Participation of a Novel 88-kD Protein in the Biogenesis of Murine Class I Histocompatibility Molecules

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Abstract. Chemical cross-linking and gel permeation chromatography were used to examine early events in the biogenesis of class I histocompatibility molecules. We show that newly synthesized class I heavy chains associate rapidly and quantitatively with an 88-kD protein in three murine tumor cell lines. This protein (p88) does not appear to possess Asn-linked glycans and it is not the abundant ER protein, GRP94. The class I–p88 complex exists transiently ($t_{1/2}$ = 20–45 min depending on the specific class I heavy chain) and several lines of evidence suggest that p88 dissociates from the complex while still in the ER. Dissociation is not triggered upon binding of β_2-microglobulin to the heavy chain ($t_{1/2}$ = 2–5 min). However, the rate of dissociation does correlate with the characteristic rate of ER to Golgi transport for the particular class I molecule studied. Consequently, dissociation of p88 may be rate limiting for ER to Golgi transport. Class I molecules bind antigenic peptides, apparently in the ER, for subsequent presentation to cytotoxic T lymphocytes at the cell surface. p88 could promote peptide binding or it may retain class I molecules in the ER during formation of the ternary complex of heavy chain, β_2-microglobulin, and peptide.

CLASS I molecules of the major histocompatibility complex associate with peptides derived from foreign proteins resulting in a cell surface complex recognizable by cytotoxic T cells (reviewed in Townsend and Bodmer, 1989). Several studies have focused on the pathway leading to the generation of antigenic peptides (antigen processing) and their subsequent presentation in the context of class I molecules (reviewed in Germain, 1986; Long and Jacobson, 1989). Antigenic peptide–class I molecular complexes are not formed if exogenous foreign proteins are internalized by endocytosis, but are formed efficiently if foreign proteins are synthesized endogenously by cells (e.g., viral infection). Delivery of foreign proteins directly into the cytoplasm of cells by various in vitro manipulations also creates these complexes (Yewdell et al., 1988*a*; Moore et al., 1988). This latter finding, taken together with the fact that endogenous viral proteins either normally synthesized in the cytosol or deliberately misrouted to effect cytosolic localization provide a good source of antigenic peptides (Townsend et al., 1986), implicates the cytosol as a major site for the production of peptides that can associate with class I molecules.

A major topological problem arises concerning how and where in the cell processed cytoplasmic peptides cross a lipid bilayer to associate with class I molecules. Studies using brefeldin A (BFA),[1] a fungal metabolite that blocks protein transit from the ER to the Golgi apparatus, showed that class I-restricted antigen presentation could be inhibited, thereby suggesting that antigenic peptides associate with newly synthesized class I molecules en route to the cell surface (Nuchtern et al., 1989; Yewdell and Bennink, 1989). Clearly, a detailed understanding of the various stages in the biogenesis and intracellular transport of class I molecules is necessary in order to characterize biosynthetic intermediates that could potentially bind antigenic peptides as well as define possible sites where the interaction may occur.

Plasma membrane and secretory proteins are synthesized on membrane-bound polysomes of the ER and subsequently move via small transport vesicles through the Golgi apparatus to the cell surface (Palade, 1975). Transport along this exocytotic pathway occurs with distinct rates and efficiencies for different proteins. In most instances, export from the ER has been identified as the rate-limiting step (reviewed in Pfeffer and Rothman, 1987; Lodish, 1988; Rose and Doms, 1988). Studies on cellular and viral surface glycoproteins have indicated that, in many cases, ER to Golgi transport depends on the acquisition of correct tertiary or quaternary structure. For example, it appears that influenza hemagglutinin (HA) and vesicular stomatitis virus G protein must form properly folded trimers before their exit from the ER (Gething et al., 1986; Doms et al., 1987; Copeland et al., 1986, 1988). Mutant forms of these glycoproteins that fail to trimerize or that trimerize but still exhibit conformational alterations are not transported beyond the ER (Gething et al., 1986; Doms et al., 1988). Numerous additional examples exist of misfolded or unassembled membrane and secretory

1. *Abbreviations used in this paper*: BFA, brefeldin A; β_2m, β_2-microglobulin; DSP, dithiobis(succinimidyl propionate); HA, influenza hemagglutinin; Met, methionine.

proteins that accumulate intracellularly (Yu et al., 1983; Minami et al., 1987; Williams et al., 1988, 1989).

These observations could be explained if proper folding and assembly are required to create a transport signal that dictates export from the ER. However, such a mechanism has received little direct support (reviewed in Pfeffer and Rothman, 1987; Lodish, 1988; Rose and Doms, 1988). Alternatively, evidence has been presented suggesting that transport along the exocytotic pathway may be a passive process requiring only that a protein reach the site of vesicle budding (Wiedmann et al., 1984; Pfeffer and Rothman, 1987; Hiebert and Lamb, 1988). In such a passive process, the question arises as to how improperly folded proteins or unassembled subunits are discriminated and retained in the ER. In several instances retention has been correlated to an association with BiP (GRP 78), a resident ER protein (Gething et al., 1986; Hendershot et al., 1987; Dorner et al., 1987; Hurtley et al., 1989; Machamer et al., 1990). Retention may also be effected by inclusion in aggregates that are incapable of being transported (Doms et al., 1987, 1988; Machamer and Rose, 1988). Transient retention by either mechanism during folding and assembly may contribute to the distinct transport rates observed for various proteins.

Class I molecules are heterodimers consisting of a highly polymorphic, transmembrane heavy chain (45–50 kD) associated noncovalently with β_2-microglobulin (β_2m; 12 kD). These subunits associate rapidly after translation (Krangel et al., 1979; Owen et al., 1980) and, like other oligomeric proteins, correct assembly is required for efficient intracellular transport (Ploegh et al., 1979; Sege et al., 1981; Allen et al., 1986; Williams et al., 1989). We have shown previously that different class I molecules synthesized within the same cell and sharing up to 80% sequence identity are exported from the ER at distinctly different rates (Williams et al., 1985). Surprisingly, the differential transport kinetics could not be attributed to differences in rates of β_2m association. These findings prompted us to reexamine the biogenesis of class I molecules in an effort to uncover additional events in folding or assembly that might explain these observations. In this report, we show that newly synthesized class I heavy chains associate rapidly and quantitatively with an 88-kD protein (p88) to form a complex that appears to represent a key intermediate in the assembly and subsequent intracellular transport of class I molecules. The role that p88 may play in the immunological function of class I molecules is discussed.

Materials and Methods

Cells and Antibodies

Three murine cell lines were used in this study: MDAY-D2 (subclone KD2.3, a DBA/2 H-2^d anaplastic tumor of lymphoreticular origin; Lagarde et al., 1984; Degen et al., 1989), EL-4 (a C57B1 H-2^b T-cell lymphoma; Gorer, 1950), and RDM-4 (an AKR/J H-2^k T-cell lymphoma; Herrmann and Mescher, 1979). Cells were maintained at 37°C in a 5% CO_2/air atmosphere in RPMI1640 containing 10% fetal bovine serum.

A rabbit antiserum, anti-peptide 8, specific for the COOH-terminal portion of the cytoplasmic tail of H-2K locus heavy chains was used to isolate the K^d, K^b, and K^k molecules (Smith et al., 1986). Anti-peptide 8 was raised against the peptide encoded by exon 8 of the K^b gene and was generously provided by Dr. B. Barber (University of Toronto, Toronto). The murine monoclonal antibody, 28-14-8s, immobilized on Affi-Gel 10 (Bio-Rad Laboratories, Richmond, CA) was used for the isolation of the D^b and L^d molecules (Ozato et al., 1980). Both reagents recognize the appropriate heavy chains either alone or in association with β_2m (Allen et al., 1986; Williams et al., 1989).

The following murine monoclonal antibodies recognize the indicated class I heavy chains only when associated with β_2m. For K^b, the B8-24-3, Y-3, EH-144, 20-8-4s, and 5Fl.2.14 antibodies were used (for references see Williams et al., 1989). The 20-8-4s antibody (anti-K^b, K^d, D^b) was also used to isolate K^d. For D^b, the B22-249.R1 antibody was used (Hammerling et al., 1982; Allen et al., 1986) and L^d was isolated with the 30-5-7s antibody (Ozato et al., 1980; Evans et al., 1982), a gift from Dr. M. Edidin (Johns Hopkins University, Baltimore, MD).

Rabbit antiserum directed against murine β_2m (designated Y-9) was produced in the laboratory of Dr. Howard Grey (National Jewish Center for Immunology and Respiratory Medicine, Denver, CO) and was provided by Dr. B. Barber. Rabbit antiserum used to isolate GRP94 was generated against the NH_2-terminal 16 amino acids of murine GRP94 and was a gift from Dr. M. Green (St. Louis University Medical Center, St. Louis, MO). Anti-HA monoclonal antibodies (designated Y8-10C2 and H17-L2) were generously provided by Dr. J. W. Yewdell (National Institute of Allergy and Immunological Diseases, Bethesda, MD) and have been described previously (Yewdell et al., 1988*b*; Gerhard et al., 1981).

Metabolic Radiolabeling and Cross-Linking of Class I Molecules

Pulse-chase experiments were performed essentially as described by Williams et al. (1985, 1988). Briefly, after cells were washed and preincubated at 37°C for 30 min in Met-free RPMI1640, they were pulsed for 5 min in this medium containing 0.5 mCi/ml [^{35}S]methionine ([^{35}S]Met; Amersham Corp., Arlington Heights, IL). The chase was initiated by the addition of at least 9 volumes of complete RPMI1640 containing 1 mM Met and 10% fetal bovine serum. At various times, aliquots of 2–4 × 10^6 cells were chilled in dry ice for 10–15 s, washed twice 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 0.5% NP-40, 10 mM iodoacetamide, and 0.23 mM PMSF (lysis buffer) either in the presence or absence of 0.1 mM dithiobis(succinimidyl propionate) (DSP; Pierce Chemical Co., Rockford, IL). This thiol-cleavable cross-linking agent was freshly prepared as a 2 mM stock in lysis buffer and 25 μl was added simultaneously with 475 μl of lysis buffer to the cells at room temperature. After vigorous vortexing, lysis and cross-linking were allowed to proceed on ice for 30 min. Cross-linking was terminated by the addition of 50 μl of 50 mM glycine, 10% aprotinin in lysis buffer. After 10 min on ice, lysates were centrifuged at 12,000 *g* for 15 min, and the post-nuclear supernatants were precleared with fixed *Staphylococcus aureus* cells, as described previously (Williams et al., 1988).

Isolation of Class I Molecules

All procedures were performed at 4°C as described previously (Williams et al., 1988). Precleared lysates were adjusted to 5% (wt/vol) skim milk powder. For the isolation of D^b and L^d, 100 μl of a 25% suspension in NTS (0.5% NP-40, 10 mM Tris pH 7.4, 0.15 M NaCl, 1 mM $MgCl_2$) of immobilized 28-14-8s antibody was added to the lysates and the mixtures were shaken for 3 h on an orbital shaker. For the isolation of H-2K molecules (K^d, K^b, K^k), lysates were incubated for 2 h with 2 μl of anti-peptide 8 serum and immune complexes were isolated by shaking for 1 h with 90 μl of a 33% suspension (in NTS) of Affi-Gel Protein A (Bio-Rad Laboratories). In all cases, the agarose beads were washed extensively and class I molecules were eluted by heating at 60–65°C in 50 μl of 10 mM Tris pH 7.4, 1% SDS. The immunoisolated molecules were subjected to SDS-PAGE analyses under nonreducing conditions using the protocol of Laemmli (1970) with the exception that the concentration of Tris in the stacking and separating gels was halved. For cleavage of DSP cross-links 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 overnight at room temperature, and reboiled before its application onto the gel. Under these conditions, cross-links were completely cleaved. After electrophoresis, gels were fixed in 10% trichloroacetic acid, 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 (model 620; Bio-Rad Laboratories).

