

# Invariant Chain Trimers Are Sequestered in the Rough Endoplasmic Reticulum in the Absence of Association with HLA Class II Antigens

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**Abstract.** HLA class II antigens are heterodimeric cell surface glycoproteins that interact with antigenic peptides to form complexes recognizable by CD4-positive T cells. During their biosynthesis, class II antigens are retained in a post-Golgi compartment in association with the invariant chain, which dissociates before class II cell surface expression. To address whether the invariant chain mediates this post-Golgi retention, its transport and assembly were examined in cells that do not express HLA class II antigens. Pulse-chase analysis and endoglycosidase digestions showed that very little invariant chain proceeded as far as the *trans*-Golgi in class II-negative cell lines. Immunofluorescence studies suggested that in these cells the invariant chain is sequestered in the RER. Gel filtration

and cross-linking data showed that RER-localized invariant chain is present as trimers or aggregated trimers. Multimerization is mediated by luminal interactions; a proteolytic fragment of the invariant chain corresponding to the luminal domain remained trimeric as determined by cross-linking analysis. Similar transport and structural characteristics were observed for a pool of excess invariant chain in class II-positive cells, suggesting that an excess of invariant chain in the ER may be important for class II antigen function. These results have important implications for the transport of cellular proteins in general and for the role of the invariant chain in class II antigen biosynthesis.

**M**AJOR histocompatibility complex (MHC) class II antigens are heterodimeric glycoproteins found on the surface of B lymphocytes, macrophages, and a limited number of other cell types (26). Class II antigens bind peptides, generated by proteolysis and/or denaturation of foreign antigens, in such a manner that the class II/peptide complex is recognized by antigen-specific, MHC-restricted CD4-positive T lymphocytes (19, 59).

Intracellularly, the class II  $\alpha$  and  $\beta$  chains associate with a third glycoprotein called the invariant chain (13). This molecule is a nonpolymorphic, basic transmembrane glycoprotein that is coordinately expressed with class II antigens (36). The invariant chain gene, which is unlinked to the HLA complex (7), encodes at least three polypeptides. The predominant polypeptide form is referred to as p33. The p35 and p41 forms result from alternate translation of the predominant mRNA species and alternate mRNA splicing, respectively (54, 55). The invariant polypeptides are type II

transmembrane glycoproteins, with NH<sub>2</sub> termini facing the cytoplasm and COOH termini facing the lumen of intracellular compartments (6, 33, 56). They bear two *N*-linked glycans (perhaps more on p41), both of which are converted to the complex type in the *trans* Golgi, and at least two *O*-linked oligosaccharides (38, 39). In addition, invariant chains can be modified by the addition of a chondroitin sulfate side chain to generate a proteoglycan (17), and some invariant chains appear to be phosphorylated (51).

Like most transmembrane proteins, the  $\alpha$  and  $\beta$  chains of class II antigens of the human MHC, or HLA, complex and the invariant chain are synthesized on polysomes associated with the RER. They associate within 10 min in the RER (31, 38), an event which is mediated by luminal interactions (41). The complex is then transported through the Golgi apparatus with a half-time of 30–60 min, as measured by acquisition of resistance to digestion by endoglycosidase H (endo H) and of charge heterogeneity due to addition of sialic acid (31, 38). In contrast to most cell surface proteins, for which the rate limiting step in cell surface expression is exit from the RER (35, 46), class II antigens are delayed in a post-Golgi compartment; the mature  $\alpha\beta$  dimer does not arrive at the cell surface until 2–3 h after the  $\alpha\beta$ I complex has acquired sialic acid (12). During the post-Golgi lag period, the invariant chain is dissociated from the  $\alpha$  and  $\beta$  chains by proteo-

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lytic cleavage, and is generally not detectable on the cell surface (1).

The contribution of the invariant chain to the biosynthetic peculiarities of class II antigens is unknown. It is clear that it is not required for HLA class II antigen transport to the cell surface, as HLA-DR  $\alpha$  and  $\beta$  chain transfectants express class II on the cell surface equally well in the absence or presence of cotransfected invariant chain (49). The effect of invariant chain on the intracellular transport of class II antigens is less clear. Whereas invariant chain altered the transport kinetics and glycosylation of  $\alpha$  and  $\beta$  chains in microinjected *Xenopus* oocytes (8), it did not appear to affect these parameters in Balb/c 3T3 transfectants (50). On the other hand, the invariant chain has been shown to functionally affect the ability of class II transfectants to process and present at least some intact antigens to antigen-specific/MHC-restricted T lymphocytes (53). The mechanism responsible for this functional effect is not known.

