

Efficient Dissociation of the p88 Chaperone from Major Histocompatibility Complex Class I Molecules Requires Both β_2 -Microglobulin and Peptide

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Summary

Previously, we showed that an 88-kD protein (p88) associates rapidly and quantitatively with newly synthesized murine major histocompatibility complex class I molecules within the endoplasmic reticulum (ER). This interaction is transient and dissociation of p88 appears to be rate limiting for transport of class I molecules from the ER to the Golgi apparatus. In this report, we examine the relationship between p88 interaction and assembly of the ternary complex of class I heavy chain β_2 -microglobulin (β_2m), and peptide ligand. In both murine and human β_2m -deficient cells, in which little or no transport of class I heavy chains is observed, p88 remained associated with intracellular heavy chains throughout their lifetime. In murine RMA-S cells, which are apparently defective in accumulating peptide ligands for class I within the ER, prolonged association of p88 with "empty" heavy chain- β_2m heterodimers was also observed. However, p88 dissociated slowly in parallel with the slow rate of ER to Golgi transport of empty class I molecules in these cells. The close correlation between p88 association and impaired class I transport suggests that p88 functions to retain incompletely assembled class I molecules in the ER. We propose that conformational changes in class I heavy chains induced by the binding of both β_2m and peptide are required for efficient p88 dissociation and subsequent class I transport.

Plasma membrane and secretory proteins are synthesized on endoplasmic reticulum (ER)¹-bound polysomes, enter the ER, and subsequently move by a process of vesicle budding and fusion, through the Golgi apparatus to the cell surface (1). Transport along this exocytic pathway occurs with distinct rates and efficiencies for different proteins. In many instances, export from the ER is the rate-limiting step (2-4). Studies on several oligomeric cellular and viral surface glycoproteins have indicated that ER to Golgi transport often depends on the acquisition of correct tertiary or quaternary structure; inappropriately folded or unassembled membrane proteins appear to be retained intracellularly either by inclusion in aggregates or by specific ER proteins, e.g., binding protein (BiP; 2-11). Class I molecules of the MHC are no exception to these phenomena. Correct assembly of class I molecules is required for efficient intracellular transport (12-18).

Until recently, the assembly of class I molecules had been

viewed as a two-component event (13, 14). It involved the noncovalent association of the highly polymorphic, transmembrane class I H chain (45-50 kD) with β_2 -microglobulin (β_2m ; 12 kD). However, with the discovery that class I molecules bind potentially immunogenic peptide ligands intracellularly (likely in the ER; 19-27), it has become clear that the tertiary and quaternary structural requirements for efficient intracellular transport of class I molecules must be reassessed. Furthermore, we have described a novel 88-kD protein, p88, that interacts quantitatively with murine class I molecules rapidly after synthesis (28). It also appears to play an important role in the egress of class I molecules from the ER since its interaction is transient and the rate of its dissociation correlates closely with the distinct rates of ER to Golgi transport observed for different class I allotypes. These observations led us to suggest that the dissociation of p88 may be rate limiting for ER to Golgi transport of class I molecules. We further hypothesized that p88 might promote class I assembly and/or retain class I molecules in the ER until the appropriate ternary complex of H chain, β_2m , and peptide is formed.

In this study, we examine the relationship between p88 interaction and the assembly of class I molecules. To assess

¹ Abbreviations used in this paper: β_2m , β_2 -microglobulin; BiP, binding protein; DSP, dithiobis (succinimidyl propionate); endo H, endoglycosidase H; ER, endoplasmic reticulum.

the effects that β_2m and peptide ligand have on the interaction of p88 with class I molecules, we used β_2m -deficient cells of mouse and human origin as well as the mutant mouse cell line RMA-S, which appears to possess a defect in accumulating peptide ligands in the ER (21, 24, 25). In β_2m -deficient cells, the bulk of newly synthesized, β_2m -free ("free") class I H chains appear to remain in the ER until they are degraded (17, 18), whereas in RMA-S cells, ER to Golgi transport of peptide-free ("empty") class I molecules appears drastically slowed (21, 29). Our findings show that p88 associates with these molecules and that its subsequent dissociation is virtually blocked or partially impaired, respectively, closely paralleling the transport behavior of these incompletely assembled class I molecules. Thus, efficient dissociation of p88 requires the binding of both β_2m and peptide ligand. Furthermore, the close correlation between duration of p88 interaction and rate of ER to Golgi transport suggests that p88 has a chaperone-like function similar to that of BiP (11), i.e., retaining inappropriately or incompletely folded/assembled class I molecules within the ER.

Materials and Methods

Cells and Antibodies. Murine β_2m -deficient R1E cells transfected with H-2K^b or H-2D^b genes, R1E-K^b or R1E-D^b, respectively, were maintained in culture as described previously (17, 18). All other cells were maintained at 37°C in a 5% CO₂/air atmosphere in RPMI 1640 containing 10% FCS and antibiotics. EL-4 is a C57Bl H-2^b T cell lymphoma cell line (30). The parental Rauscher virus-transformed murine lymphoma T cell line, RMA, and its mutant RMA-S (29), were provided by Dr. J. W. Yewdell (National Institute of Allergy and Infectious Diseases, Bethesda, MD). The human β_2m -deficient Burkitt lymphoma cell line, Daudi (12, 14–16), was obtained from Dr. G. Mills (University of Toronto).

