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Assembly and Antigen-Presenting Function of MHC Class I Molecules in Cells Lacking the ER Chaperone Calreticulin

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Summary

MHC class I molecules expressed in a calreticulin-deficient cell line (K42) assembled with β 2-microglobulin (β2-m) normally, but their subsequent loading with optimal peptides was defective. Suboptimally loaded class I molecules were released into the secretory pathway. This occurred despite the ability of newly synthesized class I to interact with the transporter associated with antigen processing (TAP) loading complex. The efficiency of peptide loading was reduced by 50%–80%, and impaired T cell recognition was observed for three out of four antigens tested. The peptide-loading function was specific to calreticulin, since the defect in K42 could be rectified by transfection with calreticulin but not a soluble form of calnexin, which shares its lectin-like activity.

Introduction

The assembly of MHC class I molecules with antigenic peptides in the ER is a coordinated and regulated process culminating in the release of molecules which are bound to peptides of 8–10 amino acids in length (Pamer and Cresswell, 1998). These short peptides contain an allele-specific binding motif and have the ability to stabilize the class I molecule (Elliott et al., 1991), and as such are called "optimal" peptides. Peptides that are longer or shorter than this or that do not posses the full complement of "anchor residues" that make up a binding motif do not stabilize the interaction between class I heavy-chain (HC) and β2-m to the same extent. They dissociate rapidly (Cerundolo et al., 1991) and are consequently termed "suboptimal." Failure to encounter peptides in the endoplasmic reticulum (ER) results in the retention and eventual degradation of class I molecules (Townsend et al., 1989). Prior to their release from the ER, newly synthesized class I molecules can be found bound to TAP, tapasin, calreticulin (Sadasivan et al., 1996), and ERp57 (Hughes and Cresswell, 1998; Lindquist et al., 1998; Morrice and Powis, 1998). This multimolecular complex has been referred to as the class I loading complex.

Incorporation of newly synthesized class I molecules into the loading complex improves their acquisition of optimal peptides and is also important for their sorting to a degradation pathway when peptide supply is limiting. This has been illustrated by previous studies in which a class I molecule with a single nonconservative amino acid substitution at position 134 (threonine to lysine, called T134K) was unable to bind to TAP, ERp57, and tapasin (Lewis et al., 1996; Yu et al., 1999; T.E., unpublished data). As a result, T134K molecules were assembled with β2-m and released into the secretory pathway prematurely, in a peptide-receptive state, and consequently were unable to present endogenous viral epitopes to T cells (Lewis et al., 1996). These studies, therefore, clearly indicated that binding to the loading complex was essential for the regulated assembly and peptide-dependent release of class I molecules from the ER. In other studies, release of peptide-receptive class I molecules from the ER required a TAP-dependent supply of peptides to the ER (Lewis and Elliott, 1998). This apparently paradoxical observation could be explained by a two-step model for peptide loading in which MHC class I molecules first assemble with suboptimal peptides with rapid off-rates, which are eventually replaced by high-affinity stabilizing peptides. The precise function of each component of the loading complex in this process and the significance of their physical interaction with MHC class I molecules remains unclear.

Many roles have been proposed for calreticulin (Michalak et al., 1999), and it is beginning to emerge that all of these are a direct or indirect result of two basic functions: its role in intracellular calcium homeostasis and its role as a chaperone in the folding of newly synthesized glycoproteins. Calreticulin is highly but transiently expressed in the embryonic heart, and deleting the calreticulin gene is lethal during embryogenesis because of impaired cardiac development resulting from deregulated calcium homeostasis (Mesaeli et al., 1999). Calreticulin's ability to discriminate between different folding states of a glycoprotein can be achieved directly via protein-protein interactions or indirectly via binding to monoglucosylated N-linked oligosaccharides present on folding intermediates of nascent glycoproteins. The function of calreticulin in the class I loading complex is not known. Calreticulin binds to class I molecules only after HC and β2-m have assembled and the intra-heavy chain disulphide bonds have formed (Farmery et al., 2000). At this stage, therefore, class I molecules are

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properly folded according to most criteria and are recognized by conformation-sensitive monoclonal antibodies.

To investigate the precise role of calreticulin in the regulated loading of class I molecules, we have studied the assembly and antigen-presenting function of H2-K^b and H2-D^b in mouse fibroblasts derived from mouse embryos carrying a lethal, homozygous deletion of the calreticulin gene.

Results

Calreticulin-Knockout Cells Express Reduced Cell-Surface Levels of MHC Class I

Cell-surface expression of H2-D^b and H2-K^b on the calreticulin-competent cell line K41 and its calreticulin-deficient counterpart K42 was determined by flow cytometry using conformation-sensitive monoclonal antibodies. Figure 1A shows that the level of expression of both alleles on K42 was 25%–30% of the level seen in K41. Lack of a functional calreticulin gene did not affect the levels of total cytosolic, nuclear, or membrane proteins in each cell (data not shown). The supply of peptides to the ER was similar in both cell types because both the rate of cytosolic degradation of a model antigen (influenza A nucleoprotein tagged with ubiquitin-arginine) and TAP activity were the same in K42 and K41 (data not shown). Expression of both D^b and K^b was restored to normal when K42 was transfected with rabbit calreticulin (Figure 1A).

When we incubated K42 overnight in the presence of a saturating concentration of the K^b binding peptide SIINFEKL or the D^b binding peptide ASNENMDAM, levels of MHC class I expression were restored to those seen for K41 (Figure 1B). This represented a 4.5-fold increase in cell-surface expression in K42 compared to only a 1.2-fold increase in K41. Similar results were obtained for five other D^b binding peptides (data not shown). The increase in cell-surface expression was dependent on the delivery of peptide-receptive class I molecules to the cell surface, since little increase in cell-surface staining was observed when the experiments were performed at 4°C (data not shown). We found no difference in the dose-response characteristics for binding of any of the peptides added to K41 and K42 over a range of concentrations (see, for example, ASNENMDAM in Figure 1B), suggesting that the peptide-receptive molecules released to the cell surface of K42 have the same affinity for peptide ligands as they do in K41. These results suggest that the same number of MHC class I molecules were assembled and exported in K41 and K42, but in K42 a greater proportion were exported in an unstable, peptide-receptive state and fall apart either at the cell surface or en route to the cell surface unless they encounter exogenous peptides to which they can bind. In K42 transfected with calreticulin, not only did cell-surface expression of class I increase (Figure 1A), but the fold increase in cell-surface expression after incubation with exogenous peptides decreased to that seen for K41 (see, for example, the ASNENMDAM titration in Figure 1B). Since in these experiments peptide can gain access to class I molecules within the secretory pathway (Day et al., 1997) as well as at the cell surface, we next evaluated the number of class I molecules on K41 and K42 that could be stabilized with exogenously added β2-m, which is unlikely to have similar access to the secretory pathway. Figure 1C shows that β2-m produced a dose-dependent stabilization of cell-surface MHC class I molecules, resulting in a 3.1-fold increase in surface D^b expression on K42 and a 1.7-fold increase in K41. There was no indication from this experiment that the affinity of class I for β2-m was altered in K42, and with saturating levels of β2-m expression of class I on K42 and K41 was the same. Confocal microscopy on K41 and K42 did not reveal differences in intracellular distribution of class I molecules or differences in the intensity of staining in the Golgi (data not shown), consistent with the delivery of all assembled MHC class I molecules to the cell surface.