One proposed function for the invariant chain is to serve as a signal for HLA class II antigens to be transported to or retained within a post-Golgi intracellular compartment (13). This compartment may be that in which newly synthesized class II antigens intersect the endocytic pathway (11), and in doing so interact with endocytosed and processed foreign peptides (20). Alternatively, the post-Golgi compartment may serve to retain class II molecules before this point. If invariant chain mediated localization to this compartment, one might expect that it would localize there in the absence of HLA class II antigens. Experiments designed to test this hypothesis revealed that the vast majority of invariant chain is not localized to a post-Golgi compartment in the absence of class II antigens, confirming a recent report using transfectants (50), but rather is retained within the RER. Our data further suggest that RER-localized invariant chain is oligomeric, and that similarly localized and oligomerized excess invariant chain is present in class II-positive cells.

## Materials and Methods

### Materials

All chemicals and proteins were purchased from Sigma Chemical Co. (St. Louis, MO) unless otherwise noted. Chemicals for electrophoresis were purchased from Bio-Rad Laboratories (Richmond, CA), and chromatographic media and supplies were purchased either from Bio-Rad Laboratories or from Pharmacia Fine Chemicals (Piscataway, NJ).

### Cells

The B lymphoblastoid cell lines (B-LCL) Swei (DR5,5) (38), Jijoye (DR2,5) (23), and T5-1 (DR1,3) (18); the class II-negative mutant B-LCL P3HR-1 (23, 52) and 6.1.6 (18); the class II-negative T-LCL CEM; the class II-negative T-B hybrid cell line 174xCEM.T2 (T2); and its class II-positive parent 174xCEM.T1 (T1) (48) were maintained as described (40), except that in some cases, 3–10% iron/transferrin-supplemented calf serum (HyClone Laboratories, Logan, UT) was substituted for FBS. P3HR-1 was a gift of Dr. R. E. Humphreys (University of Massachusetts, Worcester, MA), and 6.1.6 and T5-1 were gifts of Dr. D. Pious (University of Washington, Seattle, WA).

### Antisera and mAbs

The rabbit antisera 247/HSB (anti-class II; 40), O14 (anti-galactosyl transferase; 47), and  $\alpha$ CD-Munster (anti-cathepsin D; 22) and mAbs DA6.147 (anti-DR $\alpha$ ; 21), XD5.A11 (anti-class II $\beta$ ; 27), L243 (anti-HLA-DR; 32), RL-77 (anti-protein disulfide isomerase; 25), and 14-4-4s (anti-I-E<sup>k</sup>; 44) have been described. mAb POP.14.3 (IgM, K) was prepared by immunizing

Balb/c mice with a preparation of whole cellular membranes from T2. By immunoprecipitation and solid-phase RIA it is reactive only with human invariant chain. Rabbit antiserum Matilda was prepared from a female NZB rabbit that was injected with material purified from T2 by affinity chromatography on POP.14.3-Bio-gel A50m (Bio-Rad Laboratories) followed by gel filtration on Sephacryl S300 in 3-(3-cholamidopropyl) dimethylammonio-1-propanesulfonate detergent. For some experiments, Matilda was absorbed with the T-LCL CEM ( $5 \times 10^8$  cells/ml) to remove nonspecific antibodies, resulting in Matilda<sub>CEM</sub>. Rabbit antiserum EQLP was prepared from a rabbit injected with a peptide corresponding to amino acids 12–28 of the invariant chain cytoplasmic domain, covalently coupled to keyhole limpet hemocyanin (Calbiochem-Behring Corp., La Jolla, CA). O14,  $\alpha$ CD-Munster, and RL-77 were generous gifts of Dr. E. Berger (Universitat Zurich, Switzerland), Dr. T. Bräulke (Georg-August-Universität, Göttingen, FRG), and Dr. C. Kaetzel (Case Western Reserve School of Medicine, Cleveland, OH), respectively. We thank Dr. R. B. Corley and Ms. D. White (Duke University, Durham, NC) for the gift of 14-4-4s supernatant.

### Radiolabeling

Continuous labeling with [<sup>35</sup>S]methionine ([<sup>35</sup>S]met) was performed essentially as described (38), except that met-free DME (Gibco Laboratories, Gaithersburg, MD) and Tran<sup>35</sup>S-label (>1,000 Ci/mmol; ICN Radiochemicals, Irvine, CA) were used as medium and radiolabel, respectively. Labeling was performed for 8 h, and cells were either frozen at  $-70^\circ\text{C}$  or used immediately. Pulse-chase studies were performed as described (1).