Rabbit antiserum raised against the peptide encoded by exon 8 of the K^b gene (anti-peptide 8; reference 31) was a gift from Dr. B. Barber (University of Toronto). The mAb 28-14-8s, purified with the MAPS system (Bio-Rad Laboratories, Richmond, CA), was used for the isolation of D^b molecules (32). Both reagents recognize the appropriate H chains either alone or in association with β_2m (17, 18). For immunoisolation of K^b molecules associated with β_2m , the mAb Y-3 was used (33). Human class I H chains were isolated using a rabbit anti-HLA-A, -B, -C H chain serum. It was raised against a mixture of denatured HLA-B7 and B40 papain fragments (34), and was donated by Dr. T. Delovitch (University of Toronto).

Metabolic Radiolabeling and Crosslinking of Class I Molecules. Pulse-chase experiments coupled with chemical crosslinking were performed essentially as detailed by Degen and Williams (28). After a preincubation step, cells were pulsed for 10 min at 37°C in methionine (Met)-free RPMI 1640 containing 0.5 mCi/ml [³⁵S]Met (Amersham Corp., Oakville, Canada). The chase was initiated by the addition of at least 9 vol of complete RPMI 1640 containing 1 mM Met and 10% FCS. At various times, aliquots of 3–5 × 10⁶ cells were washed with ice-cold PBS, pH 8, and kept on ice until crosslinking and/or lysis. Typically, cells were lysed in 0.5 ml PBS, pH 8, containing 1% NP-40, 10 mM iodoacetamide, and 0.23 mM PMSF (lysis buffer) either in the presence or absence of 0.12 mM of the homobifunctional crosslinker, dithiobis(succinimidyl propionate) (DSP; Pierce Chemical Co., Rockford, IL). This thiol-

cleavable crosslinking agent was freshly prepared as a 2 mM stock in lysis buffer, and 30 μ l was added simultaneously with 470 μ l of lysis buffer to the cells at room temperature. After vigorous vortexing, lysis and crosslinking were allowed to proceed on ice for 30 min. Crosslinking was terminated by the addition of 50 μ l of 100 mM glycine, 10% aprotinin in lysis buffer. After 10 min on ice, lysates were subjected to centrifugation (100,000 g for 45 min), and the postnuclear supernatants were usually precleared with fixed *Staphylococcus aureus* cells.

Long-term, steady-state radiolabeling was performed exactly as described previously (28). Subsequently, these cells were washed twice in ice cold Met-free RPMI 1640, pulse labeled for 10 min to increase the radioactive signal of newly synthesized class I molecules, and subjected to crosslinking.

Isolation of Class I Molecules and Crosslinked Class I-containing Species. All procedures were performed at 4°C and have been well documented (28). For the isolation of murine or human class I molecules, cell lysates were incubated for 2 h with either 2–3 μ l of a rabbit antiserum or 15 μ g of a purified mAb, and immune complexes were isolated by shaking for 1 h with 90 μ l of a 33% suspension of Affi-Gel protein A (Bio-Rad Laboratories). In all cases, the Affi-Gel beads were washed extensively and class I molecules were eluted by heating at 60–65°C in a small volume of Laemmli sample buffer with or without (in the case of crosslinked samples) the reducing agent. The immunisolated molecules were subjected to SDS-PAGE analyses using the protocol of Laemmli (35), with the exception that the concentration of Tris in the stacking and separating gels was halved. For cleavage of DSP crosslinks before SDS-PAGE analysis under reducing conditions, the Affi-Gel protein A eluate was boiled in the presence of reducing agents (80 mM DTT and 0.2% β -mercaptoethanol), incubated for 1 h at 65°C, and reboiled before its application onto the gel to ensure complete DSP cleavage. After electrophoresis, gels were fixed in 10% TCA, treated with an autoradiographic image enhancer (Autofluor; National Diagnostics, Mannville, NJ), dried, and subjected to fluorography. The relative amounts of radiolabeled proteins were measured by densitometric analyses of weakly exposed x-ray films using a densitometer (620; Bio-Rad Laboratories).

The above procedure was modified when immunisolated molecules were subjected to digestion with endoglycosidase H (endo H; Boehringer Mannheim, Mannheim, FRG). Class I molecules were eluted from the Affi-Gel beads with 0.14 ml of 10 mM Tris, pH 7.4, 1% SDS, precipitated with acetone, and resuspended in 0.1 M sodium citrate, pH 6, containing 0.075% SDS and 0.2% β -mercaptoethanol. The β -mercaptoethanol was omitted from digests of class I molecules isolated from DSP-treated cells. After the addition of aprotinin (1%), the solutions were typically divided in half, one half receiving endo H (0.05 mU/ml) and the other serving as an undigested control. Digestions were carried out for 6 h at 30°C and were terminated by the addition of Laemmli sample buffer with or without the reducing agent.

For the isolation of the crosslinked, 145-kD class I-containing species, class I molecules were immunisolated from DSP-treated cell lysates and subjected to SDS-PAGE under nonreducing conditions. After electrophoresis, the gels were dried immediately without fixation and analyzed by autoradiography. The 145-kD (crosslinked class I p88) species was excised from the dried gel and eluted from the gel sections by shaking for 24 h at 22°C in 100–200 μ l PBS, pH 7.4, containing 1% SDS with occasional boiling. Eluates were precipitated with acetone, treated with or without endo H, and the DSP crosslinks cleaved before reanalysis by reducing SDS-PAGE as described above.