The above procedure was modified when immunoisolated molecules were subjected to digestion with endo H (ICN Biomedicals, Inc., Irvine, CA). Class I molecules were eluted from the agarose beads with 0.14 ml

of Tris/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, since the cross-linking agent is thiol cleavable. After the addition of aprotinin (1%), the solutions were divided into two equal aliquots, one of which received 1.8 mU endo H and the other served as an undigested control. Digestions were carried out for 6 h at 30°C and were terminated by the addition of SDS-PAGE sample buffer with or without the reducing agent, DTT. Periodically, ribonuclease B (10 μg) was added as an internal control to assess the completeness of endo H digestion (data not shown).

Detection of p88 Cross-Linked to Class I Molecules

Steady-state radiolabeling and radioiodination were used to detect the association of p88 with class I molecules. In steady-state labeling experiments, MDAY-D2 or EL4 cells were washed and preincubated in Met-free RPMI1640, and then incubated for 24 h in labeling medium that consisted of RPMI1640 mixed with Met-free RPMI1640 (1:5) and supplemented with 2% fetal bovine serum and 0.1 mCi/ml [^{35}S]Met. Subsequently, these cells were washed twice in ice-cold Met-free RPMI1640, pulse labeled for 10 min to increase the radioactive signal of newly synthesized class I molecules, and subjected to cross-linking as previously described. Cross-linked K^d or K^b complexes were immunoisolated using anti-peptide 8 and subjected to SDS-PAGE under nonreducing conditions. After electrophoresis, the gels were dried immediately without fixation and analyzed by autoradiography. Class I-p88 complexes, cross-linked heavy chain-β_2m heterodimers, and uncross-linked heavy chains were excised from the dried gel and eluted from the gel sections by shaking for 24 h at 22°C in 100–200 μl SDS-PAGE sample buffer without reducing agent. Subsequently, cleavage of the cross-links was performed as described above and the samples reanalyzed by reducing SDS-PAGE.

In a similar fashion, class I-p88 complexes and class I heavy chains were isolated for radioiodination. These species were initially isolated from DSP-treated extracts of pulse-labeled (10 min) MDAY-D2 cells using an immobilized monoclonal antibody, 20-8-4s, specific for K^d heavy chains associated with β_2m. This antibody was used rather than anti-peptide 8 serum because it was covalently bound to Affi-Gel and did not possess the anti-albumin component of the serum (Smith et al., 1986). These properties should reduce the amount of unlabeled Ig and oligomeric albumin that migrate in nonreducing gels near the 145-kD class I-p88 complex and that would be radioiodinated after excision and elution of the 145-kD region of the gel. To control for the presence of these proteins, a gel section of the 145-kD region from an uncross-linked sample was included in this procedure. Elution of the appropriate gel sections was done by shaking for 24 h at 22°C in 100–200 μl PBS, pH 7.4, containing 1% SDS.

Radioiodination was effected by the addition of 0.5 mCi carrier-free $Na^{125}I$ and adjusting each sample to 1 mM iodoacetamide and 1 mM chloramine T. After 2 min, the labeling was stopped by the addition of excess sodium metabisulfite (15 mM). To purify the radioiodinated K^d complexes, 1–1.5 ml of PBS, pH 7.4, containing 1% NP-40 was added to each sample and the K^d molecules were immunoprecipitated using anti-peptide 8. The purified molecules were subjected to SDS-PAGE under nonreducing conditions and the appropriate bands were excised, the proteins eluted, and subsequently reanalyzed by SDS-PAGE under nonreducing and reducing conditions as described above.

Gel Permeation HPLC Chromatography of Radiolabeled Cell Lysates

Cells that had been pulsed for 5 min with [^{35}S]Met or pulsed and then chased for various periods were lysed in 0.5 ml of 50 mM Na_2SO_4, 20 mM NaH_2PO_4, pH 7, 0.15 M NaCl, 0.6% CHAPS, and 0.05% NaN_3. After centrifugation at 15,000 *g*, the supernatant fraction was passed through a 0.45-μm disk filter (Millipore Corp., Bedford, MA) and injected onto a TSK 3000SW gel filtration column (7.5 × 600 mm; Toyo Soda Co., Japan) equilibrated in lysis buffer. A Gilson HPLC system was used to run the column and fractions (0.5 or 1 ml) were collected. Class I molecules were immunoisolated from each fraction and examined by SDS-PAGE (10–15% gradient gel). Dextran blue and β-mercaptoethanol were used as void volume (V_o) and total column volume (V_t) markers, respectively.

Virus Infection and HA Isolation

EL-4 cells (10^7) were washed three times with RPMI1640 medium and then were incubated for 1 h at 37°C in ~0.5 ml of this medium containing 500 hemagglutinating units of infectious A/Puerto Rico/8/34 (PR8) influenza virus. PR8 was generously provided by Dr. J. W. Yewdell (National Institute of Allergy and Immunological Diseases). 20 ml of RPMI1640 supplemented with 10% fetal bovine serum was added, and the incubation continued for 3 h. The infected cells were subsequently radiolabeled with [^{35}S]Met and lysates were prepared for HPLC gel filtration analysis as described above. HA was detected in the column eluate by immunoprecipitation with hybridoma culture supernatants (100 μl) containing monoclonal antibodies specific for HA monomers (Y8-10C2) or HA trimers (H17-L2). Immunoprecipitation was carried essentially as described for class I molecules except analysis by SDS-PAGE was conducted using a 7.5% gel.

Results

Detection of a Novel 88-kD Protein Complexed to Class I Molecules

To investigate events that may influence the ER to Golgi transport of class I molecules (other than the association of heavy chain with β_2m), a chemical cross-linking approach was taken using the thiol-cleavable, homobifunctional reagent, DSP. Initially, mouse tumor cell lines that express allelic forms of class I molecules were pulsed for 5 min with [^{35}S]methionine and then were solubilized (0.5% NP-40) in the absence or presence of 0.1 mM DSP. The concentration of cross-linker was kept low to reduce nonspecific coupling. Unless otherwise specified, the various class I molecules were immunoisolated from detergent lysates of EL4 cells (H-2K^b and D^b), MDAY-D2 cells (H-2K^d and L^d), and RDM-4 cells (H-2K^k) using either anti-peptide 8, a polyclonal antiserum that recognizes the cytoplasmic tail of H-2K locus molecules (i.e., K^b, K^d, and K^k), or the 28-14-8s monoclonal antibody specific for D^b and L^d. Both reagents were capable of immunoprecipitating class I heavy chains either "free" or associated with β_2m.

As shown in Fig. 1, radiolabeled class I heavy chains and β_2m were recovered from cells lysed in the absence or presence of DSP. Some heterogeneity in the heavy chain was apparent for each class I molecule under the non-reducing conditions of the SDS-PAGE analyses (most notable for K^d and K^b) suggesting variability in disulfide bonding. The addition of iodoacetamide did not alter this pattern indicating that disulfide formation or exchange during cell lysis was not responsible for these observations. In pulse-chase experiments (see Fig. 3) the heterogeneity observed in the K^d heavy chains was gradually lost during the chase, whereas, the K^b heavy chain heterogeneity was not. This suggests that for K^d, these transient heavy chain forms may represent folding intermediates but, in the case of K^b, there may be a minor population of heavy chains that retains a distinct conformation. Unique to the DSP-treated samples of all five immunoprecipitated class I molecules was a prominent band having an apparent molecular mass of 145 kD (Fig. 1, **). This same species was observed when DSP (1 mM) was added directly to viable cells before lysis indicating that its appearance was not an artifact induced by the lysis procedure. The 145-kD species was also detected, albeit somewhat less efficiently, using the heterobifunctional cross-linking agents, *N*-succinimidyl (4-azidophenyl)-1,3′-dithiopropionate and *N*-succinimidyl 3-(2-pyridyldithio) propionate, which possess not only a reactive site for primary amino groups as does DSP but also a photoactivatable or a sulfhydryl-specific site, respectively (data not shown). Notably absent from the DSP-

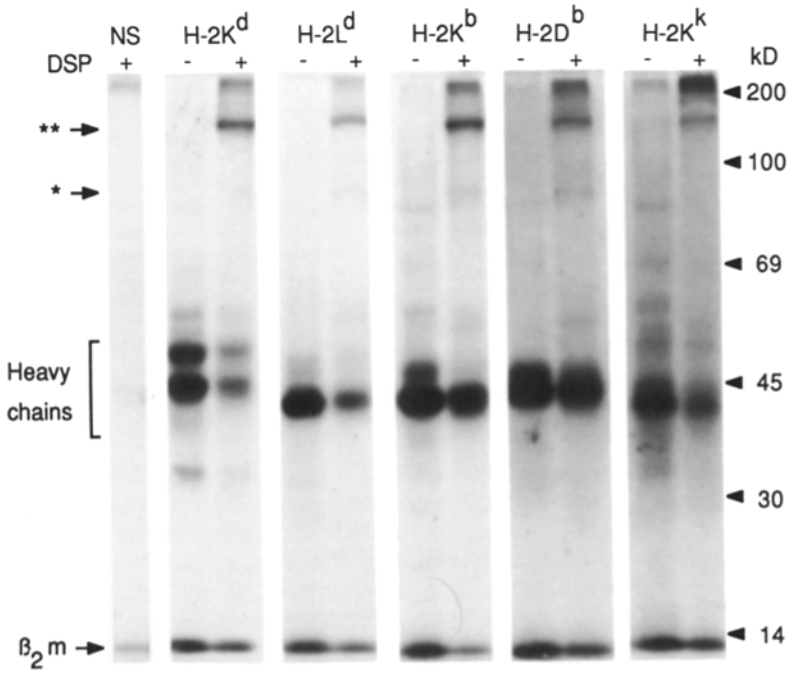


Figure 1. Cross-linking of pulse-labeled class I molecules. Mouse tumor cell lines were radiolabeled with [^{35}S]Met for 5 min and then lysed in the absence or presence of 0.1 mM DSP (see Materials and Methods). Class I molecules were immunoisolated and subjected to nonreducing SDS-PAGE (10% gel). Radiolabeled bands specific to the DSP-treated samples having apparent molecular masses of 145 and 90 kD are indicated by the symbols ** and *, respectively. NS refers to a nonspecific immunoprecipitate in which a parallel sample of DSP-treated EL4 cell lysate was incubated with preimmune rabbit serum. A similar pattern was observed when MDAY-D2 or RDM-4 lysates were incubated with this serum or an inappropriate monoclonal antibody (data not shown). The dye front is labeled β_2m since, in higher percentage acrylamide gels, the majority of radioactivity in this region was observed as a discrete β_2m band with little radioactivity at the front (not shown).

treated samples was a unique band that corresponded to heavy chain cross-linked to β_2m (Fig. 1; ~60 kD). However, such a band was readily detectable in steady-state labeling experiments or upon subsequent chase of the pulse-labeled cells, presumably reflecting the kinetics of heavy chain–β_2m association (see below).

