells.

These data strongly suggest that an ER-localized chaperone that is not presently known to interact with MHC class I is mutated, or expressed at inappropriate levels, in 4S8.12 cells and that as a result assembly of MHC class I complexes is abnormal in these cells.

Discussion

In this article, we describe a mutant cell line, 4S8.12, with a defect in quality control that manifests differently for primate and mouse MHC class I molecules. In 4S8.12 cells, monkey and human MHC class I H chains fail to assemble with β2m so that few peptide-receptive complexes form in the ER. In contrast, mouse MHC class I H chains apparently assemble normally with β2m and can bind to peptide as well as in normal cells. 4S8.12 cells show no growth abnormalities, and other glycoproteins (e.g., influenza hemagglutinin; Fig. 1D) are apparently unaffected by these defects, reaching the cell surface at wild-type levels and in a mature conformation.

Assembly and quality control in the ER is the function of chaperones, and we suggest that the phenotype of 4S8.12 cells is the result of a defect in a chaperone that facilitates assembly of MHC class I complexes. This putative chaperone has not yet been identified but does not appear to be one of the chaperones known to associate with MHC class I during its maturation: those chaperones are all present at normal levels and do not rescue the defect when transfected.

Mutations in several genes known to be involved in MHC class I Ag presentation (e.g., TAP and tapasin) prevent peptide from associating with H chain/β2m complexes in the ER. These peptide-empty complexes, which are retained in the ER, are unstable and consequently can be difficult to detect under some conditions. However, we confirmed that in our experiments we were able to detect peptide-empty H chain/β2m complexes in the ER. Therefore, the failure to detect such complexes in 4S8.12 cells was because H chain does not assemble with β2m in these cells.

Free HC in 4S8.12 cells is degraded more rapidly than in COS-Kb cells (Fig. 2, B and C; compare HC10 lanes). ER proteins that are misfolded or that fail to assemble with their appropriate binding partner are translocated normally from the ER and destroyed by ER-associated degradation pathways. The rapid degradation seen in 4S8.12 is probably because H chain fails to fold properly in these cells and is treated like any other misfolded protein. In fact, at very early time points (e.g., <10 min; data not shown), H chain in normal COS-Kb cells is degraded rapidly, at a rate very similar to that in 4S8.12, but in the normal cells, this rapid degradation quickly slows down, probably as H chain folds into a more native conformation. It is also conceivable that rapid degradation is the primary defect in 4S8.12 cells, analogous to the situation in human CMV-infected cells expressing the viral immune evasion proteins US2 and US11, which induce rapid ubiquitin-proteasome-mediated degradation of MHC class I H chain (47, 48). However, because a significant amount of free H chain is still present in 4S8.12 cells

**FIGURE 5.** MHC class I-associated chaperones are normal in 4S8.12 cells. A, Three-fold serial dilutions of lysates from COS-Kb or 4S8.12 cells were separated by SDS-PAGE, transferred to nitrocellulose, and probed with Abs against gp96, CNX, GRP78/BiP, CRT, ERp57, and PDI. B, COS-Kb and 4S8.12 cells were labeled for 1 h with 35S, lysed with 1% CHAPS in PBS, and immunoprecipitated with Abs against MHC class I (W6/32), β2m, TAP1, or, as a control, HSP70. TAP1, and HSP70 immunoprecipitates were reprecipitated with anti-β2m as described in Materials and Methods. C–G, 4S8.12 cells were transfected with plasmids (thick traces) expressing influenza hemagglutinin as a control (thin traces) or with plasmids (thick traces) expressing CNX (C), CRT (D), GRP78/BiP (E), ERp57 (F), or tapasin (G). After 3 days, the cells were stained with anti-HLA-A, -B, and -C (mAb PA2.6) and analyzed by flow cytometry. PA2.6 staining of hemagglutinin-transfected cells did not differ significantly from untransfected cells (data not shown). Transfection efficiency, judged by hemagglutinin expression, was >80% (data not shown).
even after 45 min of chase (e.g., Fig. 2A), yet very few H chain/β2m complexes are formed at that time, it is unlikely that rapid degradation is the sole abnormality in these cells.

The defect in 4S8.12 cells is partial, because a low level (~5% of wild type) of apparently properly assembled monkey MHC class I complexes are present at the cell surface. As well, a minority of cells in the population express normal levels of endogenous MHC class I on their surface and some environmental conditions, such as growth in glucose-depleted medium or treatment with deoxyglucose or the calcium ionophore A23187 (data not shown), all treatments that strongly induce many ER chaperones, at least partially reverse the defect. We do not know whether these normal-appearing cells are genuinely completely normal or whether they may still have subtle defects in MHC conformation or function. As well, the reversal of the phenotype under these conditions may be due to correction of the underlying defect or may involve overexpression of other chaperones that compensate for the defect: redundancy in chaperone function is common.