Gel Permeation HPLC Chromatography of Radiolabeled Cell Lysates. Preparation of cell lysates for injection onto a TSK 3000SW gel filtration column (7.5 × 600 mm; Toyo Soda Co., Japan) was performed exactly as described previously (28). The column was equilibrated in 50 mM Na₂SO₄, 20 mM NaH₂PO₄, pH 7, 0.15 M NaCl, 0.6% CHAPS, and 0.05% NaN₃. An HPLC system (Gilson, Middleton, WI) was used to run the column, and fractions (1 ml) were collected. Class I molecules were immunoprecipitated from each fraction and examined by reducing SDS-PAGE.

Results and Discussion

In β_2 -microglobulin-deficient murine R1E cells that are transfected with class I H-2K^b or H-2D^b genes, transport from the ER to the Golgi apparatus of class I H chains is completely (K^b) or extensively (D^b) inhibited (17, 18). To assess whether p88 interacts with free H chains, these cells were subjected to pulse-chase radiolabeling coupled with chemical crosslinking. Class I H chains were immunoprecipitated and subjected to nonreducing SDS-PAGE analysis. In Fig. 1, *a* and *b*, a prominent 145-kD species was observed in both cell lines upon crosslinking (compare -DSP to +DSP lanes). A similar crosslinked species was observed previously in β_2 m-containing murine tumor cells (28), as well as in freshly isolated murine splenocytes (our unpublished observations), and we showed that it consisted of p88 crosslinked to class I molecules. Evidence that the 145-kD species in Fig. 1 represents class I H chains complexed with p88 is presented for R1E-K^b cells later (see Fig. 3 *d*). These data demonstrate that the association of p88 with class I H chains is independent of β_2 m.

The fate of the H chain-p88 complex in R1E-K^b and R1E-D^b cells differed from that in β_2 m-containing cells. Whereas this complex is transient in β_2 m-containing cells ($t_{1/2}$ ~20 min for K^b, 45 min for D^b; reference 28), it could be detected throughout the 160-min chase period in both β_2 m-deficient cell lines (Fig. 1, *a* and *b*). Neither the uncrosslinked class I H chains nor those crosslinked to p88 acquired resistance to digestion with endo H. This enzyme cleaves the immature, high mannose-type N-linked oligosaccharides present on newly synthesized glycoproteins. It does not digest oligosaccharides after their processing to complex-type structures in the medial Golgi apparatus (17, 18, 28). Consequently, these data confirm the fact that in the absence of β_2 m, most H chains are not transported as far as the medial Golgi apparatus. A relatively rapid decrease in radiolabeled class I H chains during the chase period occurred, probably related to the ER degradation detected by Klausner and others (36). Despite this degradation, densitometric analyses of these gels (Fig. 1 *c*) revealed that the percentage of radiolabeled K^b (~50%) or D^b (~25%) H chains that could be crosslinked to p88 remained constant throughout their lifetime. These percentages are accurate since no radioactivity is contributed by p88, which is difficult to radiolabel with a short pulse (28).

To assess the extent of H chain-p88 complex formation more quantitatively, lysates of the β_2 m-deficient cells were subjected to gel permeation HPLC. For comparison, the be-