### Cell Extracts

Cells were extracted in 0.15 M NaCl, 0.01 M Tris, pH7.4 (TS) containing proteinase inhibitors and either 1% Triton X-100 for immunoprecipitations (38) or 1% polyoxyethylene-9-lauryl ether (C<sub>12</sub>E<sub>9</sub>) for affinity purifications (27), as described. In most experiments, freshly prepared 20 mM iodoacetamide was included in the extraction buffer.

### Immunoprecipitations

Preclearing of extracts with normal rabbit serum (NRS)<sup>1</sup> and Pansorbin (Calbiochem-Behring Corp., La Jolla, CA) was performed as described (38) for 30–90 min at 4°C. Extracts were immunoprecipitated as described for 3–15 h at 4°C with rabbit antisera or mAb supernatants, followed by Protein A-Agarose (Calbiochem-Behring Corp.) or rabbit anti-mouse Ig-coated Protein A-agarose. Immunoprecipitation with NRS or nonspecific monoclonal antibody was performed simultaneously as a negative control and showed no bands or spots in all cases. For preclearing with anti-class II antibodies, extracts were precipitated for 4–15 h each with Bio-gel A50m beads to which the antibodies XD5.A11 and DA6.147 were conjugated by cyanogen bromide activation (27).

### Electrophoresis

One-dimensional SDS-PAGE and two-dimensional PAGE (with nonequilibrium pH gradient electrophoresis in the first dimension and SDS-PAGE in the second dimension) were performed as described (38). Molecular weight markers were either radiolabeled with <sup>14</sup>C (Amersham Corp., Arlington Heights, IL) or prestained (Bio-Rad Laboratories or Sigma Chemical Co.), and were calibrated against the migration of Coomassie blue-labeled protein standards. In cross-linking experiments,  $\alpha_2$ -macroglobin, a generous gift of Dr. Paul Roche (Duke University, Durham, NC), was used under nonreducing (360,000 D) and reducing conditions (180,000 D) as a high molecular weight marker.

### Fixation, Fluorography, and Autoradiography

Gels were fixed and fluorographed with Enlightning (New England Nuclear, Boston, MA) as described (1), and dried and exposed to Kodak X-OMAT AR film at  $-70^\circ\text{C}$ .

### Western Blotting

The procedure was performed as described (58), except that gels were trans-

1. *Abbreviations used in this paper:* DOC, deoxycholate; DSP, dithio-bispropionic acid *N*-hydroxysuccinimide ester; DSS, disuccinimidyl suberimide; NRS, normal rabbit serum.

ferred immediately after electrophoresis to nitrocellulose, and blots were blocked in Blotto (5% [wt/vol] powdered milk [Cost-Cutter; Kroger, Cincinnati, OH]/0.05% [vol/vol] Tween 20/0.02% [wt/vol] sodium azide in PBS) for at least 1 h at room temperature. Blots were then incubated at 4°C overnight with primary antibody diluted in Blotto. The secondary reagent was an mAb that recognizes rabbit IgG, LORGI (Sera-Lab, Westbury, NY), labeled with  $^{125}\text{I}$  using chloramine T (27).

### Ethanol Precipitations

Glycogen was added as a carrier to 0.1–0.2 mg/ml, and samples were precipitated with a 7–10 times excess of 100% ethanol at  $-20^\circ\text{C}$  overnight.

### Affinity Chromatography

The procedure was essentially as described by Kølner and Cresswell (27). Purified mAbs or mouse IgG were coupled at 1–2 mg/ml to cyanogen bromide-activated Biogel A50m, and a precolumn of mouse IgG–Biogel A50m was used in tandem with the desired affinity column to remove nonspecific material. After washing, bound material was eluted with elution buffer (0.1 M Tris/5% [wt/vol] glycerol pH 11.0 containing detergent), and immediately neutralized with solid glycine.

### Endoglycosidase Treatments

Digestions with endoglycosidases H and F (endo H; ICN Immunochemicals, endo F; Boehringer Mannheim Biochemicals, Indianapolis, IN) were performed on ethanol-precipitated samples as described (1, 38).