It should be noted that a minor species of ~90 kD was observed in immunoprecipitates of class I molecules from DSP-treated cell extracts (indicated by * in Fig. 1). Treatment with DTT to cleave the cross-links revealed that this species corresponded to class I heavy chain dimers (data not shown). However, because the amount of the 90-kD species was very low and, in some cases, undetectable (e.g., Fig. 3 and Fig. 8), it was not studied further. Radioactive material could also be detected near the top of the gel in several cross-linking experiments. It is doubtful that this material contains a substantial amount of higher class I oligomers or large aggregates because its presence was variable (compare Figs. 3 and 8) and was detectable, albeit to a lesser degree, in preimmune immunoprecipitates (e.g., Fig. 1, lane *1*). Moreover, subsequent sucrose gradient centrifugation and gel filtration studies failed to detect any large class I oligomeric forms (see below). We also tested whether any class I heavy chains remained as insoluble aggregates in material that sedimented (100,000 *g*) after lysis of radiolabeled cells (10 min pulse) with either NP-40 or CHAPS detergents. This material was solubilized with SDS, a 10-fold weight excess of NP-40 was added, and the resulting solution was subjected to immunoprecipitation with the anti-peptide 8 serum that is capable of recognizing denatured K^d, K^b, or K^k heavy chains. Only trace amounts of heavy chains (<5%) could be recovered (data not shown).

To determine the nature of the 145-kD cross-linked species, each 145-kD band in Fig. 1 was excised, eluted from the gel, and the cross-links cleaved by reduction before reanalysis by SDS-PAGE. Class I heavy chains were the only radiolabeled proteins detected. This was the case even when the cross-linking was performed on cells that had been metabolically labeled with [^{35}S]Met for 1 h (data not shown). Although this finding suggested that the 145-kD species is a homooligomer (possibly a trimer) of class I heavy chains, it was conceivable that the heavy chains were interacting with a different protein(s) that was poorly radiolabeled.

To address this possibility, two approaches were used. First, MDAY-D2 cells were labeled with [^{35}S]Met under steady-state conditions for 24 h in order to prelabel molecules that turn over slowly. These cells were then pulse-labeled for 10 min to increase the radioactive signal of newly synthesized class I molecules, lysed in the absence or presence of DSP, and the K^d molecules immunoprecipitated and subjected to SDS-PAGE under nonreducing conditions (Fig. 2 *A*, lanes *1* and *2*). Two prominent bands were unique to the DSP-treated sample (Fig. 2 *A*, lane *2*), the 145-kD species (**) as well as a 60-kD species (•). The 145-, 60-kD, and K^d heavy chain bands were excised from the dried gel, eluted, and analyzed individually by SDS-PAGE after reduction (Fig. 2 *A*, lanes *3*, *4*, and *5*). Reduction of the 60-kD band (Fig. 2 *A*, lane *5*) revealed that it consisted of the K^d heavy chain cross-linked to β_2m. Unexpectedly, reduction of the 145-kD species (Fig. 2 *A*, lane *3*) revealed that it consisted of the K^d heavy chain cross-linked to a protein having an apparent molecular mass of 88 kD. Upon longer exposure of this gel to x-ray film, β_2m could also be detected as part of the 145-kD complex (not shown). Identical results have been obtained for the K^b molecule from EL4 cells.

As an independent way to characterize components of the 145-kD complex, it was subjected to radioiodination and analysis by reducing SDS-PAGE. Initially, MDAY-D2 cells (labeled for 10 min with [^{35}S]Met) were lysed in the presence of DSP and the K^d molecule immunoprecipitated using the 20-8-4s monoclonal antibody (see Materials and Methods). Fig. 2 *B*, lane *1*, depicts a portion of the immunoprecipitated material subjected to nonreducing SDS-PAGE. The [^{35}S]Met-labeled 145-kD species (Fig. 2 *B*, **) was ex-

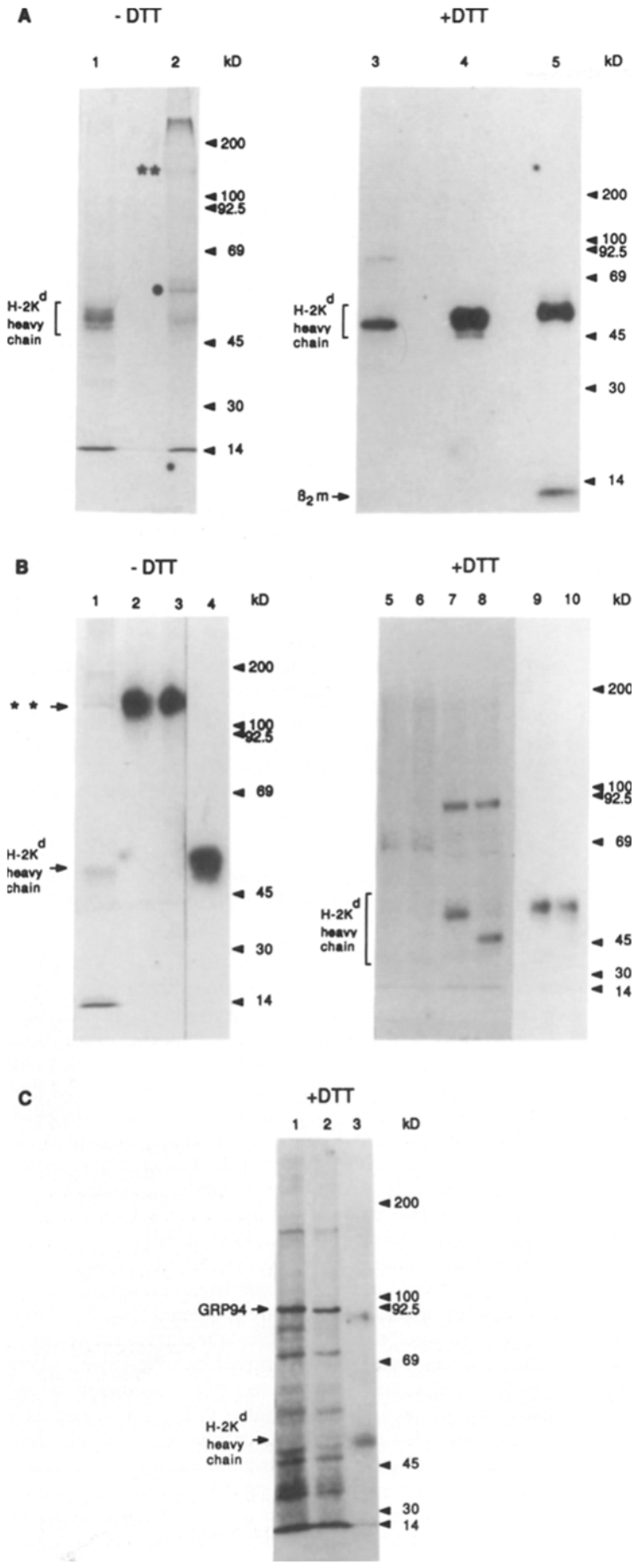


Figure 2. Identification of an 88-kD protein (p88) cross-linked to H-2K^d molecules. (*A*) After steady-state labeling with [^{35}S]Met, MDAY-D2 cells were lysed in the absence or presence of DSP and the K^d molecules were isolated and analyzed by SDS-PAGE under nonreducing conditions as in Fig. 1 (lanes *1* and *2* are − and + DSP, respectively). The 145- (**) and 60-kD (●) species specific to the DSP-treated sample (lane *2*) were excised from the dried unfixed gel, as was the K^d heavy chain band, and the proteins eluted from the individual gel slices as described in Materials and Methods. Cross-links were cleaved by reduction and the resulting radioactive material analyzed by SDS-PAGE (10–17.5%) under reducing conditions (lane *3*, reduced 145-kD species; lane *4*, reduced K^d heavy chains; lane *5*, reduced 60-kD species). (*B*) Pulse-labeled MDAY-D2 cells were lysed in the absence or presence of DSP and the K^d molecules were isolated and analyzed as above with the exception that the immobilized 20-8-4s monoclonal antibody was used for this initial immunoprecipitation. Only an aliquot of K^d isolated from the DSP-treated sample is shown (lane *1*). The 145 kD (**) and the K^d heavy chain bands were excised from the gel and the proteins eluted and subjected to radioiodination as described in Materials and Methods. An eluted sample from the 145-kD region of an uncross-linked sample was also radioiodinated to control for nonspecific contamination. The radioiodinated K^d molecules were subsequently purified using anti-peptide 8 and subjected to nonreducing SDS-PAGE. The radioiodinated 145-kD species from both the DSP-treated and untreated samples as well as the K^d heavy chains were excised and eluted from this gel and an aliquot was reanalyzed by nonreducing SDS-PAGE (lane *2*, 145-kD control region; lane *3*, 145-kD cross-linked species; lane *4*, K^d heavy chain). Each sample was also analyzed (7.5% gel) under reducing conditions before or after incubation with endo H (lanes *5* and *6*, 145-kD control region; lanes *7* and *8*, 145-kD cross-linked species; lanes *9* and *10*, K^d heavy chain). Lanes *6*, *8*, and *10* were endo H treated. (*C*) GRP94 was isolated using rabbit anti-mouse GRP94 serum from the uncross-linked cell extract prepared as described in *B*. The immunoprecipitate was divided in half, incubated in the absence (lane *1*) or presence (lane *2*) of endo H, and analyzed by SDS-PAGE (7.5% gel) under reducing conditions. A sample, identical to that depicted in lane *7*, *B* containing reduced 145-kD cross-linked species, was also run on this gel (lane *3*).

cised and eluted from such a gel lane, as was the K^d heavy chain. A section of a parallel lane corresponding to the 145-kD region obtained from an uncross-linked sample was also excised and eluted to control for the presence of nonspecific unlabeled proteins (such as Ig and oligomeric albumin introduced during immunoprecipitation) that might coelute with the 145-kD species and become radioiodinated.

The three eluted samples were subsequently subjected to radioiodination. The radioiodinated K^d heavy chain, 145-kD complex, and control region were purified by immunoisolation with anti-peptide 8, followed by SDS-PAGE under nonreducing conditions and excision and elution of the appropriate bands. The purified, radioiodinated 145-kD species and the K^d heavy chain reanalyzed by nonreducing SDS-PAGE are illustrated in Fig. 2 *B*, lanes *3* and *4*, respectively. The uncross-linked control region sample also possessed a radioiodinated 145-kD species after these purification steps (Fig. 2 *B*, lane *2*).

Each of these purified samples was characterized by SDS-PAGE after cross-links were cleaved by extensive reduction. In addition, an equal aliquot was subjected to endo H digestion (Fig. 2 *B*, lanes *5–10*). Endo H cleaves between the two core *N*-acetylglucosamine residues of immature, high mannose type asparagine (Asn)-linked oligosaccharides that are present on newly synthesized glycoproteins in the ER. It does not digest Asn-linked oligosaccharides that have undergone terminal processing by enzymes located in the medial cisternae of the Golgi apparatus (Dunphy et al., 1985; Novikoff et al., 1983). Different murine class I heavy chains possess either two or three Asn-linked oligosaccharides (Kimball and Coligan, 1983).

As can be seen in Fig. 2 *B*, lanes *5* and *6*, the radioiodinated species obtained from the uncross-linked 145-kD control region consisted of a single band upon reduction that is likely albumin because it comigrated with the 69-kD bovine serum albumin marker and was not sensitive to endo H. It was probably isolated due to some anti-albumin reactivity of the anti-peptide 8 serum (Smith et al., 1986). This band also was present upon reduction of the cross-linked K^d-specific 145-kD species (Fig. 2 *B*, lane *7*). However, two prominent bands possessing apparent molecular masses of 88 and 52 kD were also observed. Whereas the 52-kD species was sensitive to digestion by endo H (shifted to lower molecular mass), the 88-kD species was insensitive (Fig. 2 *B*, compare lane *7* with lane *8*). That the 52-kD band corresponded to the K^d heavy chain was evidenced by the fact that it comigrated with the small endo H sensitive subpopulation of heavy chains present in the purified radioiodinated K^d heavy chain preparation (Fig. 2 *B*, compare lane *8* with lane *10*). It is noteworthy that no endo H-resistant heavy chains could be detected as part of the 145-kD complex. Under the SDS-PAGE conditions used in this experiment, it was not possible to resolve β_2m from the dye front. However, β_2m must be a component of the complex since an antibody (20-8-4s) that only recognizes class I heavy chains in association with β_2m was used in the initial isolation of the complex. Further evidence supporting this point will be presented below (Fig. 8).