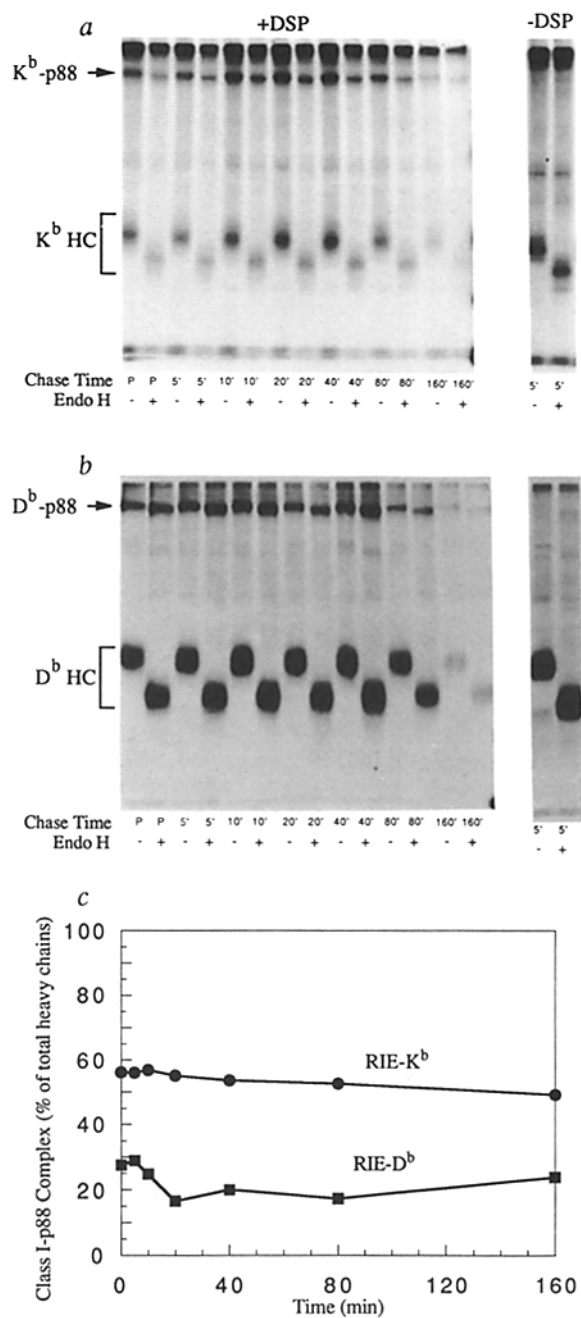


Figure 1. Kinetics of p88 interaction with class I H chains in murine β_2 m-deficient R1E cells. R1E cells transfected with either the K^b or D^b gene (R1E-K^b or R1E-D^b) were pulsed (lane P) for 10 min with [³⁵S]Met and chased in the presence of excess Met. At the indicated intervals, two equivalent aliquots from each cell population were removed and lysed in the presence or absence of DSP. The K^b (*a*) or D^b (*b*) H chains were subsequently immunoprecipitated from the appropriate cell lysates using either the anti-peptide 8 serum (anti-K^b) or the α_3 domain-specific 28-14-8s (anti-D^b) mAb, respectively. Immunoprecipitates were divided in half, incubated in the presence or absence of endo H, and analyzed by SDS-PAGE (10% gel) under nonreducing conditions. The mobilities of the 145-kD K^b- or D^b-p88 complexes and the H chains (HC) are indicated. For cells lysed in the absence of DSP (-DSP), only the results of the 5-min chase time are presented for comparison to crosslinked (+DSP) samples. (*c*) The amounts of K^b or D^b H chains crosslinked to p88 were measured as a percentage of total H chains at each time point by densitometric analysis of weakly exposed versions of the fluorograms in *a* and *b*.

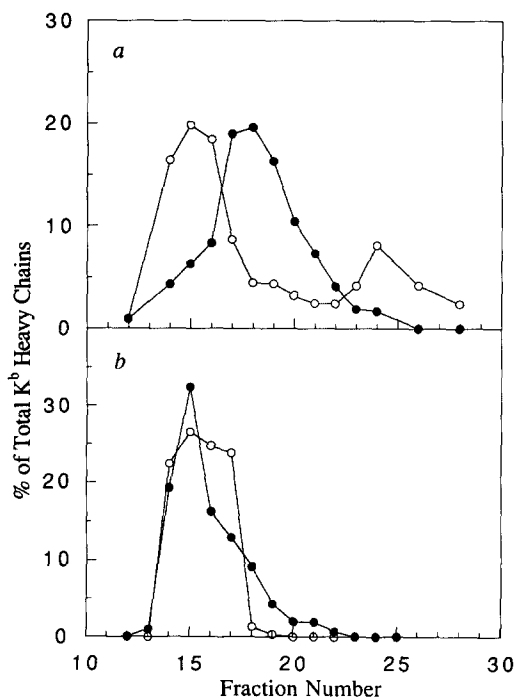


Figure 2. Quantitative assessment of class I-p88 complexes in control versus β_2m -deficient murine cells by gel permeation HPLC. (a) Profiles of K^b molecules present in lysates of EL-4 cells (possessing β_2m). (b) Profiles of K^b H chains present in lysates of R1E- K^b cells (lacking β_2m). Cells were either pulsed for 10 min with [³⁵S]Met (○) or pulsed and then chased for 2 h (●). Lysates were prepared and injected onto a TSK 3000SW gel filtration HPLC column (see Materials and Methods). The K^b molecules were immunoprecipitated from collected fractions (1 ml) using anti-peptide 8 serum and subsequently examined by reducing SDS-PAGE. The relative amount of K^b H chains in each fraction was determined by densitometry and plotted as a percentage of total K^b H chains recovered. A small amount of K^b H chains present in the pulse-labeled EL-4 lysate (a) was detected as a more included peak (fractions 23–26). The basis for the chromatographic behavior of this minor species is unclear. It was not detected in a pulse-labeled R1E- K^b lysate (b) and was virtually absent in similar analyses of other class I molecules in β_2m -containing cells (28).

behavior of K^b molecules present in lysates of a β_2m -containing tumor cell, EL-4, was also examined (Fig. 2 a). As observed previously, the majority of K^b molecules eluted from the HPLC column between fractions 13 and 17 after a 10-min pulse labeling. After a 2-h chase, when all K^b molecules have been transported through the Golgi apparatus, they eluted as a smaller species (fractions 16–21). The different elution patterns correspond to K^b molecules either associated or unassociated with p88, respectively (28). The data confirm our previous findings that only untransported, endo H-sensitive class I molecules are associated with p88 (28). In comparison, such an analysis on lysates obtained from R1E- K^b cells revealed that at either time point, the bulk of K^b H chains eluted from the column in association with p88; i.e., as a peak between fractions 13 and 17 (Fig. 2 b). Similar results have been obtained for D^b H chains present in R1E- D^b cells (data not shown). The minor shoulder observed in fractions 18 and 19 in the chase sample (Fig. 2 b) suggested that a small portion of K^b molecules may have dissociated from

p88 either in vivo or during HPLC analysis. Nevertheless, the combined crosslinking and gel filtration data indicate that p88 remains stably associated with class I H chains in the absence of β_2m . Thus, β_2m association appears to be a critical event leading to both the dissociation of p88 and the intracellular transport of class I molecules.

We also asked whether a molecule like p88 interacts with class I H chains in human cells by examining the β_2m -deficient human cell line, (Daudi (12, 14–16)). As shown in Fig. 3 a, upon crosslinking, a 145-kD species was detected throughout the lifetime of the human class I H chains. Both the 145-kD species and the class I H chains remained sensitive to endo H digestion, reflecting their inability to reach the medial Golgi apparatus. Densitometric analysis (Fig. 3 b) revealed that the percentage of radiolabeled class I H chains (~10%) found in the 145-kD species remained constant. Given that chemical crosslinking is an inefficient process, the remarkable reproducibility in the amount of class I H chains detected in the 145-kD species suggests that, without β_2m present, human H chains, like murine H chains, are stably associated with another molecule(s) throughout their lifetime.