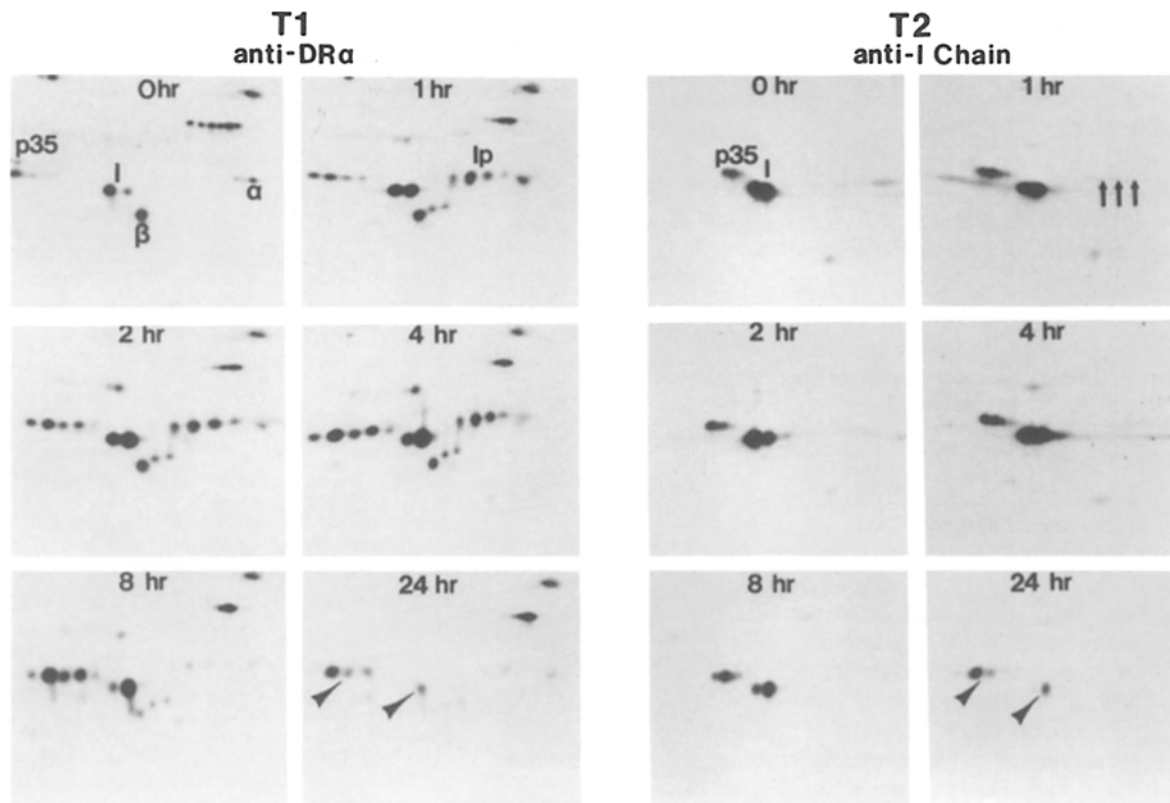
### Gel Filtration

Affinity-purified radiolabeled material or whole cell extracts in 1%  $\text{C}_{12}\text{E}_9$ ,

with [ $^3\text{H}$ ]glucosamine added as an inclusion marker and blue dextran 2000 as an exclusion marker, were applied directly to a  $50 \times 2.5$  cm column of Sephacryl S300 equilibrated in 0.02 M bicine pH 8.1/0.13 M NaCl (BS) containing either 0.1%  $\text{C}_{12}\text{E}_9$  or 0.5% (wt/vol) recrystallized sodium deoxycholate (DOC; Fisher Scientific, Lexington, MA; 27). The Sephacryl S300 column was calibrated in each detergent with blue dextran 2000 (D; excluded), bovine thyroglobulin (TG or Th; 667,000), mouse IgG (I; 155,000), human transferrin (Tf; 80,500), ovalbumin (O; 44,500), and [ $^{14}\text{C}$ ]lysine or [ $^3\text{H}$ ]glucosamine (included volume). The elution positions of detergent micelles were determined by the mobility of [9,10(*n*)- $^3\text{H}$ ]palmitic acid (500 mCi/mmol; Amersham Corp.). By this method, the deoxycholate (DOC) micelle eluted in the position of a 10,000-D globular protein, and the  $\text{C}_{12}\text{E}_9$  micelle as a 140,000-D globular protein.