In order to determine whether the intracellular loading of D^b and K^b with peptides was indeed impaired in K42, we immunoprecipitated a cohort of newly synthesized class I molecules in the presence or absence of added peptide ligand using either a conformation-sensitive mAb (which recognizes fully folded and assembled class I molecules: B22 for D^b and Y3 for K^b) or an antiserum (T18) which only recognizes the β2-m free conformation of D^b and K^b. The increase in the amount of class I precipitated with the conformation-sensitive antibodies in the presence of added peptide is directly related to the amount of peptide-receptive class I in the cell (Lewis et al., 1996). Molecules that are assembled but not stabilized by bound peptide dissociate during the preclearing step and can only be recovered with T18. Figure 1D shows that the amount of D^b and K^b that could be recovered with conformation-sensitive mAb in the presence of peptides, and therefore the total amount of K^b synthesized and assembled during the pulse label was similar in K41 and K42. However, the increase in recovery of both D^b and K^b that was observed when peptide was added to the lysate was greater in K42 than in K41. This was accompanied by a corresponding decrease in T18-recoverable heavy chains. This result indicates, therefore, that in K42, while assembly of the HC:β2-m heterodimer was normal, a greater proportion of these class I molecules were peptide receptive. Thus, the loading of class I molecules with stabilizing peptide ligands in the ER was defective in the absence of calreticulin.

Antigen Presentation Is Impaired in Calreticulin-Knockout Cells

To determine the extent to which the reduction in intracellular peptide loading in K42 affected the presentation of epitopes at the cell surface, we assessed the presentation of four viral epitopes in K41 and K42. The first of these, the D^b-restricted LCMV gp160-derived epitope KAVYNFATC, was not presented by K42 following infection with a vaccinia expressing gp160, although the synthetic peptide epitope was well presented (Figure 2A). Both the viral protein and the peptide epitope were well presented by K41. The fact that exogenous peptide presentation by K42 was normal indicated that the lack of virus-induced sensitization to lysis was not due to any adverse antigen-independent effects on CTL recognition or killing. We next tested the ability of K42 to present the K^b-restricted ovalbumin-derived epitope SIINFEKL following infection with vaccinia-expressing ovalbumin

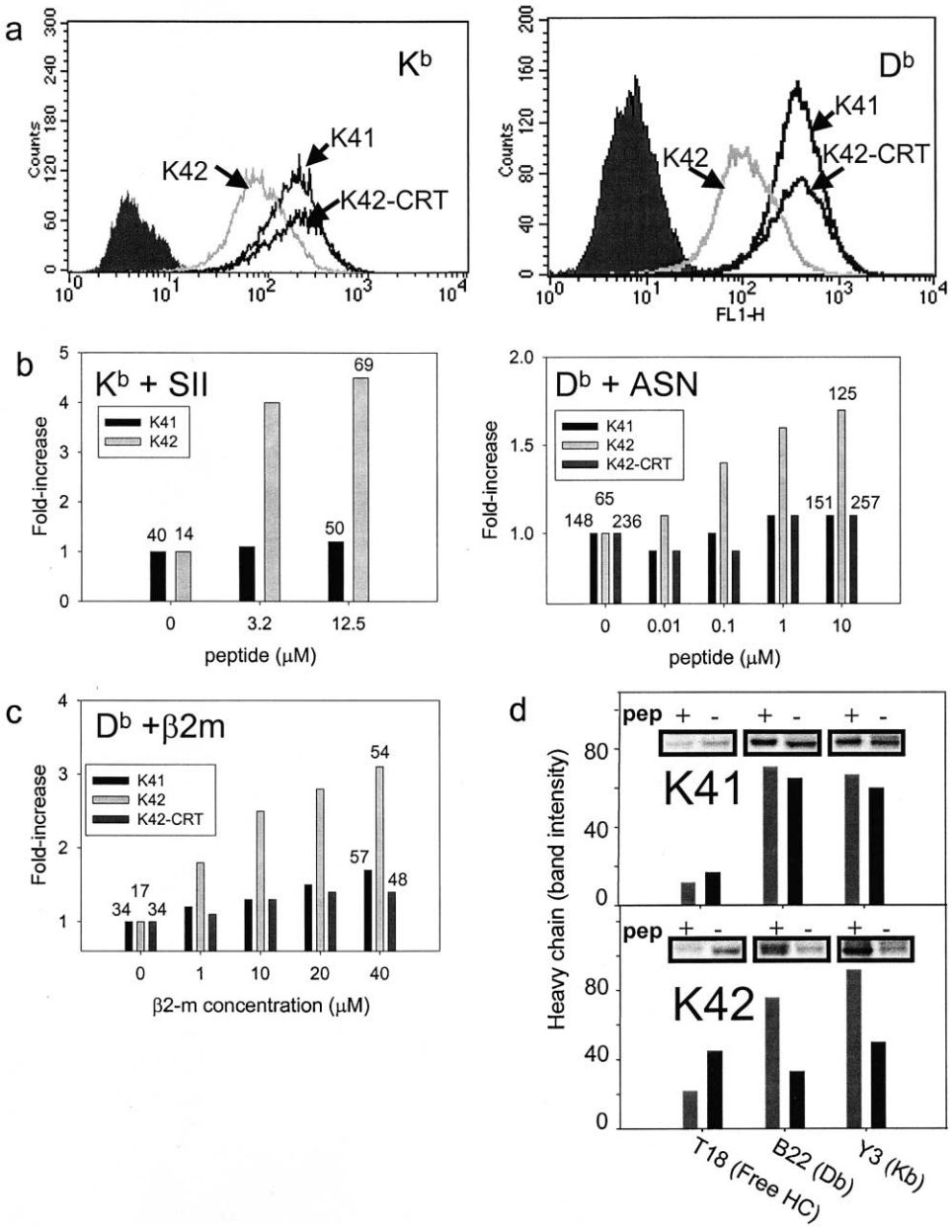

Figure 1. Reduced Expression of MHC Class I Molecules on the Surface of K42 Compared to K41

(A) Cell-surface expression of K^b and D^b was detected on the surface of K41, K42, and K42 transfected with calreticulin (K42-CRT) using monoclonal antibodies Y3 and B22, respectively. NFA, no first antibody.