The identification of a novel 88-kD protein in the K^d-specific 145-kD complex by radioiodination corroborates the results of the steady-state labeling experiment (Fig. 2 *A*). That each experiment identified the same molecule is evidenced by the fact that the two 88-kD species comigrated on SDS-PAGE gels under reducing conditions, did not shift upon endo H digestion, and were exclusively associated with endo H-sensitive K^d heavy chains (data not shown). These data, combined with the fact that the 145-kD complex is readily detected after a short pulse labeling (Fig. 1), indicate that a significant portion of class I molecules associate with an 88-kD protein rapidly after synthesis.

The apparent molecular mass of this 88-kD protein could be influenced by the presence of covalently associated DSP molecules. Fortunately, we have had some limited success in coimmunoprecipitating this protein with class I molecules in the absence of cross-linking when cells were solubilized in CHAPS detergent. There was no significant difference in its mobility upon SDS-PAGE analysis relative to the DSP-modified protein (data not shown). Consequently, we have designated this protein, p88.

As a first attempt to identify the nature of p88, its mobility on reducing SDS-PAGE gels was compared to the abundant ER protein, GRP94 (Mazzarella and Green, 1987). We reasoned that p88 might be GRP94 because: (*a*) p88 had a similar molecular mass, (*b*) it appeared to interact solely with endo H-sensitive class I heavy chains suggesting that it might be ER localized, and (*c*) the extreme measures required to detect it suggested that it might be abundant and thus difficult to radiolabel metabolically. However, Fig. 2 *C* shows that p88 had a greater mobility than did GRP94 (compare lane *1* with lane *3*). Moreover, whereas p88 was unaffected by digestion with endo H (Fig. 2 *B*), a slight but discernable shift characteristic of the presence of one high mannose type Asn-linked oligosaccharide could be observed for GRP94 (Fig. 2 *C*, lanes *1* and *2*). These data indicate that p88 is not GRP94 and likely does not possess Asn-linked glycans.

The Class I-p88 Complex Is Transient and p88 Dissociation Is Kinetically Related to ER to Golgi Transport

In an effort to determine the fate of the class I-p88 complex and the possible influence p88 might have on the ER to Golgi transport of class I molecules, the cross-linking protocol was incorporated into a pulse-chase biosynthetic experiment (Fig. 3). At various chase times, cell lysis was carried out in the presence or absence of DSP and then class I molecules were immunoisolated, subjected to endo H digestion and analyzed by SDS-PAGE under nonreducing conditions. It has been well established that the rate at which class I molecules acquire resistance to endo H digestion provides a measure of their rate of transport from the ER to the medial Golgi cisterna (Williams et al., 1985, 1988).

Four class I molecules (H-$2K^b$, D^b, K^d, and L^d) were analyzed in this fashion and only the data obtained from cells lysed in the presence of DSP are presented (Fig. 3). After the pulse (0 min chase time), each of the class I heavy chains, either uncross-linked or cross-linked into the 145-kD class I–p88 complex, was sensitive to endo H treatment as expected for glycoproteins shortly after their translocation into the ER. Endo H-sensitive heavy chains cross-linked to β_2m could also be observed just above the heterogeneous heavy chain region of these nonreducing gels, usually by 5 min of chase. Interestingly, only a trace amount of this species was detected for K^b, however, this was highly variable (see Fig.

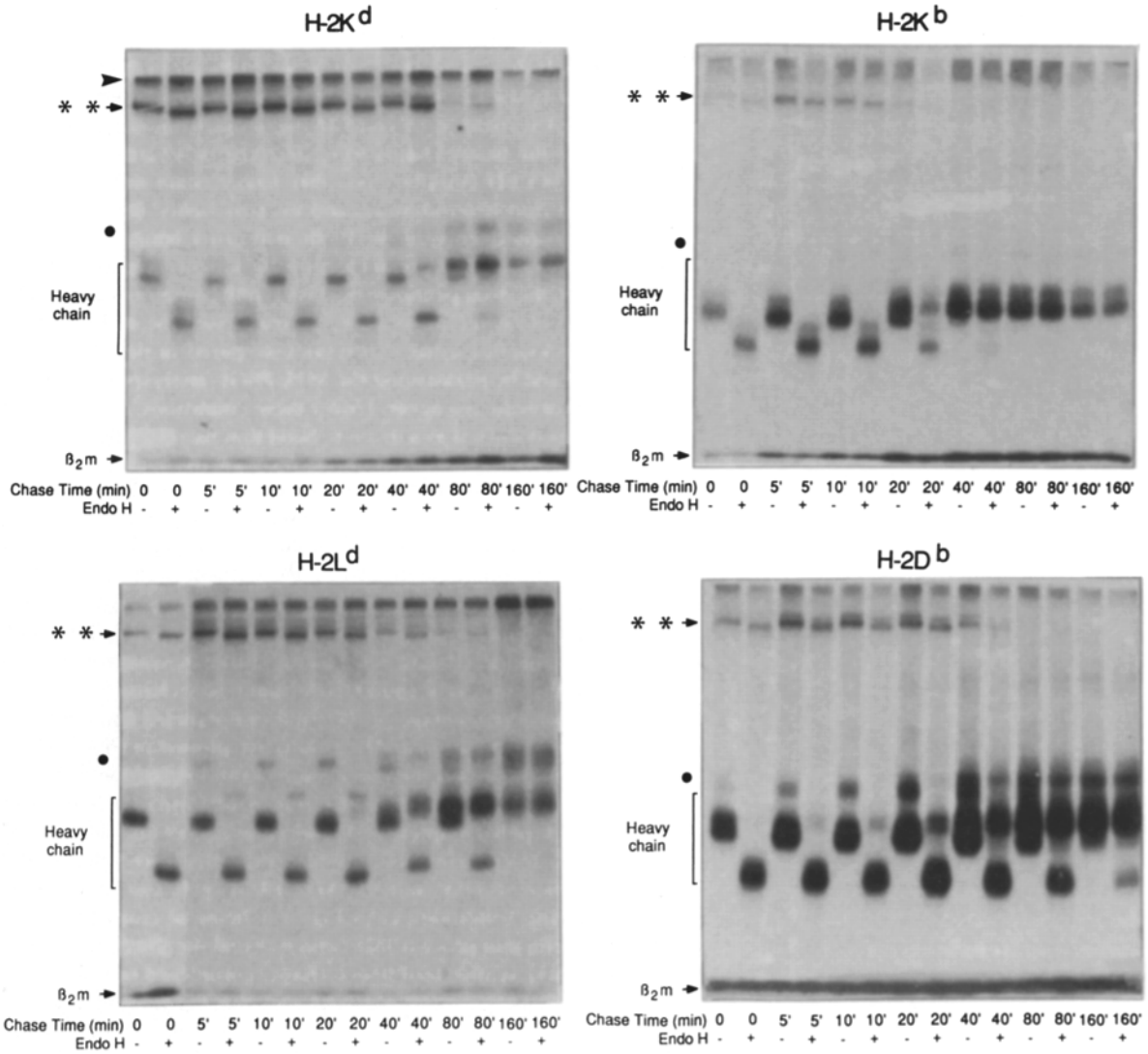


Figure 3. Pulse-chase analysis of class I-p88 association and intracellular transport. MDAY-D2 and EL4 cells were pulsed for 5 min with [^{35}S]Met and chased for the indicated intervals. At each chase time, an equivalent aliquot of cells was removed and lysed in the presence of DSP. The K^d and L^d molecules were isolated sequentially from lysates of MDAY-D2 cells and K^b and D^b were isolated sequentially from EL4 lysates. 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 class I–p88 complex and of heavy chain cross-linked to β_2m are indicated (** and •, respectively). The arrowhead points to the top of the separating gel in the K^d panel. The pulse labeled L^d sample (0 min chase) was isolated in a separate experiment in which radiolabeling of both the heavy chain and β_2m was more extensive.

7 *B*). That this band corresponded to heavy chain-β_2m heterodimers was evidenced by its appropriate molecular mass (~60 kD), and its increase in intensity during the chase period presumably due to increased β_2m association (see Fig. 9). Furthermore, upon excision from the gel and cleavage of the cross-links, two radioactive bands were detected: heavy chain and β_2m (see Fig. 2 *B*, lane 5). As in Fig. 1, no class I-p88 complexes or heavy chain-β_2m heterodimers were detected in the absence of DSP.

As the duration of the chase increased, the class I heavy chains acquired resistance to endo H digestion (Fig. 3). Remarkably, during this period, the ability to form the cross-linked class I–p88 complex decreased such that by the time all class I molecules had acquired endo H resistance, no complexes were detectable. At no time were any endo H-resistant class I-p88 complexes observed. This result is consistent with the previous finding (Fig. 2 *B*) that exclusively endo H-sensitive class I heavy chains were present in the isolated class I–p88 complexes. In addition, no class I–p88 complexes could be detected at the cell surface as revealed by cross-linking of surface radioiodinated cells (data not shown). It should be noted that, for most class I molecules, some heterogeneity in the region of the 145-kD complex was detectable, usually after 5–10 min of chase. This may reflect different degrees of cross-linking to β_2m or alternative complex forms reflecting the heavy chain heterogeneity observed in Fig. 1. The data indicates that the existence of class I–p88 complexes is transient and, based on their complete endo H sensitivity and lack of surface expression, that they are not extensively transported; at most reaching only the *cis*-cisternae of the Golgi apparatus.

The rates at which the cross-linked class I–p88 complexes

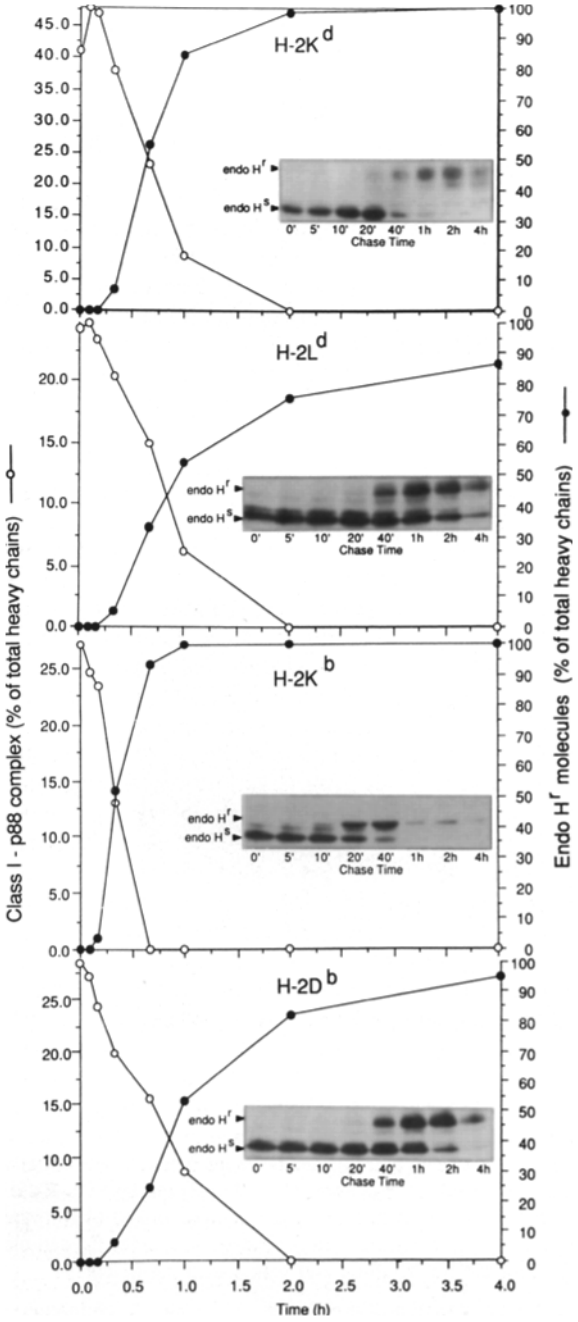


Figure 4. Kinetic relationship between class I–p88 complex disappearance and ER to Golgi transport. The rate of class I–p88 complex disappearance was assessed by densitometric scanning of weakly exposed fluorograms similar to those shown in Fig. 3. The amount of heavy chain in the complex is expressed as a percentage of the total heavy chain present in the gel lane. Each point represents the average of two independent experiments (○). The rate of ER to Golgi transport, as measured by acquisition of endo H resistance, was determined in parallel experiments in which cells were lysed at the indicated times in the absence of DSP and class I molecules were isolated, digested with endo H, and analyzed under reducing conditions (*insets*). The heavy chain region of the gels was scanned and the amount of endo H resistant heavy chain expressed as a percentage of the total heavy chain in the gel lane. Each point is the average of two independent experiments (●). In each panel, the maximum amount of class I–p88 complex obtained is set to correspond to 100% on the scale of percentage endo H resistance. This facilitates a comparison of the half-times for the two processes.