Characterization of the 145-kD species was subsequently performed. Daudi cells were radiolabeled for 24 h before lysis in the absence or presence of DSP and subsequent isolation of class I molecules. Again, upon crosslinking, a 145-kD species could be observed by nonreducing SDS-PAGE analysis (Fig. 3 c, lanes 1 and 2). However, upon reduction to cleave the DSP crosslink, radioactive 90- and 78-kD proteins were unique to the DSP-treated sample (Fig. 3 c, lanes 3 and 4). These same two molecules, along with class I H chains, could be detected upon isolation of the 145-kD species and subsequent cleavage of the crosslinks by reduction (Fig. 3 d, lanes 3 and 4). By comparison, a similar treatment of the 145-kD crosslinked species (K^b -p88) isolated from murine R1E- K^b cells radiolabeled for 6 h revealed the presence of only p88 and K^b H chains (Fig. 3 d, lanes 1 and 2). In each case, the class I H chains were sensitive to endo H digestion, whereas p88 as well as the 90- and 78-kD molecules were not, suggesting that, like p88, these molecules do not possess N-linked oligosaccharides. We have also established that p88 is resistant to digestion with peptide: N-glycosidase F, an enzyme that cleaves all forms of N-linked oligosaccharides from glycoproteins (37), further confirming that it is not N-glycosylated (data not shown).

The similar molecular mass and insensitivity to endo H digestion suggest that the 90-kD species is the human equivalent of murine p88. Indeed, each of these molecules can be coimmunoprecipitated with their respective class I H chains in the absence of crosslinking when cells (β_2m deficient or not) are solubilized in CHAPS detergent (data not shown). The size of the 78-kD protein and its insensitivity to endo H digestion suggest that it may be the resident ER chaperone protein, BiP, which has been implicated in ER retention as well as in the folding and assembly of other proteins (5–11). This was confirmed by demonstrating that the 78-kD protein comigrates precisely with an authentic BiP standard and also by coimmunoprecipitation of free class I H chains with

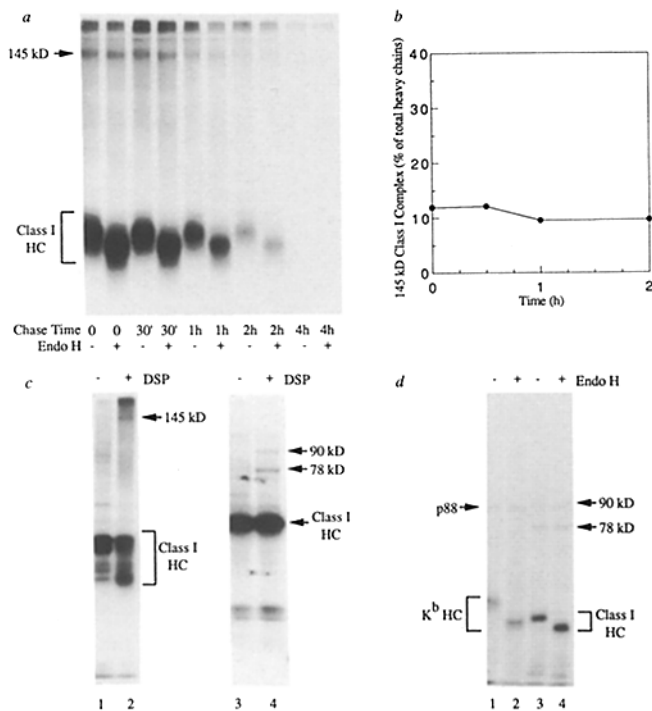


Figure 3. Association of a human p88 homologue with class I H chains in β_2m -deficient Daudi cells. (a) Detection of a crosslinked, class I-containing, 145-kD species in lysates of Daudi cells during a pulse-chase experiment coupled with chemical crosslinking. The radiolabeling, crosslinking and nonreducing SDS-PAGE analysis was performed as in Fig. 1. Anti-HLA-A, B, C H chain serum was used to immunoprecipitate the class I H chains. The mobilities of the 145-kD species and the H chains (HC) are indicated; the gel front is omitted. (b) Quantitation of the crosslinked 145-kD species by densitometric analysis. (c) Identification of molecules crosslinked to class I H chains. Steady-state labeling of Daudi cells for 24 h with [35 S]Met followed by a 10-min pulse was performed. Two small aliquots of cells were then lysed in the absence (-) or presence (+) of DSP, and the class I H chains were immunoprecipitated and analyzed by SDS-PAGE under nonreducing (lanes 1 and 2) or reducing conditions (lanes 3 and 4); 10%, and 10–17.5% gels were used, respectively. (d) The remaining Daudi cells were lysed in the presence of DSP and the crosslinked 145-kD species isolated (see Materials and Methods). After endo H digestion of half the sample, crosslinks were cleaved by reduction and the constituents of the 145-kD species analyzed (7.5% gel) by reducing SDS-PAGE (lanes 3 and 4). The crosslinked 145-kD K^b -p88 species was isolated from murine R1E- K^b cells and analyzed in a similar manner (lanes 1 and 2). For these cells, radiolabeling was performed for 6 h; labeling beyond 6 h resulted in reduced cell viability. In c, lane 4, and in d, lanes 3 and 4, a 78- and a 90-kD species are crosslinked to class I H chains in Daudi cells, whereas only p88 is crosslinked to K^b H chains in R1E- K^b cells (d, lanes 1 and 2).