(B and C) After incubation with the K^b binding peptide SIINFEKL ([B], left panel), the D^b binding peptide ASNENMDAM ([B], right panel), or β2-microglobulin (C), class I expression was evaluated by flow cytometry. Results are shown as the fractional increase in expression with increasing amounts of peptide, with the actual mean fluorescence value indicated above individual bars.

(D) K41 (upper panel) and K42 (lower panel) were pulse labeled, and aliquots were lysed in the presence (+) or absence (−) of 20 μM FAPGNYPAL, which binds to both D^b and K^b. Lysates were immunoprecipitated with either antiserum T18, which recognizes free H2 heavy chains (left insets), mAb B22, which recognizes folded and assembled D^b (middle insets), or mAb Y3, which recognizes folded and assembled K^b (right insets).

(vacc-OVA) in a system that was independent of target cell lysis as a readout. Figure 2B shows that unlike the LCMV epitope, SIINFEKL was presented equally well by K41 and K42 following a 16 hr infection using the T cell hybridoma B3Z to detect presentation. However, we observed a difference in presentation when the duration of vaccinia infection was limited. Thus, after 1 hr of infection when presentation by K41 was half-maximal, presentation by K42 was barely detectable, and after 5 hr of infection, by which time presentation by K41 was maximal, presentation by K42 was only half-maximal. Transfection of K42 with calreticulin restored presentation

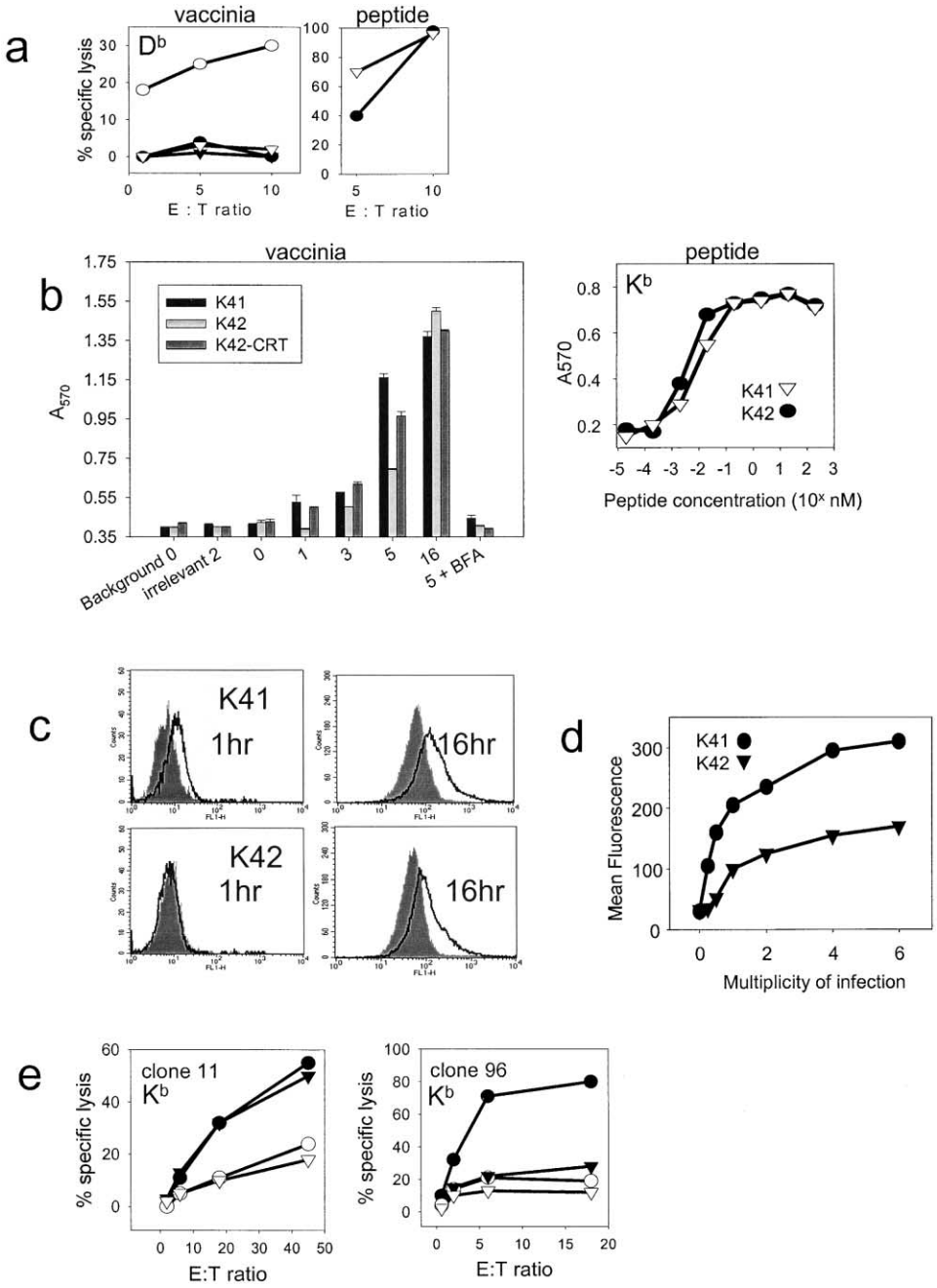

Figure 2. Antigen-Presenting Function of Class I Molecules Is Impaired in K42

(A) The left panel shows recognition of the D^b-restricted gp160 KAVYNFATC by CTL following infection of K41 (circles) or K42 (triangles) with vacc-gp160 (open symbols) or control vaccinia (closed symbols). The right panel shows recognition of K41 (circle) and K42 (triangle) pulsed with 1 μM KAVYNFATC.

(B) The left panel shows recognition of the K^b-restricted ovalbumin epitope by the T cell hybridoma B3Z following infection of K41, K42, and K42-CRT with vacc-OVA or irrelevant vaccinia. This hybridoma has a copy of the β-galactosidase gene which is activated following T cell receptor engagement. Background was assessed with uninfected target cells. Numbers refer to the length of time allowed for processing and presentation to occur prior to fixation, following a 30 min infection. One sample of vacc-OVA-infected cells was cultured in the presence of brefeldin A for 5 hr prior to fixation (5 + BFA) to show that the observed increase in staining was due to delivery of new Kb:SIINFEKL complexes to the cell surface. The right panel shows recognition of K41 (circles) and K42 (triangles) pulsed with increasing concentrations of peptide.