### Cross-linking

For purified material, pooled fractions from gel filtration in BS/ $\text{C}_{12}\text{E}_9$  were treated for 30 min at  $4^\circ\text{C}$  with 3,3'-dithio-bis-propionic acid *N*-hydroxysuccinimide ester (DSP) at concentrations ranging from 1.5–100  $\mu\text{g}/\text{ml}$ . In some experiments, DSP was purchased from Pierce Chemical Co. (Rockford, IL), and required higher concentrations than the Sigma material to achieve similar cross-linking. Cross-linking reactions were stopped by adding glycine or ammonium chloride to 10 mM, and samples were precipitated with ethanol, subjected to nonreducing SDS-PAGE and analyzed by autoradiography or Western blotting. For homogenized cellular membranes, log phase cells were pelleted, washed three times with PBS, and resuspended at  $50\text{--}100 \times 10^6/\text{ml}$  in 0.25 M sucrose (Ultra-Pure; Bethesda Research Laboratories, Gaithersburg, MD), 1 mM sodium bicarbonate/0.5 mM calcium chloride/1 mM magnesium chloride/0.02% (wt/vol) sodium azide. Cells were lysed on ice by 50–100 strokes of a 15 ml Potter-Elvehjem glass/Teflon homogenizer (Kontes Co., Vineland, NJ) that was driven by a drive with an extendible drive shaft attachment (Tri-R Stir-R model K43; Cole-Parmer Instrument Co., Chicago, IL). Lysis efficiency was monitored



**Figure 1.** Pulse-chase analysis of invariant chain in presence or absence of HLA class II antigens. T1 (left) or T2 (right) cells were pulse labeled with [ $^{35}\text{S}$ ]met for 10 min and then chased for the times indicated. T1 cell extracts from each time point were immunoprecipitated with DA6.147 (anti-DR $\alpha$ , left) and T2 cell extracts with Matilda<sub>CEM</sub> (anti-invariant chain, right). Immunoprecipitates were analyzed by two-dimensional PAGE. Arrows indicate position expected for processed invariant chain (*Ip*) in T2 immunoprecipitates. Arrowheads indicate p35 and p33 (*I*) forms of invariant chain present after 24 h.

by staining with trypan blue, and homogenization was continued until <5% of cells were intact. Nuclei and cell debris were pelleted by centrifugation at 900 g for 10 min. The supernatant was layered over a 0.2-ml cushion of 1.58 M sucrose in 3 mM imidazole and mitochondria were pelleted by centrifugation at 2,200 g for 15 min. Supernatant and interface were pooled, and cellular membranes were pelleted by ultracentrifugation at 100,000 g. Supernatants, consisting of soluble cytoplasmic proteins, were discarded. Membranes were resuspended in BS and treated with 1.5–400  $\mu$ g/ml disuccinimidyl suberimide (DSS; Pierce Chemical Co.), a non-reducible analogue of DSP, for 30 min at 4°C. After stopping the reaction with ammonium chloride as described above, membranes were solubilized in Triton X-100 (1% wt/vol) and analyzed by one-dimensional SDS-PAGE and Western blotting.

### Proteinase K Treatment

[<sup>35</sup>S]met-labeled affinity-purified invariant chain was dialyzed into T2 7.4/0.1% C<sub>12</sub>E<sub>9</sub> and digested with 10  $\mu$ g/ml proteinase K as described (41). Samples were either precipitated with ethanol or fractionated by gel filtration prior to analysis.

### Intracellular Immunofluorescence

Log-phase cells were analyzed essentially as described by Burkhardt et al. (4). Briefly, 10<sup>3</sup>–10<sup>4</sup> cells were allowed to adhere to poly-L-lysine-coated wells of immunofluorescence slides (Polysciences, Inc., Warrington, PA), fixed with 2% (wt/vol) paraformaldehyde in PBS, quenched with ammonium chloride, and permeabilized for 1 h with PBS containing 0.01% (wt/vol) saponin and 0.25% (wt/vol) bovine skin gelatin (PBS/sap/gel). Wells were incubated with mAb supernatant or with ascites or serum diluted in PBS/sap/gel for 1 h, washed with PBS/sap/gel, and incubated with fluoresceinated goat anti-mouse immunoglobulin or rhodaminylated goat anti-rabbit IgG (Cappel Laboratories, Malvern, PA) diluted in PBS/sap/gel. Slides were washed with PBS/sap/gel, sealed under 10–50% glycerol in PBS containing 0.1% phenylenediamine or 1,4-diazabicyclo-(2,2,2) octane (Polysciences, Inc.) to minimize quenching, and examined under a MRC-5000 confocal microscope (Bio-Rad Laboratories) using the EHS filter for fluorescein or the GHS filter for rhodamine and photographed from the computer-enhanced image using Kodak TMAX-125 film.

## Results

### Invariant Chain Is Synthesized But Not Processed in a Class II-negative Hybrid Cell Line

Invariant chain synthesis was initially characterized by pulse-chase analysis in the hybrid cell line T2 (48). T2 expresses no MHC class II antigens due to the deletion of all functional genes from the HLA-D region. However, the cell does express very high levels of invariant chain. For comparison, invariant chain associated with HLA-DR antigens was analyzed in T1 cells, the HLA class II-positive parental cell line of T2. Two-dimensional PAGE analysis of immunoprecipitates (Fig. 1) indicates that the p33 and p35 forms of invariant chain are synthesized in T2. However, very little processed invariant chain is observed, even after 24 h of chase. Thus, invariant chain oligosaccharides are relatively unprocessed in T2 compared with the highly processed glycans of class II antigen-associated invariant chain in T1.