MHC class I molecules undergo a precisely orchestrated program of maturation in the ER that results in expression of a mature complex at the cell surface. If any of the three components of the complex (H chain, β2m, or peptide) is missing, the incomplete complex is normally retained within the ER and ultimately degraded. Assembly of H chain and β2m in vivo is presumably facilitated by chaperones, and indeed, the MHC class I H chain associates with a series of ER chaperones during its maturation: GRP78, CNX, CRT, and ERp57. These chaperones associate with many other nascent ER proteins and glycoproteins (49, 50) and generally facilitate proper protein folding (51, 52). However, while the general function of these chaperones is clear, their precise roles in MHC class I maturation are not well understood. CNX has been shown to enhance folding and assembly of MHC class I (19) and may also help retain misfolded complexes in the ER. However, even in a cell line that lacks CNX, MHC maturation proceeds apparently normally (53). After H chain binds to β2m, CNX dissociates from human H chain, and the H chain/β2m dimer associates with several other proteins to form a peptide-binding complex, containing at least CRT, ERp57, tapasin, and TAP. CRT plays a role in loading MHC complexes with optimal peptide (11). ERp57, which has PDI motifs, is required for the production of a disulfide bond between tapasin and the MHC class I peptide-binding groove (54); however, the physiological importance of this disulfide link is not clear. ERp57 has also been implicated in the production of intrachain disulfide bonds in the H chain (55), but again, the importance of this is not clear.

We tested 4S8.12 cells for the presence and function of multiple chaperones. All those tested showed equal levels by Western blots (Fig. 5A), and transfection of 4S8.12 cells with wild-type chaperones did not restore expression of MHC class I (Fig. 5, C–F). In addition, we confirmed that the primary sequence of several genes critical to MHC class I folding and assembly (ERp57, CRT, and β2m) were all wild type.

Tapasin is involved in loading of H chain/β2m complexes with peptide and in retention of peptide-empty complexes in the ER (13, 14, 18, 45, 46). Because tapasin deficiency leads to a marked reduction in peptide binding to newly synthesized MHC class I, it was a strong candidate as the mutant gene in 4S8.12 cells. However, several lines of evidence rule this possibility out. First, presentation of SIINFEKL on H-2Kb in 4S8.12 cells was similar to, or higher than, that in parent cells (Fig. 3, D and E), whether the SIINFEKL was delivered directly into the ER (bypassing TAP) or generated by proteasome-mediated degradation of full-length OVA. Efficient generation of H-2Kb-SIINFEKL complexes has been shown previously to be dependent on tapasin (45, 46). Second, MHC class I complexes are associated physically with TAP in parent and mutant cells (Fig. 5B), which requires tapasin (13, 14). Third, sequencing of cDNA from parent and mutant cells showed no change in the primary sequencing of tapasin. Finally, transfection of 4S8.12 cells with wild-type tapasin did not restore surface MHC class I to wild-type levels (Fig. 5G).

Primate and mouse MHC class I H chains behaved very differently in the 4S8.12 cell line. Whereas human and monkey MHC class I complexes failed to associate with β2m and were retained in the ER until they were degraded, mouse MHC class I H chains reached the cell surface at least as well as in the wild-type COS-Kb cells; in fact, the mouse alleles tested reached higher levels at the surface of 4S8.12 than on COS-Kb cells (Fig. 4, A–C). As well, although human MHC class I failed to interact with binding peptide (Fig. 3), mouse H-2Kb in 4S8.12 cells presented peptide at least as well as did wild-type cells (Fig. 4, D and E). Therefore, the putative chaperone that is defective in 4S8.12 cells is essential for folding and assembly of human MHC class I but is not required (or is redundant) for the assembly of mouse MHC class I. Mice and human MHC class I alleles are known to interact differently with ER chaperones; in particular, mouse alleles remain associated with CNX even after β2m association, whereas CNX dissociates from human alleles at this stage (6). However, CNX is present in 4S8.12 at normal levels (Fig. 5A), and transfection of wild-type CNX into 4S8.12 does not alter expression of human MHC class I (Fig. 5C) or of H-2Kb (data not shown); as well, the phenotype of a mutant human cell line lacking CNX is quite different from that of 4S8.12 (53), and disruption of CNX association with human MHC class I does not inhibit association with β2m (56) as occurs in 4S8.12.

Although most if not all glycoproteins interact with chaperones during their maturation, MHC class I complexes may be unusual in their chaperone requirements because for their normal function the complexes must achieve a nearly fully folded conformation to bind peptides in the ER. The difference in conformation between peptide-empty and peptide-loaded H chain/β2m complexes is very subtle; for example, almost all mAbs that recognize conformational determinants on MHC class I recognize both the peptide-empty and loaded complexes, although not always with equal efficiency. Yet the peptide-empty complex is normally efficiently retained within the ER, whereas the peptide-loaded form is released. Presumably, there is some subtle conformational change that permits MHC class I to exit the ER.

The gene that is affected in 4S8.12 cells is not yet identified. Most known chaperones in the MHC class I pathway were identified based on finding a physical association with MHC class I, but in immunoprecipitations, we have not detected differences in molecules that coprecipitate with MHC class I in 4S8.12 and wild-type COS-Kb cells (data not shown). However, such biochemical analyses are limited by the ability to preserve and detect stable intermolecular associations. In contrast, this is not a limitation of mutational analysis, which allows genetic screens to identify essential new steps in complex pathways. The 4S8.12 cells provide strong genetic evidence for a new component in the MHC class I pathway. This novel gene is important in the folding and assembly of primate but not mouse MHC class I.

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Disclosures
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References