(C) K41 (top panels) and K42 (bottom panels) were infected with vacc-OVA at a moi of 1 for 1 hr (left) or 16 hr (right) before staining with the epitope-specific mAb 25-D1.16.

(D) K41 (circles) and K42 (triangles) were infected with vacc-OVA for 16 hr at increasing moi, stained with mAb 25-D1.16, and analyzed by FACS.

(E) Chromium release assay for the K^b-restricted MCMV-specific CTL clones 11 (left panel) and 96 (right panel) on K41 (circles) and K42 (triangles) infected (closed symbols) or mock infected (open symbols) with MCMV.

to that of K41. In order to determine to what extent the difference in recognition by B3Z of vacc-OVA-infected K41 and K42 correlated with epitope density at the cell surface, we measured the appearance of the SIINFEKL:K^b complex at the surface of infected cells with the antibody 25-D1.16. Figure 2C shows that although the epitope can be detected on both K41 and K42 after overnight infection, less of the epitope is delivered to the surface of K42. No epitope could be detected on the surface of K42 when cells were analyzed one hour after infection, whereas low amounts of the epitope were clearly visible on the surface of K41. Increasing the multiplicity of infection increased the amount of epitope that was delivered to the surface of both cells after overnight infection (Figure 2D), but at all doses of virus between 2- to 3-fold more SIINFEKL:K^b complexes were found on the surface of K41. In order to extend these findings to other CTL specificities, we assessed the presentation of epitopes derived from two MCMV-derived proteins to MCMV-specific CTL clones. Figure 2E shows that one of these did not recognize K42 infected with MCMV, whereas another CTL clone (recognizing an early protein) did recognize MCMV-infected K42 (Kavanagh et al., 2001). It is possible that the differences in CTL recognition we see reflect quantitative differences in epitope presentation in the presence and absence of calreticulin and that some CTL are sensitive to the lower levels of antigen presentation in the absence of calreticulin while others are not. However, clone 96, which was very sensitive to the lack of calreticulin, also had a high apparent avidity, recognizing target cells at an effector-to-target ratio as low as 2:1. It is therefore also possible that calreticulin-assisted antigen presentation is epitope specific.

Rapid Transport of Unstable Class I Molecules in Calreticulin-Knockout Cells

We have previously observed a correlation between the length of time that newly synthesized class I molecules dwell in the ER and the extent to which they become loaded with peptides (Lewis et al., 1996). To determine whether the absence of calreticulin affected the rate of D^b and K^b transport in K42, we performed a pulse-chase analysis. The fraction of peptide-receptive class I molecules at each time point was assessed by immunoprecipitating with conformation-sensitive antibodies in the presence or absence of a stabilizing peptide. Figures 3A and 3B show that the rate of export of total (loaded and unloaded) D^b and K^b molecules is considerably enhanced in K42. For D^b, this faster trafficking is similar to that seen for the T134K mutant D^b molecule (data not shown). Another noticeable feature of this experiment was that in K42, class I molecules were rapidly lost from the assay after having left the ER (and consequently acquired endo H resistance), and this was particularly dramatic for K^b. We have interpreted this phenomenon before as the disappearance of unstable class I molecules from the cell surface (Lewis et al., 1996), and this most probably reflects degradation following endocytosis. Export of a greater proportion of peptide-receptive K^b molecules in K42 is shown in Figure 3C. An equivalent result was obtained for D^b (data not shown). By comparing the difference in endo H-resistant class I molecules recoverable with conformation-sensitive mAb in the presence and absence of peptide, we estimate that around 50% of both K^b and D^b that assembles in K42 is exported in a peptide-receptive state, compared to less than 5% in K41. These figures are consistent with data shown in Figures 1 and 3 and with the reduced presentation of the ovalbumin-derived epitope (Figures 2B–2D).

MHC Class I Is Incorporated into the Peptide-Loading Complex in Calreticulin-Knockout Cells

We and others have shown previously that in human cells assembly of the peptide-loading complex appears to be cooperative, in that the interaction between class I, calreticulin, and ERp57 is dependent on the presence of TAP and tapasin (Sadasivan et al., 1996; Lewis and Elliott, 1998; Hughes and Cresswell, 1998). This led us to determine whether class I molecules become incorporated into the multimolecular loading complex when calreticulin is absent. We immunoprecipitated TAP1+2 from a digitonin lysate and Western blotted for the other components of the loading complex. Calnexin, ERp57, tapasin, and class I heavy chain were identified in coimmunoprecipitates from K42 (Figure 4A), indicating that class I is incorporated into the loading complex even in the absence of calreticulin. In sequential immunoprecipitation experiments (Figure 4B), both D^b and K^b could be recovered from anti-TAP1+2 coimmunoprecipitates. After dissociating MHC class I molecules from immunoprecipitated TAP with Triton X-100, some of the K^b molecules were sufficiently stable to be immunoprecipitated with a conformation-specific antibody. These molecules presumably represent the previously described pool of tapasin-associated molecules that carry bound peptides (Li et al., 1999). Thus, although it has been shown that MHC class I binding to calreticulin and Erp57 is dramatically reduced in the absence of TAP or tapasin, these results indicate that the opposite is not true.

Interestingly, abundant ERp57 coprecipitated with TAP in K42, indicating that ERp57 incorporation into the loading complex is not simply a consequence of its interaction with calreticulin (Oliver et al., 1999). Although it is still possible that the ERp57 we detect in Figure 4A was recruited by calnexin in the complex, we have also detected high levels of ERp57 coimmunoprecipitating with TAP from K42 even in cases where there was no detectable calnexin (data not shown). These data imply that ERp57 may interact independently with class I, tapasin, or TAP. Relevant to this point is the observation made by Lindquist et al. (1998) that the half-life of ERp57 in the loading complex is considerably longer than that for class I. One interpretation of this result is that ERp57 interacts with components of the loading complex independently of class I (Lindquist et al., 1998), and it is significant that a covalent complex of a mutant ERp57 and tapasin has recently been found (P. Cresswell, Abstract at the 12th International Congress for Immunology, Stockholm, 2001). Deidrich et al. have recently reported that newly assembled class I molecules bind to a "preloading complex" of TAP, tapasin, ERp57, and calnexin (Diedrich et al., 2001). The immunoprecipitates analyzed in Figure 4A will therefore represent a mixture of preloading complexes and mature loading complexes (containing TAP, tapasin, ERp57, calnexin, and MHC class I). The ratio of HC to either ERp57 or tapasin in these