To confirm the lack of N-linked glycan processing, 8-h [<sup>35</sup>S]met-labeled invariant chain from T2 and from another invariant chain-positive class II-negative mutant cell line, P3HR-1, was treated with endo H. As shown in Fig. 2, virtually all of the detectable invariant chain in both cell lines is as sensitive to endo H as it is to endo F. Identical results were obtained in T2 by Western blotting (not shown), demonstrating that the 8-h [<sup>35</sup>S]met label reflected invariant chain steady state levels. In contrast, most of the class II-associated invariant chain from similarly labelled class II-

positive cells was resistant to digestion with endo H, as found previously (39, results not shown).

### Invariant Chain Is Localized to the RER in the Absence of HLA Class II Antigens

The lack of oligosaccharide processing suggested that invariant chain was retained in a subcellular compartment proximal to the medial Golgi in cells that lack class II antigens. To further localize this compartment, class II-negative cell lines were analyzed by indirect intracellular immunofluorescence using the anti-invariant chain serum Matilda<sub>CEM</sub>. The patterns that emerged in T2, P3HR-1, and a third invariant chain-positive class II-negative mutant, 6.1.6, were identical, showing intense staining encircling the cell nucleus (Fig. 3 a). An invariant chain-negative T cell line, CEM, showed no such staining. To determine the subcellular compartment to which this pattern corresponded, T2 was stained with antibodies to proteins previously localized to RER, Golgi, and lysosomes/endosomes (Fig. 3 b). The invariant chain pattern