disappeared seemed to correlate with the characteristic rates at which the class I heavy chains acquired resistance to endo H digestion. However, it was difficult to determine accurately the kinetics with which the class I heavy chains acquired resistance to endo H digestion from these gels because the heavy chains were detected in a variety of forms (the 145-kD complex, possibly heavy chain dimers, heavy chain-β_2m heterodimers, and uncross-linked heavy chains) as a consequence of the inefficient cross-linking reaction.

To examine in greater detail the kinetic relationship between the disappearance of class I–p88 complexes and acquisition of endo H resistance, we reassessed the endo H resistance kinetics using cell lysates prepared in the absence of DSP and by performing the SDS-PAGE analyses in the presence of reducing agent. Under these conditions, class I heavy chains occurred as a discrete band that was initially sensitive to endo H digestion, becoming resistant to enzyme treatment at a rate characteristic for the particular heavy chain (see Fig. 4, *insets*). This reflects the differential rates of ER to Golgi transport observed previously for closely related class I molecules (Williams et al., 1985, 1988; Beck et al., 1986). The bands observed between the endo H-sensitive and -resistant heavy chains were present in nonimmune precipitates and were considered to be nonspecific. From densitometric analysis, the amount of endo H-resistant heavy chains was calculated as a percentage of the total radiolabeled heavy chains at a given chase time. The results are plotted in Fig. 4. Also shown in Fig. 4 is the percentage of total radiolabeled heavy chains at each chase time that was in the form of cross-linked class I–p88 complexes. These values were the average of duplicate experiments, in which the cells were lysed in the presence of DSP and the SDS-PAGE gels run under nonreducing conditions as in Fig. 3. Chemical cross-linking is an inefficient process that cannot reveal the absolute amount of newly synthesized heavy chains complexed to p88. However, this experiment required only that the amount of cross-linked complexes obtained reflects the relative size of the radiolabeled class I–p88 complex pool. Support for this assumption was provided by the remarkable reproducibility of the rates of complex disappearance determined in replicate experiments (~10% variation). Furthermore, independent measurement of K^d–p88 complexes at three time points using a quantitative gel filtration assay completely corroborated the cross-linking results (see next section).

As shown in Fig. 4, ~40–50% of the total labeled K^d heavy chains could be cross-linked to p88 immediately after a 5-min pulse, whereas, 25–30% of the other three class I heavy chains were initially observed in this form. A comparison of the $t_{1/2}$ for the disappearance of the complex with that for the acquisition of endo H resistance of the total class I heavy chains confirmed that the two processes were closely linked kinetically. The K^d– and K^b–p88 complexes disappeared with half-times after the pulse of 38 and 20 min, respectively, coinciding exactly with the time at which half the K^d and K^b heavy chains became resistant to endo H digestion. For the L^d– and D^b–p88 complexes, both disappeared with a $t_{1/2}$ of 45 min, slightly preceding the $t_{1/2}$ for acquisition of endo H resistance of the heavy chains ($t_{1/2}$ = 55 min). This correlation was not simply a consequence of complex Asn-linked oligosaccharides interfering with the reactivity of the cross-linker. As shown in Fig. 7, altering the rate of oligosaccharide processing by treating cells with BFA

had no influence on the rates of class I–p88 complex disappearance. Because the complexes disappeared with characteristically different kinetics ($t_{1/2}$ of 20, 38, 45 min) that closely coincided with the rates at which the respective class I heavy chains become endo H resistant, these data suggest that the disappearance of the class I–p88 complexes is somehow related to the transport of class I heavy chains from the ER to the medial Golgi cisterna.

Class I–p88 Complexes Are Precursors of Mature Class I Molecules

For the class I–p88 complex to be relevant to the process of class I intracellular transport, it was necessary to show that it was, in fact, a precursor of mature, endo H-resistant molecules and not simply a dead-end population that was degraded intracellularly at a characteristic rate. This required a quantitative technique to separate complexes from other forms of class I heavy chains, e.g., sedimentation through sucrose density gradients. However, when lysates prepared from pulse-labeled cells in the absence of DSP were applied to sucrose gradients, we were unable to detect any class I–p88 complexes or large aggregates despite the ability of the gradient to resolve cross-linked class I–p88 complexes from other class I heavy chain forms. The same results were obtained when the lysis and gradient analysis were performed at various pH values and with different detergents. As observed for other oligomeric proteins (Vogel et al., 1986; Copeland et al., 1986; Doms et al., 1987), this was probably due to the instability of the class I–p88 complex in sucrose gradients.

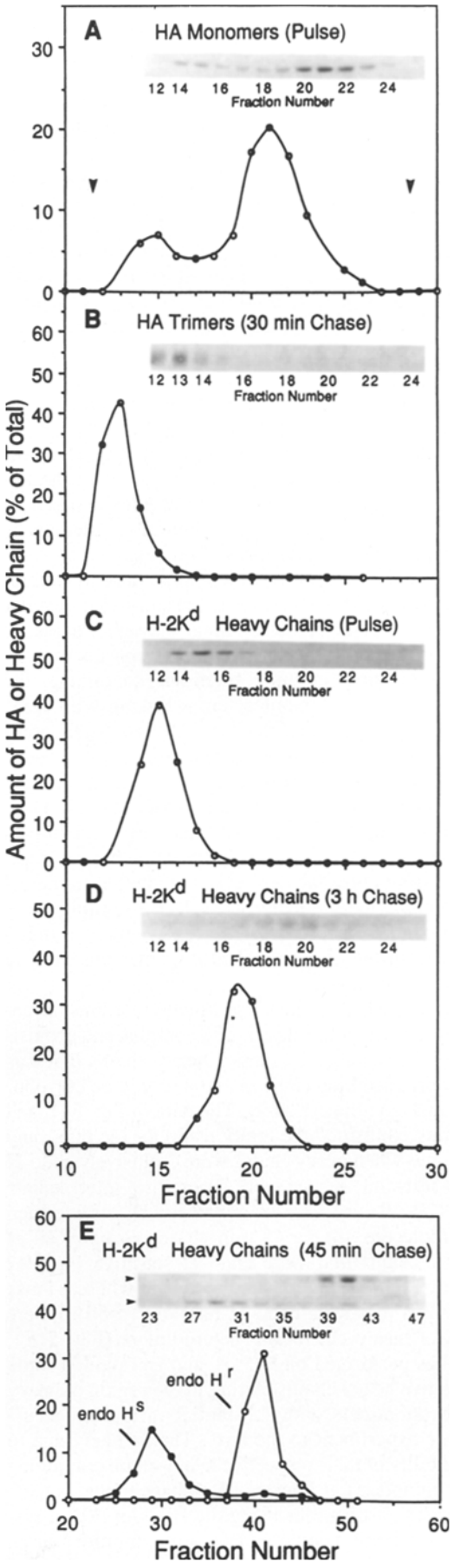
An alternative methodology was tested that used gel permeation chromatography through Bio-Gel A0.5m in the presence of Triton X-100 (Morrison et al., 1988). Using this technique, the bulk of K^d heavy chains from pulse-labeled MDAY-D2 cells (presumably enriched in K^d–p88 complexes) eluted from the column before the K^d heavy chain-β_2m heterodimers from cells pulsed and then chased for 3 h (data not shown). However, studies performed with the other three class I molecules revealed a lower and highly variable proportion of this oligomeric species (typically 20–60%) in the pulse-labeled sample. Apparently, class I–p88 complexes of these molecules were less stable over the 8–10-h column run than those of the K^d molecule.

A system that was more rapid (30 min) and that involved less sample dilution was subsequently developed using gel filtration HPLC in the presence of 0.6% CHAPS detergent. CHAPS was used because it is known to preserve the integrity of a variety of oligomeric proteins (Brenner et al., 1987; Hemler et al., 1987) and because it contributes less to the apparent molecular mass of a protein (~9 kD micelle) than does Triton X-100 (~100 kD micelle; Neugebauer, 1987). The system was initially tested for its ability to resolve monomers and trimers of HA, a transmembrane glycoprotein like the class I molecule (Fig. 5, *A* and *B*). It is well-established that after a 5-min pulse, the majority of HA is in monomeric form (~68 kD) whereas after 30 min of chase, all HA is trimeric (Gething et al., 1986; Copeland et al., 1986, 1988; Yewdell et al., 1988*b*). HA trimers (Fig. 5 *B*) eluted from the column just after the V_o whereas HA monomers (*A*) eluted as a smaller species well separated from trimers. The small amount of a larger HA form seen in Fig. 5 *A* probably represents HA dimers or incompletely folded trimers that are still detectable with the monomer-specific antibody used for immunoprecipitation.

When the K^d heavy chains in a pulse-labeled sample were analyzed, they eluted as a single symmetrical peak well resolved from the smaller K^d heavy chain-β_2m heterodimers present in a 3-h chased sample (compare Fig. 5, *C* and *D*). Similar results were obtained for K^b, D^b, and L^d (data not shown). These findings provide compelling evidence that, for four different class I molecules, the entire population of newly synthesized heavy chains associate to form an oligomeric species that is a precursor of mature, heavy chain-β_2m heterodimers. It should be noted that radiolabeled p88 could not be detected in the immunoprecipitates depicted in Fig. 5 due to the short labeling time used in this experiment.

The molecular mass of the heavy chain containing oligomer observed by gel filtration cannot be ascertained unambiguously without a detailed analysis of its hydrodynamic properties. Unfortunately, this is not possible with the minute amounts of intracellular class I molecules that can be obtained, even from large-scale cell cultures. Attempts to derive the apparent molecular mass of the oligomer by comparison with the HA trimer or with soluble protein calibration standards are of little value due to variation in molecular shape and degree of detergent binding. However, evidence that the oligomer represents class I–p88 complexes was obtained by subjecting column fractions containing the oligomer (Fig. 5 *C*) to chemical cross-linking with DSP. SDS-PAGE analysis revealed the 145-kD class I–p88 complex as the largest cross-linked species (data not shown). Significantly, when this experiment was repeated on fractions containing heavy chains in the chased sample (Fig. 5 *D*), no cross-linked species other than heavy chain-β_2m heterodimers were detected. Furthermore, when a cross-linked lysate from pulse-labeled cells was applied to the gel filtration column, the authentic class I–p88 complex (detected by nonreducing SDS-PAGE) eluted in a manner indistinguishable from the uncross-linked oligomer observed in Fig. 5 *C* (data not shown).