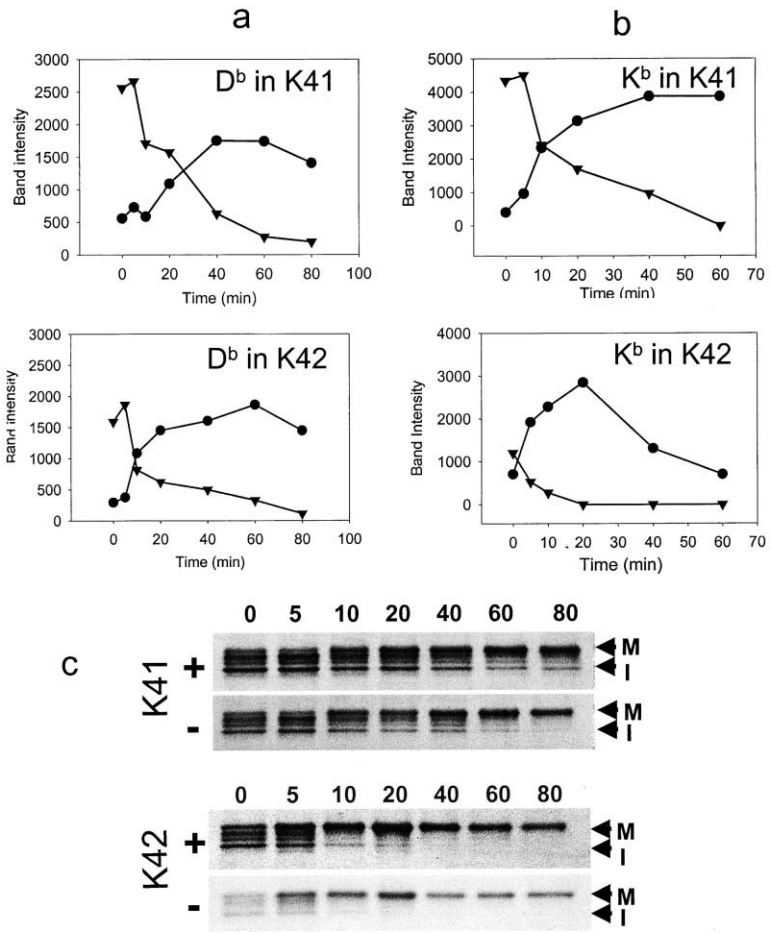

Figure 3. Class I Molecules Are Transported Rapidly and in a Peptide-Receptive State in the Absence of Calreticulin

Pulse-chase analysis was carried out on D^b (A) and K^b ([B] and [C]) synthesized in K41 (top panels) and K42 (bottom panels). Aliquots of labeled cells were lysed in the presence or absence of 20 μM FAPGNYPAL at each time point. Immunoprecipitates recognized by either mAb B22 (for D^b) or mAb Y3 (for K^b) were digested with endoglycosidase H, fractionated by SDS-PAGE, and the heavy-chain band was quantitated by phosphorimaging. For cells lysed in the absence of peptide, the band intensity of immature, endo H-sensitive (I, triangles) and mature, endo H-resistant (M, circles) heavy chains recovered from K41 (upper panels, [A] and [B]) and K42 (lower panels, [A] and [B]) was plotted against time. An example pulse-chase experiment for K^b is shown in (C), where heavy chains recovered by mAb Y3 from cells lysed in the presence (+) or absence (−) of peptide to determine the fraction of peptide-receptive class I molecules were endo H treated to determine the fraction of mature (M) and immature (I) class I molecules at each time point.

immunoprecipitates gives a rough estimation of the relative abundance of pre- and mature-loading complexes. Because of the relative sensitivity of tapasin to proteases, we felt that this was a less reliable denominator in estimating such ratios compared to ERp57. The HC:ERp57 ratio was lower for K42 in experiments shown in Figure 4A, suggesting that in this cell line less MHC class I was incorporated into mature loading complexes, although this conclusion can only be viewed as tentative given the semiquantitative nature of the immunoblotting technique. To investigate this issue further, we analyzed the composition of coimmunoprecipitates using the anti-$H2D^b$ antibody 28-14-8s. The immunoprecipitates analyzed in Figure 4C represent a mixture of D^b HC bound to calnexin and D^b bound to the mature loading complex. Figure 4C clearly indicates the presence of ERp57 and tapasin in 28-14-8s immunoprecipitates from both K41 and K42, confirming that in the absence of calreticulin MHC class I can assemble with the mature loading complex. Here, the ratio of HC:ERp57 gives a rough estimate of the distribution of class I molecules (HC) between calnexin and the mature loading complex. This ratio is similar for K41 and K42, indicating that the pool of calnexin-bound HC is similar and consequently that assembly with β2-m is unlikely to be impaired in the absence of calreticulin. This is consistent with data shown in Figures 1 and 3.

Peptide Loading of Class I Molecules Is a Specific Function of Calreticulin

There is approximately 40% identity between calnexin and calreticulin at the amino acid level. Both have identical lectin activity, but their interaction with class I HC is different. Calnexin binds rapidly to newly synthesized HC, whereas calreticulin has only ever been detected bound to HC:β2-m heterodimers (Harris et al., 1998). Danilczyk et al. have recently demonstrated considerable redundancy in the function of calreticulin and calnexin in the early biogenesis of class I HC:β2-m heterodimers in insect cells and that calnexin lacking its transmembrane anchor has a similar substrate specificity to calreticulin (Danilczyk et al., 2000). This led us to determine whether soluble calnexin could restore normal class I assembly in K42. We transfected K42 with the lumenal portion of calnexin tagged with the ER retrieval sequence KDEL (kindly provided by D.B.Williams, Toronto) and compared it to K42 transfected with full-length rabbit calreticulin. The transfected proteins were expressed in similar amounts as assessed by immunoblotting (see Figure 5 inset). Whereas calreticulin was able to restore class I expression to the level seen in K41 (see Figure 1A for example), soluble calnexin was not (Figure 5). The soluble calnexin construct has been shown to be functional with respect to lectin binding and chaperone function in mammalian cells (Vassilakos