BiP using an anti-BiP mAb (kindly provided by Dr. L. Hendershot, St. Jude Children's Research Hospital, Memphis, TN; data not shown). Interestingly, in Daudi cells, free class I H chains were nearly quantitatively precipitated in association with BiP, whereas in murine R1E cells, only trace amounts of free H chains could be coimmunoprecipitated. This suggests that in human cells chaperone interaction with free class I H chains is shared by both BiP and the putative p88 homologue, whereas in murine cells BiP appears to play only a minor role.

The results obtained from these β_2m -deficient cells indicate that β_2m binding is a necessary step leading to p88 dissociation and class I intracellular transport. Yet, the rapid kinetics of β_2m binding ($t_{1/2} \sim 2$ –5 min) as measured in murine (28) or human (13, 14) cells suggests that β_2m binding does not trigger p88 dissociation ($t_{1/2} = 20$ –45 min). Thus, an additional event likely influences dissociation, possibly the binding of peptide ligand. Studies with both human (174, T2) and murine (RMA-S) mutant cells, defective in the ability to create class I-antigenic peptide target structures endogenously (21–25), suggest that a link between peptide binding and p88 dissociation may exist. These cells exhibit impaired ER to Golgi transport of class I molecules, which, because of the relationship between p88 dissociation and class I transport, may be due to an impairment in p88 dissociation.

To test this possibility, we subjected RMA-S cells and parental RMA cells to pulse-chase radiolabeling coupled with chemical crosslinking. After a 10-min pulse with [35 S]Met, K^b molecules (Fig. 4, a and b) could be detected in association with p88 upon crosslinking in both cell types. Consequently, it appears that p88 association is independent of the presence of peptide ligands. Also specific to DSP-treated lanes were radioactive bands of ~ 60 , ~ 90 , and ~ 115 kD. The 60- and 90-kD species have been shown to consist of H chain crosslinked to β_2m and H chain dimers, respectively (28). Based on the molecular mass, the 115-kD species probably represents crosslinked dimers of H chain- β_2m molecules. We have detected the 60-kD species in varying amounts in every β_2m -containing cell line tested. However, the 90- and 115-kD species have only been detected at low levels, if at all. Thus, it is unlikely that these latter two species contribute substantially to the biogenesis of class I molecules.

We next focused our attention on the fate of the crosslinked K^b -p88 complexes. Whereas K^b -p88 complexes disappeared in parental cells by 30 min to 1 h of chase (Fig. 4, a and e), complexes were detected even after a 2–4-h chase period in mutant cells (Fig. 4, b and f; detection at 4 h required longer exposure). Assessment of the kinetics of ER to Golgi transport of the K^b molecules in these cells in the absence of crosslinking was also performed (Fig. 4, c and d). In RMA cells, K^b molecules became quantitatively resistant to digestion with endo H by 1 h of chase, whereas a large proportion of K^b molecules in RMA-S cells were still sensitive to digestion even after a 4-h chase period. We showed previously that the crosslinking technique, although inefficient, could be used reliably to estimate rates of p88 dissociation (28). Comparison of the rates of p88 dissociation obtained in this way with K^b transport revealed that a close kinetic relationship existed between these events in both cell types (Fig. 4, e and f). Dissociation of p88 from K^b molecules occurred rapidly in RMA cells ($t_{1/2} \sim 15$ min) slightly preceding the arrival of the K^b molecules in the medial Golgi apparatus ($t_{1/2} \sim 21$ min). In RMA-S cells, the slower rate of p88 dissociation closely resembled the slower ER to Golgi transport of the K^b molecules, both processes proceeding to $\sim 40\%$ completion by 2 h and apparently ceasing thereafter (Fig. 4, solid lines). This apparent cessation is misleading be-