We also tested whether the close kinetic relationship between disappearance of the class I–p88 complex and acquisition of endo H resistance by class I heavy chains that was observed by cross-linking (Figs. 3 and 4) could be corroborated using this quantitative assay. The data in Fig. 5 (*C* and *D*) had already confirmed the relationship for the pulse and 3-h chase, times when heavy chains were exclusively endo H sensitive or resistant, respectively. Therefore, intermediate chase times (20–45 min) were examined as well. As expected for the K^d molecule after a 45-min chase, virtually all of the K^d heavy chains that were endo H sensitive (~40%) were in oligomeric form (associated with p88) whereas those K^d heavy chains that were endo H resistant (~60%) were in the form of heavy chain-β_2m heterodimers (Fig. 5 *E*). Similar studies performed on K^b, D^b, and L^d revealed some endo H-sensitive heavy chains eluting in the region of heavy chain-β_2m heterodimers with substantial variability occurring from one experiment to the next. This is likely due to the lesser stability of their respective heavy chain containing class I–p88 complexes at intermediate chase times, consistent with earlier observations using the Bio-Gel A0.5m system. We are currently testing a variety of conditions in an effort to minimize this instability. In spite of this problem,



the cumulative cross-linking and gel filtration data indicates that class I–p88 complex dissociation is closely linked to the transport of heavy chains from the ER to the medial Golgi.

Subcellular Location of Class I–p88 Complex Dissociation

To better understand the close kinetic relationship between the processes of class I–p88 complex dissociation and intracellular transport, it was necessary to ascertain the intracellular site of p88 dissociation. In principle, dissociation of the endo H-sensitive complexes could occur before, during, or immediately after transport to the *cis*-Golgi cisterna. To address this question we determined whether p88 dissociation could occur under conditions that block ER to Golgi transport.

Initial experiments focused on the use of CCCP or oxygen deprivation to deplete cellular ATP and thereby block vesicular transport. EL4 cells were subjected to a standard pulse-chase radiolabeling with the exception that the chase was performed in the presence of 50 μM CCCP in the absence of glucose. Subsequent cross-linking of the K^b molecule revealed that the K^b–p88 complex dissociated completely between 30–60 min of chase. However, under these conditions, intracellular transport was not totally blocked; ~5–15% of the K^b molecules acquired resistance to endo H digestion over the same period (data not shown). Although this data suggests that disassembly occurs in the ER, it cannot be considered conclusive due to the low level of intracellular transport. Unfortunately, the alternative of using oxygen deprivation to deplete ATP resulted in extensive cell death within the time period of class I–p88 complex dissociation (data not shown).

Subsequently, we investigated whether p88 could dissoci-

Figure 5. Gel filtration HPLC analyses of HA and class I molecules. EL-4 cells infected with the PR8 strain of influenza were either pulsed for 5 min with [^{35}S]Met or pulsed and then chased for 30 min (see Materials and Methods). Lysates were prepared and injected onto a TSK 3000SW gel filtration HPLC column. An HA monomer-specific antibody (Y8-10C2) was employed to isolate HA from column fractions obtained from the pulse-labeled lysate (*A*) whereas an HA trimer-specific antibody (H17-L2) was used to isolate HA from column fractions obtained from the 30-min chased lysate (*B*). Similarly, lysates from uninfected MDAY-D2 cells that had either been pulsed (*C*) or pulsed and then chased for 45 min (*E*) or 3 h (*D*) were injected onto the TSK 3000SW column. K^d molecules in appropriate column fractions were isolated using the anti-peptide 8 serum. For each time point, the isolated HA or K^d molecules were analyzed by reducing SDS-PAGE. Presented are the HA and K^d heavy chain regions of each gel (*insets*). The relative amount of HA or K^d heavy chain in each fraction was determined by densitometry and plotted as percentage of the total HA or heavy chains recovered (*A–D*). In *E*, the isolated K^d molecules were treated with endo H before SDS-PAGE analysis; the mobilities of endo H sensitive (*lower arrow, inset*) and endo H-resistant K^d heavy chains (*upper arrow, inset*) are indicated. The relative amount of endo H-sensitive or -resistant K^d heavy chains in each fraction was plotted separately as a percentage of the total. Note that 0.5 ml fractions were collected during the gel filtration depicted in *E* whereas 1-ml fractions were collected in *A–D*. Dextran blue and β-mercaptoethanol were used as void volume (V_o) and total column volume (V_t) markers, respectively (*two large arrows* in *A*).

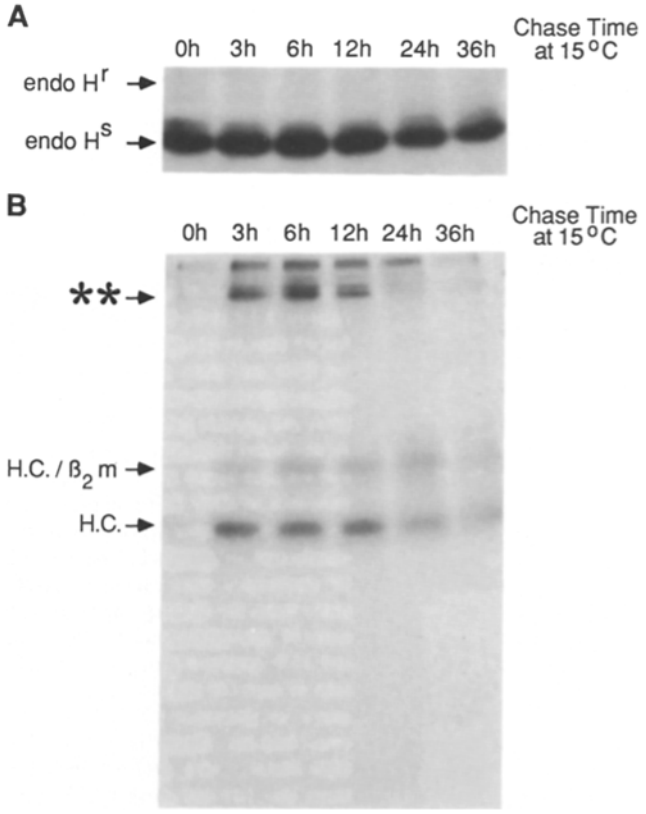


Figure 6. p88 dissociation from class I molecules at 15°C. MDAY-D2 cells were pulsed for 5 min with [^{35}S]Met, chased for 5 min at 37°C, and then chased at 15°C for periods of up to 36 h. At each chase time (15°C), two equivalent aliquots of cells were removed and lysed in the absence or presence of DSP. The L^d molecules were subsequently immunoisolated and subjected to endo H digestion. Uncross-linked L^d molecules were analyzed by SDS-PAGE (10–15% gradient gel) under reducing conditions (*A*). Only the heavy chain region of the gel is shown and the mobilities of L^d heavy chains resistant or sensitive to endo H digestion are indicated. Cross-linked L^d molecules were analyzed in a similar fashion with the omission of reducing agent (*B*). The mobilities of the cross-linked class I–p88 complex (**), cross-linked heavy chain-β_2m heterodimer (*H.C./β_2m*), and heavy chain (*H.C.*) are indicated.

ate from the complex at 15°C. At this temperature, protein transport from the ER to the Golgi apparatus is blocked or dramatically slowed resulting in accumulations in both the ER and a pre-Golgi intermediate compartment (Saraste and Kuismanen, 1984; Balch et al., 1986; Tartakoff, 1986; Lippincott-Schwartz et al., 1990). Both MDAY-D2 and EL4 cells were subjected to a pulse-chase experiment in which the cells were radiolabeled with [^{35}S]Met for 5 min, chased for 5 min at 37°C to ensure extensive association of β_2m with class I heavy chains (see Fig. 9), and then chased at 15°C for 36 h. At various times during the chase, aliquots of cells were removed, lysed in the presence or absence of DSP, and the amounts of endo H-resistant class I heavy chains and cross-linked class I–p88 complexes were determined. Under these conditions, the class I heavy chains acquired endo H resistance at substantially reduced rates relative to those at 37°C (~20-fold reduction in EL-4 cells and ~70-fold reduction in MDAY-D2 cells). Only L^d heavy chains remained completely endo H sensitive over the 36-h chase (Fig. 6 *A*). By comparison, the rates of class I–p88 complex dissociation were also reduced but, unlike the situation at 37°C, p88 dissociation substantially preceded acquisition of endo H resistance for each heavy chain examined. In particular, L^d–p88 complexes had almost entirely disappeared by the 36 h chase time (Fig. 6 *B*). These findings suggest that p88 dissociation occurs at a location before the site of acquisition of endo H resistance in the Golgi, either in the ER or in the pre-Golgi intermediate compartment.

Dissociation of the complexes was also examined when ER to Golgi transport was blocked by treatment with the fungal metabolite BFA (Misumi et al., 1986; Fujiwara et al., 1988). Initially, EL4 cells were incubated in the absence or presence of BFA in a standard pulse-chase experiment to assess its effects on oligosaccharide processing of the H-2K^b and D^b molecules (Fig. 7 *A*). In the absence of BFA treatment, these molecules acquired resistance to endo H digestion at their characteristic rates ($t_{1/2}$ ~20 min for K^b and ~55 min for D^b). In BFA-treated cells, both molecules became endo H resistant at comparable rates ($t_{1/2}$ ~60 min) and their endo H-resistant forms exhibited greater electrophoretic mobilities than in control cells. Comparable oligosaccharide processing rates and altered electrophoretic mobilities were also observed for the K^d and L^d molecules in BFA-treated MDAY-D2 cells (data not shown). Intermediate forms of the K^b and D^b molecules were also observed at the 1- and 2-h time points. They likely represent K^b with one of its two oligosaccharides endo H resistant and D^b with one or two of its three oligosaccharides endo H resistant. These findings are consistent with previous studies on the action of BFA which showed that, in addition to its effect on intracellular transport, BFA causes redistribution of Golgi processing enzymes to the ER resulting in the partial processing of glycoproteins in the ER (Lippincott-Schwartz et al., 1989, 1990; Doms et al., 1989). Class I–p88 complex dissociation was examined under these conditions as assessed by chemical cross-linking (Fig. 7 *B*). Despite the BFA-induced block in transport, K^b– and D^b–p88 complexes disappeared at rates comparable to those observed in control cells. Densitometric analysis revealed half-times for p88 dissociation in the presence of BFA of 23 and 42 min for K^b and D^b, respectively, vs. 20 and 45 min in the controls (see Fig. 4 for controls).

The redistribution of resident Golgi proteins to the ER caused by BFA treatment complicates the interpretation of these results. It could be argued that class I–p88 complexes normally are transported at characteristic rates to the Golgi where p88 dissociates as a consequence of exposure to some Golgi disassembly-promoting factors. Treatment with BFA might redistribute such factors to the ER causing dissociation of class I–p88 complexes that are blocked in this organelle. We consider this possibility unlikely because in such a scenario one would expect the rates of dissociation of the various class I–p88 complexes to become similar just as we observed for their rates of oligosaccharide processing due to redistribution of Golgi processing enzymes. In addition, Lippincott-Schwartz et al. (1990) have found that Golgi proteins redistributed to the ER can be transported to a pre-Golgi intermediate compartment in the presence of BFA; whether this also occurs for the class I–p88 complex is uncertain.