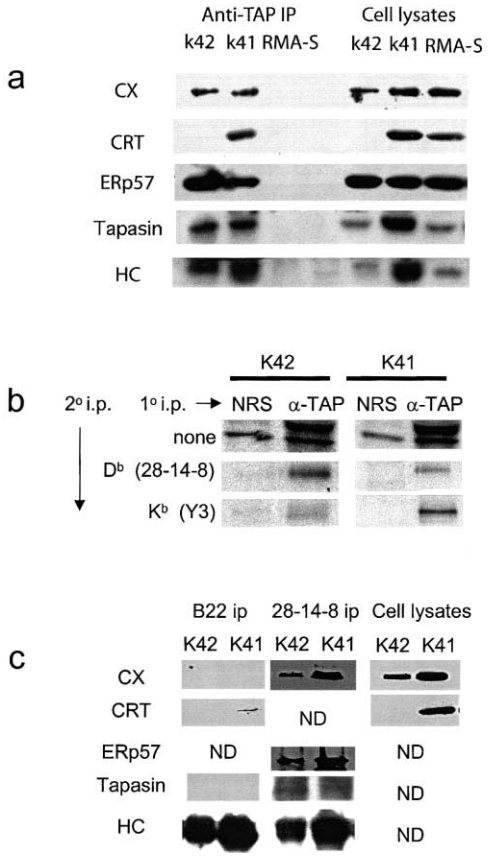

Figure 4. Assembly of the Peptide-Loading Complex in the Absence of Calreticulin

(A) Immunoprecipitates of TAP2 and associated proteins were made (left panel) from K41, K42, and the TAP 2 mutant cell line RMA-S, fractionated by SDS-PAGE, and immunoblotted with antibodies to calnexin (CX), calreticulin (CRT), ERp57, tapasin, and class I MHC heavy chain (HC). The presence of each component in these cells was evaluated qualitatively by immunoblotting a sample of cell lysate. The relatively poor detection of HC and tapasin in lysates of K42 and RMA-S is probably due to some degradation prior to adding SDS-containing sample buffer. Detection of calnexin in the loading complex of K41 and K42 is variable, suggesting that it is present in the complex at the limit of our ability to detect it. The experiment shown here is an example in which calnexin was detectable in complexes from both K41 and K42. Other experiments have yielded examples where calnexin was present in coimmunoprecipitates from K41 or K42 only.

(B) Primary immunoprecipitates (1° i.p.) of TAP 1+2 and associated proteins (α-TAP) or control immunoprecipitates using normal rabbit serum (NRS) from K41 and K42 were solublized with Triton X-100 and reprecipitated (2° i.p) with antibodies to either K^b (Y3) or D^b (28-14-8s) to demonstrate the presence of class I molecules in the loading complex of both K41 and K42.

(C) Primary immunoprecipitates of D^b and associated proteins were made from digitonin lysates with the antibody 28-14-8s after extensive preclearing of non-TAP-associated D^b with the antibody B22 and probed for the presence of calnexin (CX), calreticulin (CRT), ERp57, tapasin, and TAP 2, as in (A).

et al., 1998) and was not secreted, indicating that the C-terminal KDEL tag was functional (data not shown). Whether soluble calnexin failed to restore class I expression because of a different or absent interaction with class I is not clear and is currently under investigation. These results indicate that the peptide-loading cofactor

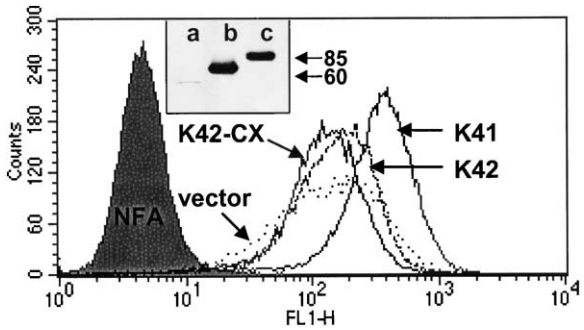

Figure 5. Soluble Calnexin Does Not Restore Class I Expression on the Surface of K42

K42 was transfected with soluble calnexin (K42-CX) or vector alone. Cell-surface expression of K^b was detected with mAb Y3. Expression of soluble calnexin in K42-CX (lane c, inset) was confirmed by Western blotting against the HA epitope tag and compared to the expression of calreticulin in K42-CRT cells (lane b, inset) in which class I expression was restored to normal (see Figure 1). Lane a (inset) is a blot of K42 lysate with the anti-HA antiserum.

function of calreticulin cannot be replaced by a homologous protein with overlapping glycoprotein-substrate specificities. Consequently, this function is probably specific to calreticulin and opens up the possibility that the functional interaction between calreticulin and class I resides outside the lectin site (Saito et al., 1999).

Discussion

Our study of the calreticulin-knockout cell line K42 has demonstrated a crucial role for calreticulin in the loading of MHC class I molecules with optimal peptide cargo. Newly synthesized HC binds to calnexin in the ER (Salter, 1994), where it is protected from degradation (Wilson et al., 2000) and during which time intramolecular disulphide bonds are formed (Tector and Salter, 1995; Farmery et al., 2000). Upon β2-m association, HC undergoes a conformational change (Elliott et al., 1991), and for heavy chains with just one glycosylation site at asparagine 86 calnexin dissociates and is replaced by calreticulin (Sadasivan et al., 1996; Zhang and Salter, 1998; Harris et al., 1998). These newly assembled MHC class I molecules are likely to be truly empty of peptide ligand and are not competent for export from the ER. This is suggested by the fact that class I molecules that are expressed in the absence of a peptide supply, assemble with β2-m normally but tend to be retained in the ER (Townsend et al., 1989). Soon after assembly, empty class I molecules load with suboptimal peptides and become competent for export from the ER. (Lewis et al., 1996). Class I molecules and calreticulin then bind to a preloading complex comprising TAP, tapasin, ERp57, and calnexin, with the resulting dissociation of the latter, at least in human cells (Diedrich et al., 2001). It is not known whether a class I:calreticulin complex forms first, but our results indicate that the formation of a class I:calreticulin complex is not obligatory for class I binding to the preloading complex, although the extent of binding may be impaired in the absence of calreticulin. The suboptimal peptide cargo is then optimized (Lewis and

Elliott, 1998), during which peptides with fast off-rates are replaced by (or converted into) high-affinity peptides. This process is tapasin dependent (Androlewicz, 1999), and our results indicate that it is also dependent on calreticulin. The rate-determining step for the transport of MHC class I molecules to the cell surface probably follows dissociation of class I molecules from the loading complex (Spiliotis et al., 2001) and can be bypassed by introducing mutations into the class I molecule that prevent it from binding to the loading complex (Lewis and Elliott, 1998; Pentcheva and Edidin, 2001).

Overall, the assembly and antigen-presenting function of MHC class I molecules expressed in the absence of calreticulin share some features with MHC class I molecules that are unable to associate with the TAP complex because of either the absence of tapasin or a point mutation in the MHC class I heavy chain. This phenotype is consistent with an inability to optimize the MHC class I peptide cargo efficiently. However, there is a surprising and significant difference in that we have shown that D^b and K^b can interact with the peptide-loading complex in the absence of calreticulin. There are several possible contributions of calreticulin to the observed phenotype.