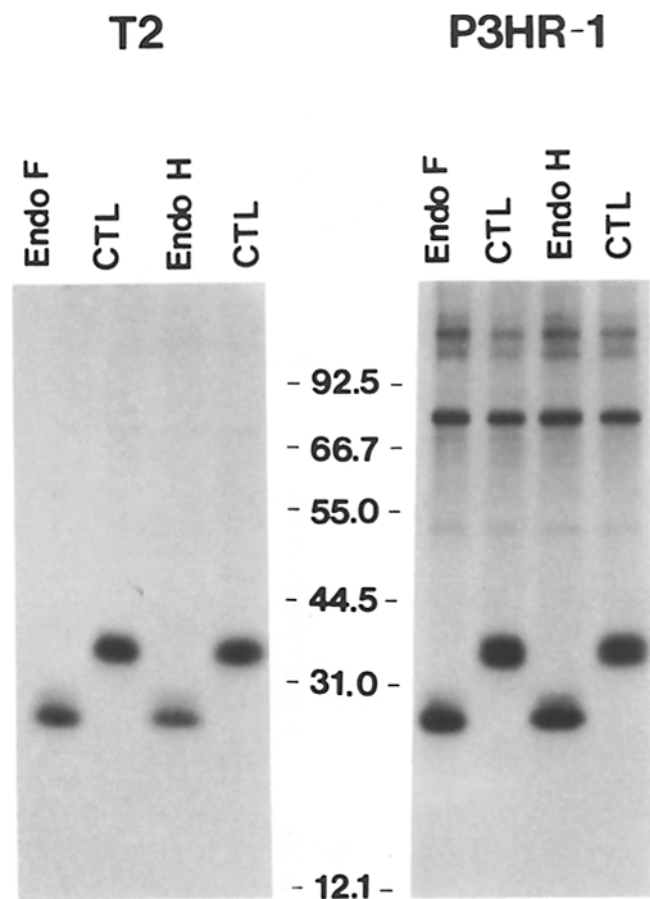
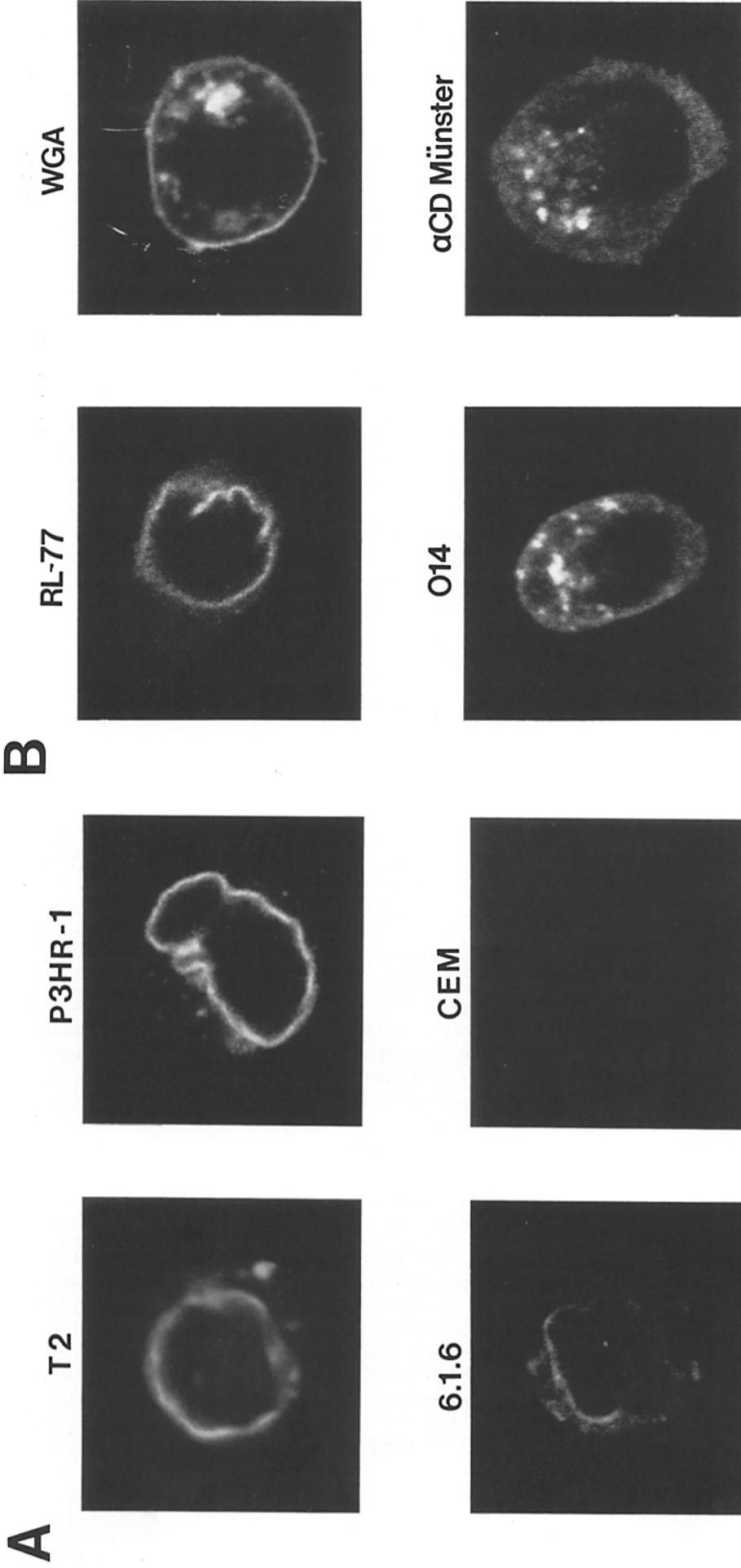
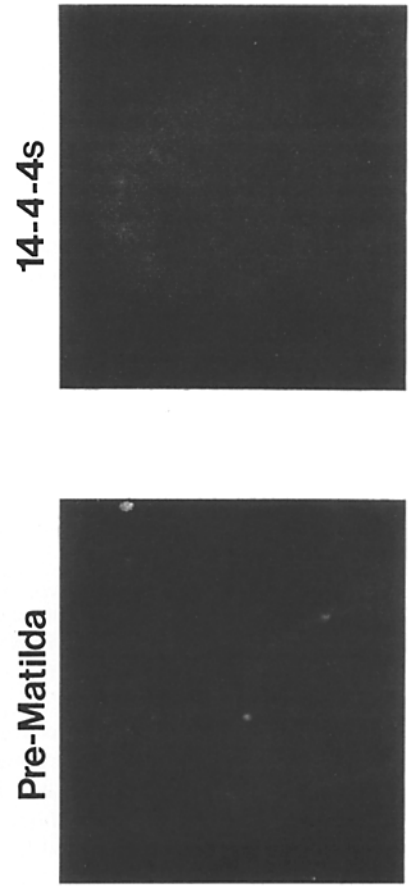


Figure 2. Invariant chain synthesized in the absence of HLA class II antigens remains sensitive to endo H. Invariant chain was affinity purified on POP.14.3-Biogel from T2 or P3HR-1 cells labeled for 8 h with [<sup>35</sup>S]met, and eluates were further purified by gel filtration on Sephacryl S300 in C<sub>12</sub>E<sub>9</sub>. Peak fractions were ethanol precipitated and either mock-treated (CTL) or digested with endo H or endo F. Products were reprecipitated with ethanol and analyzed by one-dimensional SDS-PAGE. Positions of molecular weight markers (kilodaltons) are indicated.