Collectively, these data suggest that class I–p88 complex

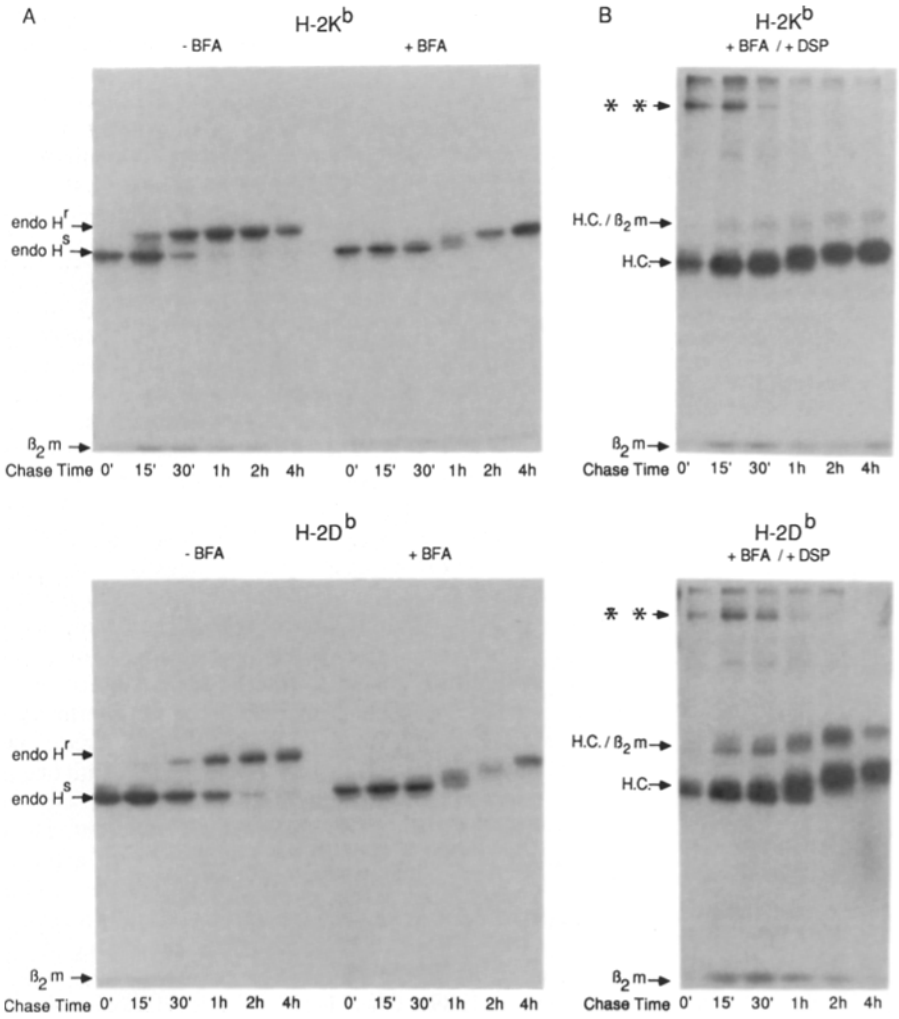


Figure 7. Effect of BFA on p88 dissociation. EL4 cells were preincubated for 45 min in the presence of 10 μg/ml BFA and then a standard pulse-chase experiment was conducted with BFA (10 μg/ml) present throughout. At the times indicated, cells were removed and lysed in the absence or presence of DSP. For comparison, a second experiment without BFA was conducted in parallel. The K^b and D^b molecules were isolated sequentially from the lysates, digested with endo H, and analyzed by SDS-PAGE (10–15% gradient gel). (*A*) Acquisition of endo H resistance in the absence or presence of BFA without cross-linking. Analysis by reducing SDS-PAGE. (*B*) p88 dissociation from the 145-kD class I–p88 complex in the presence of BFA as assessed by cross-linking. Analysis by SDS-PAGE under nonreducing conditions. The mobilities of the cross-linked class I-p88 complex (**), cross-linked heavy chain-β_2m heterodimer (*H.C./β_2m*), heavy chain (*H.C.*), and β_2m are indicated.

dissociation occurs before the Golgi, most likely in the ER. This finding, coupled with the similarity between the kinetics of dissociation and ER to Golgi transport observed both in cross-linking (Fig. 4) and gel filtration (Fig. 5) experiments, suggests that p88 dissociation from class I molecules may represent the rate limiting step beyond which rapid transport of class I molecules to the Golgi can occur.

Relationship of β_2m to Class I–p88 Complex Formation and Dissociation

Characterization of the class I–p88 complexes by the steady-state labeling and radioiodination techniques described earlier (Fig. 2, *A* and *B*), suggested that β_2m was present as part of the complex. To confirm this finding, five conformation-dependent monoclonal antibodies that are capable of recognizing H-2K^b heavy chains only when associated with β_2m were tested for their ability to immunoprecipitate cross-linked K^b–p88 complexes from DSP-treated lysates of pulse-labeled EL4 cells. As shown in Fig. 8 *A* (lanes *2–6*), the K^b–p88 complexes could be detected readily with each of these antibodies. Furthermore, anti-β_2m serum was also capable of immunoprecipitating the endo H-sensitive class I–p88 complexes from lysates of MDAY-D2 cells (Fig. 8 *B*, lanes *2* and *3*). Therefore, a significant portion of class I–p88 complexes contains β_2m.

It was of interest to determine whether p88 interacts with the class I heavy chain before β_2m association or only subsequent to the formation of heavy chain-β_2m heterodimers. To investigate this question, the kinetics of β_2m association were compared to the kinetics of class I–p88 complex formation. To determine the kinetics of heavy chain-β_2m association, β_2m-dependent monoclonal antibodies were used to immunoprecipitate only those H-2K^b, D^b, K^d, and L^d heavy chains associated with β_2m during a pulse-chase experiment (Fig. 9). As shown in Fig. 9 *A*, the amount of [^{35}S]Met-labeled heavy chains immunoisolated by these antibodies increased during the chase period reflecting an increase in β_2m association. In this experiment, processing of the heavy chains from the endo H sensitive form to the larger endo H-resistant form was detectable even without enzyme treatment. Densitometric analyses (Fig. 9 *B*) revealed that 30–40% of the class I heavy chains were already associated with β_2m after a 5-min pulse and that maximum association was reached after about 20 min of chase ($t_{1/2}$ = 2–5 min post pulse). The amount of heavy chain diminished at later times as a consequence of turnover at the cell surface (Williams et al., 1989). These findings were confirmed for the K^b, K^d, and D^b molecules in parallel experiments by clearing lysates first with the β_2m-dependent antibody and then quantifying heavy chains not associated with β_2m using the β_2m-inde-

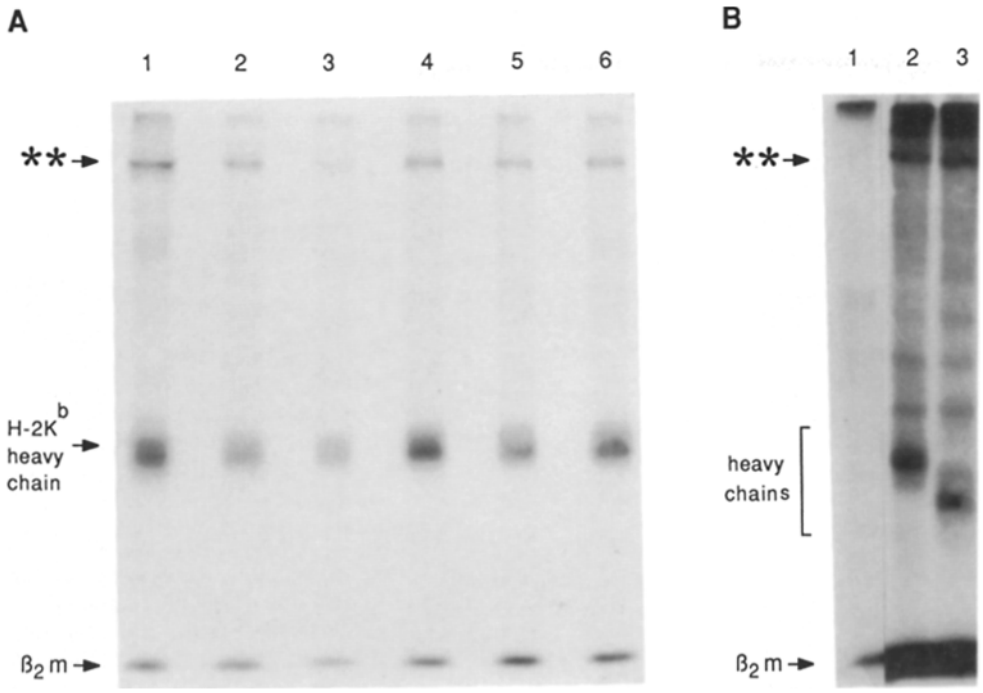


Figure 8. Detection of β_2m associated with class I–p88 complexes. (*A*) EL4 cells were radiolabeled for 10 min with [^{35}S]Met, divided into six equal aliquots, and lysed in the presence of DSP. Lysates were immunoprecipitated with either anti-peptide 8 which recognizes the K^b heavy chain whether or not β_2m is associated (lane *1*) or the following anti-K^b monoclonal antibodies that require β_2m for reactivity: EH-144 (lane *2*), 5F1.2.14 (lane *3*), B8-24-3 (lane *4*), Y-3 (lane *5*), and 20-8-4S (lane *6*). (*B*) Three DSP-treated lysates were prepared from MDAY-D2 cells radiolabeled for 5 min with [^{35}S]Met, and were subjected to immunoprecipitation with preimmune rabbit serum (lane *1*) or with Y-9, a rabbit anti-mouse β_2m serum (lanes *2* and *3*). In addition, anti-β_2m immunoprecipitates were incubated in the absence (lane *2*) or presence (lane *3*) of endo H. The anti-β_2m antiserum will react with the K^d, L^d, and D^d heavy chains associated with β_2m in MDAY-D2 lysates.

pendent antibody. For each class I molecule, only trace amounts of free heavy chain could be detected between 15 and 30 min of chase indicating near quantitative association of subunits (data not shown). By comparison, the rate of p88 association with class I molecules was much more rapid. Data presented previously (Fig. 5 *C*) showed that all heavy chains were complexed with p88 after a 5-min pulse labeling. Consequently, p88 interacts with the bulk of heavy chains before β_2m association.

Because β_2m association follows the formation of the heavy chain–p88 complex and since β_2m association is accompanied by a substantial conformational change in the heavy chain (Lancet et al., 1979; Yokoyama et al., 1985; Allen et al., 1986; Williams et al., 1989), we wished to establish whether β_2m plays a role in triggering the subsequent dissociation of p88 from the class I molecule. If β_2m association triggers dissociation of the class I–p88 complex, it would be expected that the kinetics of β_2m association would correlate with the disappearance of class I–p88 complexes. However, the half-times of heavy chain-β_2m association (Fig. 9 *B*) substantially preceded the characteristic half-times of disappearance of class I–p88 complexes for each class I molecule (Fig. 4). Even after 20 min of chase, when association with β_2m was essentially complete, 50–80% of the class I–p88 complexes remained. Furthermore, whereas p88 dissociated from class I–p88 complexes with kinetics characteristic for each specific heavy chain, the rates of heavy chain-β_2m association were remarkably similar for the four class I molecules examined. Taken together, these data indicate that association with β_2m substantially precedes class I–p88 complex disappearance and therefore does not appear to directly trigger p88 dissociation.

Discussion

In this report, we describe a novel interaction involving class I heavy chains that occurs rapidly following synthesis and that appears to play an important role in the egress of class I molecules from the ER. Using both chemical cross-linking and gel permeation chromatography, we have shown that, during the course of a 5-min pulse labeling, virtually all newly synthesized class I heavy chains associate with an 88-kD protein (p88) to form a complex that is a precursor of the mature class I heavy chain-β_2m heterodimer. Formation of the complex with p88 occurred more rapidly than the association of heavy chains with β_2m suggesting that the ability to associate with p88 was a property of class I heavy chains alone. Experiments performed with cell lines that are deficient in the synthesis of β_2m revealed that free heavy chains do indeed associate quantitatively with p88, confirming this suggestion (Degen, E., and D. Williams, manuscript in preparation).