(1) Calreticulin may stabilize the interaction between class I and the TAP-tapasin complex, as suggested by the observation that castanospermine (which prevents the generation of the monoglucosyl-glycan recognized by calreticulin) inhibits the interaction between class I molecules and TAP (Sadasivan et al., 1996; Lewis and Elliott, 1998). The lower incorporation of class I molecules into the mature loading complex in K42 would be consistent with this (see Figure 4A), but we have found no other evidence that loading complexes formed in K42 are any less stable than those formed in K41 which contain calreticulin (data not shown). It is equally likely, therefore, that this represents the assembly of fewer mature loading complexes rather than the assembly of an equal number of less stable mature loading complexes in K42. Interestingly, in the absence of tapasin or TAP, fewer class I molecules can be coimmunoprecipitated with calreticulin (Lewis and Elliott, 1998; Harris et al., 1998). It is possible, therefore, that TAP/tapasin initiates or stabilizes an interaction between calreticulin and HC but not the reverse.

(2) Calreticulin may promote the assembly of class I heavy chains with β2-m, thereby promoting incorporation of newly assembled class I molecules into the loading complex. Although this would be consistent with the slightly lower amount of HC we see in the absence of calreticulin, data shown in Figures 1 and 4 suggest that assembly with β2-m is normal in K42, since in these assays the addition of peptide has been shown to stabilize preassembled MHC class I molecules and not to promote de novo assembly of heavy chain with β2-m and peptide (Elliott et al., 1991).

(3) Calreticulin may chaperone peptides from TAP to the peptide binding groove of class I. There is evidence from peptide crosslinking experiments that calreticulin can bind to peptides delivered to the ER via TAP (Spee and Neefjes, 1997). In addition, calreticulin bound to peptides can be taken up by antigen-presenting cells and the immunogenic peptides transferred to MHC class I molecules (Basu and Srivastava, 1999; Nair et al., 1999). It is possible, therefore, that calreticulin could chaperone peptides between TAP and class I, although it is unclear how the lack of this function in K42 would lead to more rapid trafficking of class I molecules.

(4) Calreticulin may modulate a calcium-dependent trimmase in the ER. N-terminal trimming of long, suboptimal peptides in the ER could be important in the optimization process. A recent report identifies the ER resident gp96 as a calcium-dependent aminopeptidase capable of trimming a precursor T cell epitope in vitro (Menoret et al., 2001). The fact that the ER calcium store in K42 is reduced (Nakamura et al., 2001) may therefore be a regulating factor in this process.

(5) Another possibility is that calreticulin may augment a tapasin-dependent peptide optimization function (Lehner et al., 1998; Peh et al., 1998). This could be achieved by its ability to stabilize the interaction between HC and TAP/tapasin as outlined in (1) or via a protein-sorting function. For example, a recent paper (Pentcheva and Edidin, 2001) has shown that a mutant class I molecule which does not interact with the peptide loading complex (T134K) enters the secretory pathway prematurely at an exit site in the ER that is different for wild-type molecules. In doing so, they appear to bypass normal quality control that ensures their loading with optimal peptides. The similarities between the suboptimal loading of class I molecules in K42 and of T134K molecules in normal cells would be consistent with calreticulin being involved in the distribution of newly assembled class I molecules between these two subdomains of the ER, only one of which has access to TAP-tapasin. Another possible role for calreticulin could be in the recycling of newly synthesized but incompletely loaded class I molecules from the ERGIC/Golgi to the ER (Hsu et al., 1991). Such recycling contributes to the quality control of other glycoproteins and is mediated by the recognition of either a cytoplasmic KKXX motif or a C-terminal KDEL motif. MHC class I molecules contain neither of these motifs, so it is likely that their sorting is mediated by binding to cofactors that do contain a suitable motif. The obvious candidates for this are tapasin (KKXX), ERp57 (KDEL), and calreticulin (KDEL). Interestingly, the quality control of T cell receptor α chain has recently been shown to depend on cycling between the Golgi and the ER via KDEL-dependent retrieval (Yamamoto et al., 2001). One implication of this model of quality control for class I molecules is that the cofactor mediating retrieval would need to sense the difference between a class I molecule that is incompletely assembled and one which is sufficiently stable to proceed beyond this checkpoint. In this respect, it is intriguing that calreticulin has been shown to associate predominantly with empty class I molecules, whereas tapasin has been shown to interact with both empty and peptide-loaded class I molecules (Li et al., 1999).

Optimization of the class I peptide cargo may therefore have two components. The first is catalyzed peptide exchange or trimming (a function of tapasin and perhaps ERp57) in the ER; and the second is retrieval to the loading complex by calreticulin of class I molecules that have dissociated from TAP prior to achieving an optimal peptide load. Therefore, interfering with either of these processes by deleting calreticulin or tapasin (Grandea et al., 2000; Garbi et al., 2000) might be expected to lead to an increase in the number of peptide-receptive

D^b and K^b molecules that are released into the secretory pathway, having escaped quality control.

Experimental Procedures

Cells and Antibodies

Calreticulin-deficient (K42) fibroblasts were derived by SV40 transformation of primary fibroblast cultures from mouse embryos carrying a targeted knockout of the calreticulin gene (Mesaeli et al., 1999; Nakamura et al., 2001). Wild-type (K41) fibroblasts were derived in the same way from a normal embryo. The cells were maintained in RPMI 1640 with 10% fetal calf serum supplemented with glutamine, penicillin, and streptomycin (R10). K42 was transfected with a plasmid containing full-length rabbit calreticulin cDNA tagged with a sequence encoding an HA epitope and a KDEL retrieval sequence. K42 was also transfected with plasmid pUB6v5 containing cDNA encoding residues 1–387 of mouse calnexin with a C-terminal KDEL sequence using Effectene (Qiagen) and selected with 5 μg/ml blasticidin. Anti-calnexin antiserum was raised in rabbits against a C-terminal peptide (Lewis and Elliott, 1998). Rabbit anti-human calreticulin antiserum that crossreacts with the mouse protein was kindly provided by Dr. Bob Sim (Oxford, UK). Purified sheep anti-rat TAP1 and TAP2 that crossreact with murine TAP were generous gifts from Dr. Simon Powis (Dundee, UK). Rabbit anti-murine tapasin N-terminal peptide antiserum was kindly provided by Dr. Ted Hansen (St. Louis, MO), and rabbit anti-ERp57 antiserum was a gift from Dr. Tom Wileman (Purbright, UK). Murine MHC heavy chain-specific antiserum T18 was generated against a recombinant fragment of D^b from residues 1–270 (Rigney et al., 1998). K^b and D^b conformation-sensitive monoclonal antibodies Y3 and B22 were purified with protein A affinity chromatography. 25-D1.16 recognizing the K^b/SIINFEKL complex was kindly provided by Dr. Ron Germain (Bethesda, MD), and recombinant vaccinia virus expressing ovalbumin was kindly provided by Dr. Jon Yewdell (Bethesda, MD). Peptides SIINFEKL and ASNENMDAM were from Research Genetics (Huntsville, AL). KAVYNFATC, the immunodominant peptide derived from the glycoprotein of lymphocytic choriomeningitis virus (LCMV), recombinant vaccinia virus expressing the same glycoprotein of LCMV (vacc-gp160), and KAVYNFATC-specific CTL were generous gifts from Dr. Awen Gallimore (Oxford, UK). Purified, recombinant human β2-m was provided by Dr. Mei Sun (Oxford, UK).