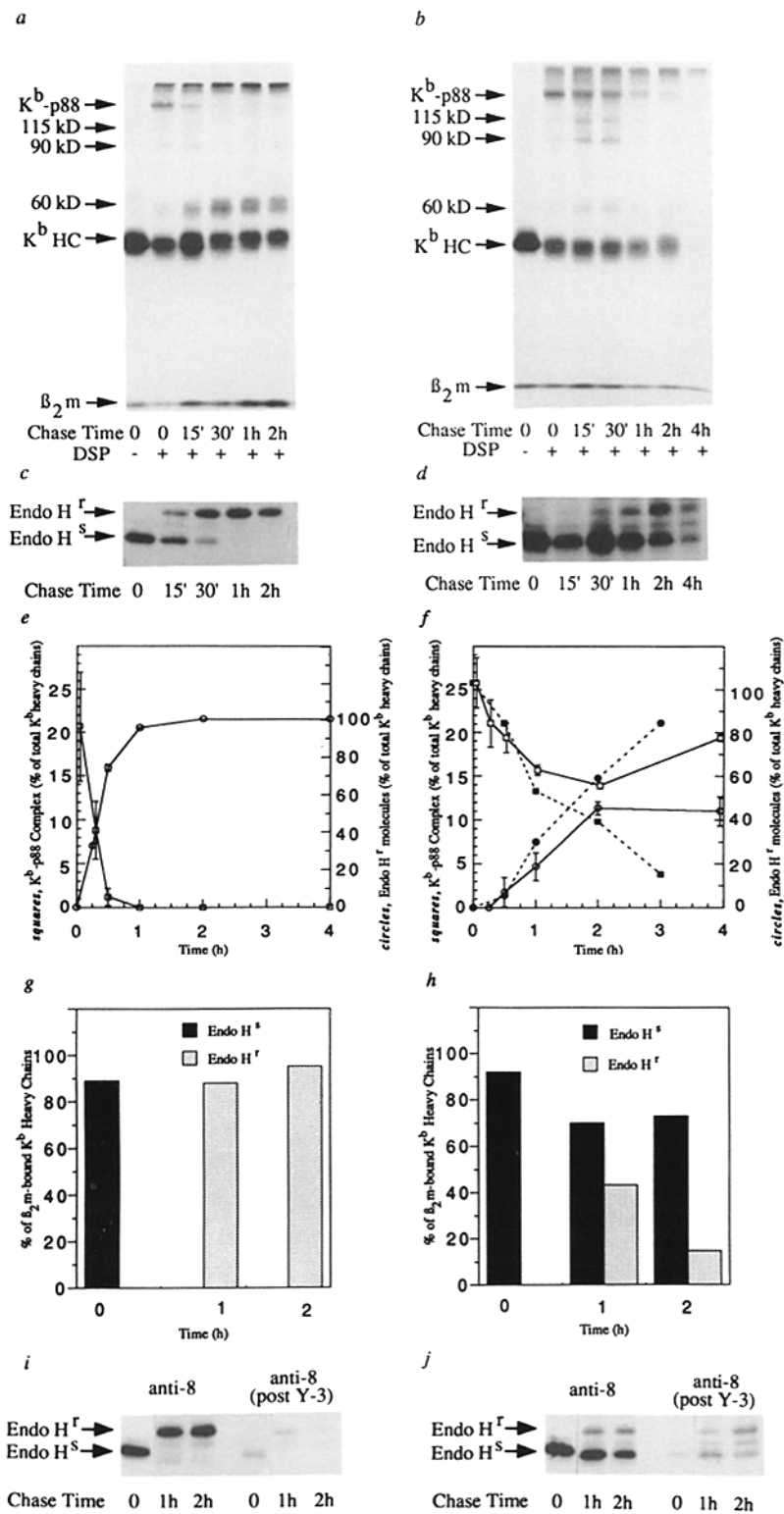


Figure 4. Fate of K^b -p88 complexes and K^b - β_2m heterodimers in murine RMA versus RMA-S cells. (*a-d*) Pulse-chase experiments were carried out on RMA (*a* and *c*) or RMA-S (*b* and *d*) cells as described in Fig. 1. Equivalent aliquots of cells were lysed in the presence (*a* and *b*) or absence (*c* and *d*) of DSP at indicated intervals and immunoprecipitated K^b molecules were analyzed by SDS-PAGE (10% gel) under nonreducing (*a* and *b*) or reducing (*c* and *d*) conditions. Under these conditions, β_2m ran with the gel front. In *c* and *d*, immunoprecipitates were subjected to endo H digestion before SDS-PAGE analysis. Shown are the endo H-resistant (endo H^r) or -sensitive (endo H^s) K^b H chains. (*e* and *f*) The kinetics of K^b -p88 complex disappearance (squares) and ER to Golgi transport of K^b molecules (circles) in RMA (*e*) and RMA-S (*f*) cells were assessed by densitometric scanning of weakly exposed fluorograms similar to those shown in *a-d*. Note that to detect the K^b -p88 complex in *b* at 4 h, a longer fluorographic exposure was used. The amount of K^b H chain in the complex or resistant to endo H digestion is expressed as a percentage of the total K^b H chain present in a gel lane. To facilitate a direct comparison of the half-times for the two processes, the maximum amount of cross-linked K^b -p88 complex obtained is set to correspond to 100% on the scale of percentage endo H resistance. Error bars represent the range obtained in duplicate experiments. In addition, values were obtained in which RMA-S cells (*f*) were preincubated (24 h) and then subjected to the above experiments in the presence of 50 μ M influenza nucleoprotein peptide NP345-360 (dashed lines). These data are representative of several independent experiments. (*g-j*) Fate of K^b - β_2m heterodimers in RMA (*g* and *i*) and RMA-S (*h* and *j*) cells. Cells were subjected to a pulse-chase experiment as above. Lysates were prepared at 0 h (pulse), 1 h, and 2 h after initiation of the chase period and divided into two equal aliquots. One of the aliquots was treated directly with the anti-peptide 8 serum to immunoprecipitate total K^b molecules. The other aliquot was first subjected to two rounds of immunoprecipitation with the mAb Y-3, which only recognizes K^b molecules bound to β_2m . The remaining K^b molecules were subsequently recovered using the anti-peptide 8 serum. The anti-peptide 8 immunoprecipitates were then digested with endo H, analyzed by reducing SDS-PAGE, and the relative amount of K^b H chains found in each immunoprecipitate either in an endo H-sensitive or -resistant form was assessed by densitometry (*i* and *j*). The amounts of endo H-sensitive or -resistant K^b H chains that were bound to β_2m (calculated from the difference between the two immunoprecipitates) are expressed as a percentage of the total endo H-sensitive or -resistant H chains at a given chase time (*g* and *h*). The data shown are representative of two independent experiments.

cause substantial degradation of endo H-resistant molecules occurred at later chase times and was not considered in calculating p88 dissociation or K^b transport. These molecules could be selectively stabilized by conducting the chase in the presence of the K^b -restricted influenza nucleoprotein peptide, NP345-360 (21). In the presence of NP345-360 (Fig. 4 *f*,

dashed lines), the transport rate did not change substantially, but by 3 h of chase, both endo H resistance and p88 dissociation proceeded to >80% completion ($t_{1/2}$ for both processes between 70 and 100 min). No stabilization of K^b molecules was observed when cells were incubated with the D^b -restricted peptide, NP366-379 (not shown). In similar experiments,

we also observed prolonged association of p88 with the D^b molecule in RMA-S cells; although like K^b, a substantial amount of the D^b molecules dissociated from p88 by 4 h of chase, were transported, and were largely degraded (data not shown). These data infer that peptide ligands play an intimate role in efficient p88 dissociation and intracellular transport of class I molecules.