The class I–p88 complex existed transiently and dissociation of p88 occurred at a distinct rate for each class I molecule examined ($t_{1/2}$ = 20–45 min). From our pulse-chase studies, it was observed that heavy chain-β_2m association for all class I molecules tested ($t_{1/2}$ = 2–5 min) substantially preceded the dissociation of p88 from class I–p88 complexes. In fact, for three of the class I molecules tested (K^d, D^b, and L^d), β_2m association was completed before a substantial degree of p88 dissociation occurred. These data suggest that the conformational changes in the heavy chain that accompany β_2m association do not disrupt the interactions stabilizing class I–p88 complexes.

By comparison, the kinetics of p88 dissociation from

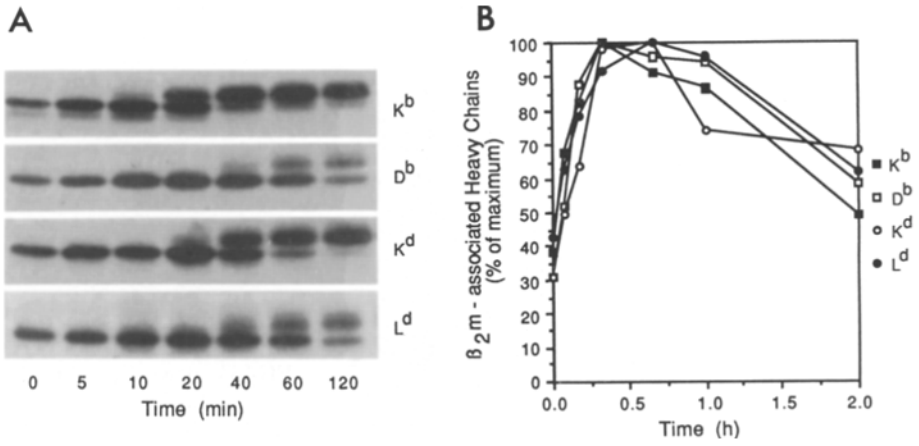


Figure 9. Kinetics of β_2m association with class I heavy chains. MDAY-D2 and EL4 cells were pulsed for 5 min with [^{35}S]Met and chased for the times indicated. Lysates prepared from MDAY-D2 cells were immunoprecipitated sequentially with the 20-8-4S (anti-K^d) and 30-5-7S (anti-L^d) monoclonal antibodies and EL4 lysates were treated sequentially with the Y-3 (anti-K^b) and B22-249.R1 (anti-D^b) monoclonal antibodies. (*A*) Analysis of immunoprecipitates by SDS-PAGE (10–15% gel) under reducing conditions. (*B*) Densitometric analysis of the relative amounts of heavy chains shown in *A*.

heavy chain-β_2m heterodimers correlated with the characteristic ER to Golgi transport rates for each specific class I molecule. Because the rate-limiting step in class I transport is export from the ER (Williams et al., 1985, 1988), dissociation of the complex could either occur in the ER and be rate-limiting for transport, or, alternatively, it could occur later in the pathway, rapidly following some other rate-limiting event in the ER. Four lines of evidence were consistent with dissociation being a pre-Golgi event, most likely occurring in the ER. First, p88 was exclusively associated with class I heavy chains that were sensitive to endo H digestion, indicating that the complex remained localized to the ER or at most reached the *cis*-Golgi cisterna. Second, class I–p88 complexes dissociated completely when ER to Golgi transport was extensively inhibited by CCCP treatment. Third, dissociation of p88 from the complex substantially preceded acquisition of endo H resistance by class I heavy chains at 15°C. Notably, p88 dissociated from L^d–p88 complexes virtually quantitatively in the absence of detectable intracellular transport. Finally, treatment of cells with BFA, a compound that blocks transport to the Golgi, had no effect on the characteristic rates of p88 dissociation. These data on the site of p88 dissociation, combined with the similarity between the characteristic dissociation and transport rates ($t_{1/2}$ p88 dissociation = 20 min K^b, 38 min K^d, 45 min D^b and L^d; $t_{1/2}$ transport to medial Golgi = 20 min K^b, 38 min K^d, 55 min D^b and L^d) suggest that the dissociation of p88 from class I–p88 complexes may represent the rate limiting step in ER to Golgi transport of class I molecules and as such appears to be a prerequisite for export of these molecules from the ER. We cannot, however, formally exclude the possibility that p88 dissociation rapidly follows some other, undefined step in the ER that limits the rate of ER to Golgi transport.

The transient association of p88 with newly synthesized class I molecules resembles the interaction of the 78-kD resident ER protein, BiP, with a number of secretory and membrane proteins. BiP is a member of the HSP70 family of proteins and it was originally identified by virtue of its stable intracellular association with Ig heavy chains in the absence of light chain synthesis. However, upon light chain association, BiP is displaced allowing mature Ig to be transported to the cell surface and secreted (reviewed in Hendershot et al., 1987). Subsequently, BiP has been reported to associate permanently with a variety of nonglycosylated, mutant, or otherwise misfolded proteins and transiently with several proteins during normal folding and/or subunit assembly in the ER (Gething et al., 1986; Dorner et al., 1987; Hurtley et al., 1989; Ng et al., 1989; Machamer et al., 1990). In these studies, it has been speculated that BiP functions to promote folding and subunit assembly of newly synthesized proteins or, alternatively, that it may retain proteins in the ER until folding or assembly are complete. Interestingly, we were unable to detect any association of class I molecules with BiP by coprecipitation either with anti-BiP antibody (kindly provided by Dr. Linda Hendershot) or anti-class I heavy chain antibody even when ATP levels were reduced to minimize ATP-induced dissociation of BiP from its associated molecules (Degen, E., unpublished observation).

BiP is thought to be a molecular chaperone; a cellular protein whose function it is to ensure that the folding of certain other polypeptide chains and their assembly into oligomeric structures occurs correctly and in so doing does not form part of the final structure (Ellis, 1987). Whereas p88 fits into the latter part of this definition, the role it may play in the folding and assembly of class I molecules is unclear. Like BiP, p88 appears to be involved in ER retention. However, whereas BiP is rapidly displaced by subunit assembly or certain folding events, neither early folding of the class I heavy chain nor the subsequent association of β_2m with the heavy chain appear to trigger displacement of p88. Although it is conceivable that p88 dissociates slowly from the complex as a direct consequence of β_2m association, it is more likely that p88 dissociation is triggered by some subsequent event. It should be noted that the well-established requirement for β_2m in class I transport suggests that its association is a necessary, although insufficient step leading to p88 dissociation.

In light of the unique immunological function of class I molecules, namely to bind antigenic peptides, a question that arises from these findings is whether peptide binding could trigger the dissociation of p88. Studies using BFA suggest that peptide binding occurs intracellularly to nascent class I molecules at some point between the ER and *trans*-Golgi complex (Nuchtern et al., 1989; Yewdell and Bennink, 1989). Furthermore, recent studies in which antigenic peptides added to detergent lysates of pulse labeled cells either promote heavy chain-β_2m assembly or stabilize preformed

heavy chain-β_2m complexes, suggest that peptide binding is an ER event (Townsend et al., 1990). Because the association of nascent heavy chain with p88 in the ER is extremely rapid and the subsequent dissociation of p88 occurs near the time of class I export from the ER, the class I–p88 complex is the most likely candidate for the form involved in peptide binding. At issue is whether peptide binding is a relatively slow event that could possibly displace p88 and permit subsequent class I transport.

The question of when peptide binding occurs has been addressed in studies on three cell lines (RMA-S, .174, and T2) that appear to possess dual defects in the presentation of endogenous (cytoplasmically processed) peptide antigens and in the intracellular assembly of class I heavy chains with β_2m (Salter and Cresswell, 1986; Ljunggren et al., 1989; Cerundolo et al., 1990; Hosken and Bevan, 1990; Townsend et al., 1989, 1990). Addition of exogenous peptides either to intact cells or to detergent lysates results in the formation of immunoprecipitable heavy chain-β_2m heterodimers. The authors suggest that the mutation in these cells somehow limits the access of endogenous peptides to newly synthesized class I molecules and that peptides bind to free heavy chains and stabilize the heavy chain in a conformation capable of associating with β_2m. In such a scenario, peptide binding is a rapid event occurring at an early stage in the biogenesis of class I molecules, before β_2m association, when class I heavy chains are complexed with p88. If this scheme can be shown to be generally applicable, then dissociation of p88 from class I–p88 complexes could not be promoted by peptide binding; it would have to be triggered by some other, later event.

A number of observations suggest, however, that peptide may not be required for heavy chain-β_2m association. First, addition of purified β_2m to detergent lysates of RMA-S cells results in stable association of heavy chain with β_2m in the absence of added peptide (Townsend et al., 1990). Second, when a purified class I molecule (H-2K^b) is dissociated in vitro, the component heavy chain and β_2m can reassociate and regain serological epitopes presumably in the absence of peptide (Yokoyama et al., 1985). Third, upon incubation of the RMA-S cell line at 23–33°C, class I heavy chains and β_2m associate into stable peptide-free heterodimers that are transported to the cell surface (Ljunggren et al., 1990). This also occurs at 37°C, albeit to a lesser degree. These surface heterodimers are relatively unstable ($t_{1/2}$ ~1 h) at 37°C but can be stabilized ($t_{1/2}$ ~6 h) by the subsequent binding of specific peptides. Finally, a recent examination of class I molecules in the T2 cell line, after radiolabeling at 37°C in the absence of added peptide, has shown that the HLA-A2 heavy chain is quantitatively associated with β_2m (Cresswell, P., personal communication).

Taken together, these findings suggest that peptide binding is not required for initial heavy chain-β_2m association, but rather it may increase the stability of the heavy chain-β_2m interaction. Therefore, the kinetics of β_2m association into class I complexes stable to immunoprecipitation with β_2m-dependent antibodies as observed in this study (Fig. 9) may not reflect corresponding peptide binding kinetics. Instead, the ability to detect these complexes (presumably less stable than peptide bound complexes) could depend on other cell-specific factors such as high concentrations of intracellular β_2m or, alternatively, the nature of the immunoprecipitation conditions. If this is the case in our experiments, then peptide binding may follow our detection of heavy chain-β_2m heterodimers. Peptide binding could induce a conformational change in the heavy chain that might stabilize heavy chain-β_2m interactions within class I–p88 complexes, trigger p88 dissociation, and thereby promote class I transport. In this view, the observed differences in transport rates for various class I molecules could be a reflection of their relative abilities to bind specific peptides, or perhaps, might reflect the supply of peptides available for association with a particular class I–p88 complex. Interestingly, the necessity of peptide binding for p88 dissociation and subsequent class I transport may be circumvented at 23–33°C. It is noteworthy that Townsend and colleagues (1990) have observed a species with an apparent molecular mass of 105 kD coimmunoprecipitating with class I molecules from RMA-S cell extracts incubated with excess β_2m but not with β_2m and peptide together. This species may be p88 that is associated with peptide-free heavy chains-β_2m complexes and that is displaced in the presence of peptide.

Regardless of the event that triggers p88 dissociation, a likely possibility for the function of p88 is that it retains class I molecules in the ER during the formation of the ternary complex of heavy chain, β_2m, and peptide. p88 could also promote the binding of either β_2m, peptide, or both to the heavy chain. Recently, we have obtained some variant class I molecules that do not form complexes with p88. It should be possible to test the above ideas through analysis of the intracellular transport and antigen presentation capabilities of these variant molecules.

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