Cloning of MCMV-Specific CTL

Mice were infected with 5×10^4 plaque-forming units (pfu) MCMV, strain Smith, or ΔMS94.5, and CTL were isolated as described (Kavanagh et al., 2001). Clone 11 was derived from a ΔMS94.5-infected mouse, and clone 96 was derived from a Smith-infected mouse. Clone 96 is K^b restricted, and clone 11 is K^b restricted.

CTL Assays

For assays with recombinant vaccinia, 2×10^6 K41 or K42 in 0.5 ml R10 was infected for 1 hr with 10^7 pfu of either vacc-gp160 or virus expressing influenza nucleoprotein as control. Infected cells were incubated overnight and then labeled for 1 hr with 50 μCi ^{51}Cr in the presence or absence of 20 μM peptide. Plates with target cells were washed four times before the T cell clones were added to 1×10^4 target cells per well at specified effector-to-target ratios. Plates were incubated for 5 hr at 37°C, after which time 25 μl of the supernatant was removed for counting. Maximum ^{51}Cr release was determined from wells that were lysed by the addition of 1% Triton X-100, and spontaneous release was the cpm released from cells incubated in medium only. Percentage of specific lysis was calculated as (cpm experimental − cpm spontaneous release) / (cpm maximum − cpm spontaneous release). For assays with MCMV-specific CTL clones, target cells were seeded in flat-bottom 96-well plates at 10,000 cells/well and incubated for 24 hr with 50 U/ml IFN-γ (Sigma). Targets were then either left uninfected or were infected with MCMV-Δ MS94.5 at a multiplicity of infection (moi) of 100 MCMV overnight (16 hr) in the presence of phosphonoacetic acid to prevent MCMV late gene expression. Target cells were also labeled overnight with 100 μCi ^{51}Cr/plate. For assays with the T cell hybridoma B3Z (Karttunen et al., 1992), a kind gift of Dr. Nilabh Shastri (Berkeley, CA), targets were washed once in DMEM, infected at a moi of 10 for 30 min at 37°C, then washed once in R10/1 mM sodium pyruvate/50 μM 2-mercaptoethanol and replated in the same containing 375 μg/ml cytosine arabinoside (Sigma) to block expression of β-galactosidase driven by the late vaccinia promoter. After incubation for varying periods of time, targets were fixed in ice-cold PBS containing 1% formaldehyde, quenched with PBS/200 mM glycine, and then washed before being exposed to B3Z at an E:T ratio of 1:1 overnight. Cells were then washed and 100 μl of chromogenic substrate (0.15 mM chlorophenol red β-galactopyranoside in PBS/0.5% NP-40) was added to each well and incubated at 37°C for 0.5–3 hr. Optical density was measured at 570 nm in a multiplate reader.

Flow Cytometry

2×10^5 cells were incubated with hybridoma culture supernatant (for 25-D1.16) or antibody at a final concentration of 5 μg/ml (for Y3 and B22) in 250 μl of R10 on ice for 1 hr then washed with 1 ml cold PBS twice. The cells were stained for 45 min at 4°C in 250 μl of R10 containing a 200-fold dilution of goat anti-mouse Ig-FITC conjugate (Sigma, St. Louis, MO). The cells were washed with 500 μl cold PBS three times and resuspended in 300 μl PBS/0.7% formaldehyde. A minimum of 20,000 cells were counted using the FACScan (Becton Dickinson). In some cases, cells were incubated for 4 hr at 37°C in serum-free medium with either peptide or recombinant β2-m before flow cytometric analysis.

Western Blot

Proteins fractionated by SDS-PAGE were blotted onto Hybond-C extra membrane (Amersham) by electrophoretic transfer. The transferred membrane was blocked with PBS containing 2.5% fat-free milk for at least 1 hr. The first antibody in PBS with 0.02% Tween 20 (PBST) and 2.5% fat-free milk was added and incubated for 1 hr at room temperature. The membrane was then washed three times with PBST and incubated with an appropriate secondary antibody conjugated to horseradish peroxidase for a further 1 hr. The membrane was washed four times with PBST, the signal was developed with a SuperSignal Dura West (Pierce) chemiluminescence kit, and the membrane was exposed onto Kodak Xomat film.

Immunoprecipitation

For detecting calreticulin, calnexin, and ERp57 associated with TAP, the following procedure was adopted. Cells were washed once with PBS and lysed on ice in 1 ml of 1% digitonin with mammalian protease inhibitor cocktail (Sigma) for 15 min. The lysates were clarified by microcentrifugation at 13,000 rpm for 10 min. One hundred microliters of Protein G-Sepharose beads (10% w/v) and 5 μg of purified sheep anti-rat TAP1 antibody were mixed with each lysate and incubated at 4°C with rotation for 1 hr. The beads were washed three times with 0.1% digitonin in PBS and mixed with 40 μl of SDS-PAGE loading buffer. The mixture was boiled for 3 min, and 20 μl of sample was loaded into each lane on a 10% SDS-PAGE gel. For probing tapasin, the same protocol was used except that anti-TAP1 antibody was added at the time of lysis. For sequential immunoprecipitations, primary immunoprecipitations made in 1% digitonin/PBS were washed three times in the same then resuspended in lysis buffer containing 0.5% Triton X-100. The beads were removed by centrifugation, and the supernatant was reprecipitated with the relevant antibody. Pulse-chase analyses were carried out exactly as described in Lewis et al. (1996). Cells were starved in cysteine/methionine-free medium for 30 min, and labeling was for 10 min with 10 μCi Promix (Amersham) per 10^7 cells. The chase was carried out in the presence of 2 mM cysteine and 2 mM methionine. Cells were lysed in 0.5 ml lysis buffer and precleared for 2 hr before immunoprecipitation with B22 (for D^b) or Y3 (for K^b).

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