The question arises from these findings as to whether peptide binding constitutes a separate event in addition to β_2m binding that is required for efficient p88 dissociation, or whether peptide binding is simply required for stable H chain- β_2m association and some subsequent event triggers p88 dissociation. Thus, it is important to determine if p88 is interacting only with free H chains in RMA-S cells or with empty H chain- β_2m heterodimers. Recent work has demonstrated that substantial β_2m binding to D^b H chains occurs intracellularly in RMA-S cells as detected by immunoprecipitation (38). To determine whether similar results can be obtained with K^b molecules, we measured β_2m binding during a pulse-chase experiment. In this experiment, steps that may affect the stability of β_2m -bound molecules or may add exogenous β_2m or peptides to prepared lysates, such as freezing, addition of skim milk, preclearance with fixed *S. aureus* cells, and washing with buffers containing elevated salt concentrations or BSA, were avoided.

The results of our efforts are summarized in Fig. 4 *g-j*. The relative amount of β_2m -bound K^b H chains was quantified by calculating the difference between the K^b H chains immunoprecipitated with a β_2m -independent antibody from two identical lysates, one of which was first precleared of β_2m -bound molecules. After a 10-min pulse-labeling [³⁵S]Met (0 time), comparable levels of radioactive, β_2m -bound K^b molecules were detected in both RMA and RMA-S cells, 89% and 92%, respectively. Consequently, virtually all K^b H chains bind β_2m rapidly after synthesis, whether peptides are present or not. In RMA cells, these K^b molecules remained stably bound to β_2m over the course of a 2-h chase period (Fig. 4, *g* and *i*), during which time they were quantitatively transported from the ER through the Golgi apparatus (became endo H resistant). In contrast, whereas those K^b molecules in RMA-S cells that were not transported through the Golgi apparatus (endo H sensitive) also remained largely β_2m bound (>70% and as high as 90% in some experiments), those K^b molecules that were transported lost β_2m rapidly (only 43% and 15% were associated with β_2m after 1 and 2 h of chase, respectively; Fig. 4, *h* and *j*).

These results are in agreement with several studies (25, 39-42), suggesting that transported class I molecules in RMA-S cells are in the form of peptide-free, H chain- β_2m heterodimers that dissociate after exit from the ER (most likely at the cell surface), but as noted above can be stabilized in the presence of appropriate exogenous peptide. In addition, these data indicate that in RMA-S cells the pool of endo H-sensitive K^b molecules that p88 interacts with is composed predominantly of empty H chain- β_2m heterodimers rather than free H chains. Independent confirmation of

prolonged interaction of p88 with empty intracellular K^b molecules was obtained by immunoprecipitating virtually all the crosslinked K^b-p88 complexes at various chase times (to 2 h) with the β_2m -dependent mAb, Y-3 (data not shown). Consequently, efficient dissociation of p88 from class I molecules requires peptide binding as a separate event in addition to association of β_2m with H chain.

Assuming that the peptide deficiency in RMA-S cells is complete, the finding that p88 dissociation and class I transport occurs at all in these cells indicates that the requirement for peptide binding in both processes is not absolute. Similarly, the requirement discussed above for β_2m in p88 dissociation and transport also cannot be considered absolute for all class I molecules. For example, in R1E-D^b cells, a low level of p88 dissociation and subsequent surface expression of free D^b H chains has been observed (17, 18, 43, and our unpublished observations). These events may be a consequence of the demonstrated ability of newly synthesized D^b H chains to bind peptide and fold in the absence of β_2m (38). Similarly, a portion of the closely related L^d molecule apparently can be transported in the absence of β_2m (44).

Our finding that prolonged p88 association occurs with incompletely assembled forms of class I molecules suggests that p88 could play a role in mediating class I folding/subunit assembly. For example, our observation that in RMA-S cells empty H chain- β_2m heterodimers become unstable after dissociation from p88 suggests that p88 may normally function to stabilize this assembly intermediate (see also reference 45). Alternatively, p88 may retain class I molecules intracellularly until folding/assembly is complete. We favor the latter possibility for two reasons. First, formation of H chain- β_2m -peptide complexes can be accomplished in the absence of p88 with purified components *in vitro* (46). Second, the close correlation between p88 dissociation and ER to Golgi transport consistently observed in all of our studies strongly implicates p88 as a retention molecule. Consistent with this idea, we have identified p88 as a resident protein of the ER (47). As a retention molecule, p88 would ensure that the bulk of newly synthesized class I molecules do not reach the cell surface without having first acquired peptide cargo for scrutiny by CTL. Failure of p88 to function in this manner could result in excessive surface expression of incompletely assembled class I molecules that may bind exogenous antigenic peptides under appropriate conditions (21, 24, 25, 40-43) and lead to the needless destruction of otherwise normal cells.

Recently, it was shown that in a mutant murine cell line (CMT) that accumulates unassembled class I molecules within the ER, the free H chains are recycled between the ER and a *cis*-Golgi compartment (48). These findings may be rationalized with our observations either by a small portion of p88 chaperoning these events or by a continuous low-level dissociation of H chains from p88 and subsequent rapid salvage from the Golgi apparatus to reassociate with p88. A detailed examination of the dynamics and localization of class I-p88 interactions in CMT and other cells should determine the role of p88 in such a recycling pathway and whether this pathway is generally applicable.

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