**Figure 3.** Immunofluorescence localization of invariant chain in class II-negative cells. (A) Class II-negative, invariant chain-positive cells T2, P3HR-1, and 6.1.6 and the class II-negative, invariant chain-negative cell CEM were analyzed by intracellular immunofluorescence, as described in Materials and Methods, using Matildacem as the primary antibody. (B) Log phase T2 cells were analyzed by intracellular immunofluorescence using the indicated primary antibodies or wheat germ agglutinin (WGA). Pre-Matilda and 14-4-4s serve as negative controls for rabbit and mouse antibodies, respectively. Antibodies and the organelles they stain are: RL-77, RER; O14, trans-Golgi;  $\alpha$ CD Münster, lysosomes and endosomes; WGA, organelles distal to medial Golgi.



is identical to that observed upon staining with RL-77, an mAb recognizing the RER resident protein disulfide isomerase. In contrast, staining with O14 (anti-galactosyl transferase; *trans*-Golgi),  $\alpha$ CD Munster (anti-cathepsin D; lysosomes/endosomes), or the lectin wheat germ agglutinin (*trans*-Golgi and distal compartments; 60) resulted in patterns that were completely distinct from that obtained with anti-invariant chain reagents. Thus, the invariant chain appears to be sequestered in the RER in the absence of HLA class II antigens.

### ***Invariant Chain Self-Associates in the Absence of HLA Class II Antigens***

Retention of invariant chain within the RER could be due to any of a variety of factors. Misfolded transmembrane proteins are often retained within the RER until they are degraded in lysosomes or in a RER-associated degradation pathway (28, 46). A similar fate is observed for incompletely complexed proteins, such as individual subunits of the T cell receptor/CD3 complex (5, 34, 42) or mutants of the vesicular stomatitis virus glycoprotein (VSV-G) or influenza hemagglutinin (flu-HA) that fail to associate into trimers (9, 10, 14, 16, 30), and for HLA-DR molecules containing a mutant  $\alpha$ -subunit (28a). It was therefore possible that invariant chain retention in the RER resulted from a failure to fold or oligomerize properly. In addition, some misfolded or incompletely complexed protein subunits have been shown to bind within the RER to the heat shock-related protein BiP (2, 16, 24) and it has been proposed that this interaction results in retention (2). We were therefore interested in determining whether invariant chain, in the absence of class II antigens, could be found in association with other molecules.

Invariant chain from T2 cells labeled for 8 h with [<sup>35</sup>S]met was affinity purified using the mAb POP.I4.3 and analyzed by gel filtration and by cross-linking with the homobifunctional, reducible cross-linking reagent DSP. DOC was used in gel filtration due to its small micelle size, but it was found that DSP-mediated cross-linking of membrane-bound proteins was inhibited in DOC. Therefore, cross-linking was performed after gel filtration in C<sub>12</sub>E<sub>9</sub>, a detergent of large micelle size in which invariant chain migrated primarily with the void volume.

The DOC gel filtration profile of the [<sup>35</sup>S]met-labeled POP.I4.3 eluate (Fig. 4 *a*) showed three overlapping peaks of surprisingly large molecular size. Invariant chain, as judged by SDS-PAGE (Fig. 4 *a*, *inset*) was seen predominantly in the second and third peaks, which eluted with the  $M_r$  of globular proteins of 550,000 and 220,000, respectively. No other radiolabeled bands migrated with the invariant chain in gel filtration, suggesting a lack of association with other proteins.

Cross-linking of the affinity eluate with increasing amounts of DSP resulted in the step-wise appearance of a number of high  $M_r$  bands on SDS-PAGE (Fig. 4 *b*). A band of  $M_r$  65,000 was present even in untreated samples under non-reducing conditions and corresponds to a disulfide-linked invariant chain dimer. The most prominent cross-linked band migrated with  $M_r$  95,000–106,000, as would be expected for a trimer. Other bands, corresponding in migration to invariant chain tetramers and, more intensely, hexamers (when analyzed on a semi-log plot: not shown) were also apparent

with the addition of 50–100  $\mu$ g/ml DSP. The end products consisted of a mixture of the  $M_r$  95,000 band and higher  $M_r$  bands. Thus, the DOC gel filtration peaks of  $M_r$  220,000 and 550,000 probably correlate with cross-linked trimers and higher order multimers of invariant chain, respectively.

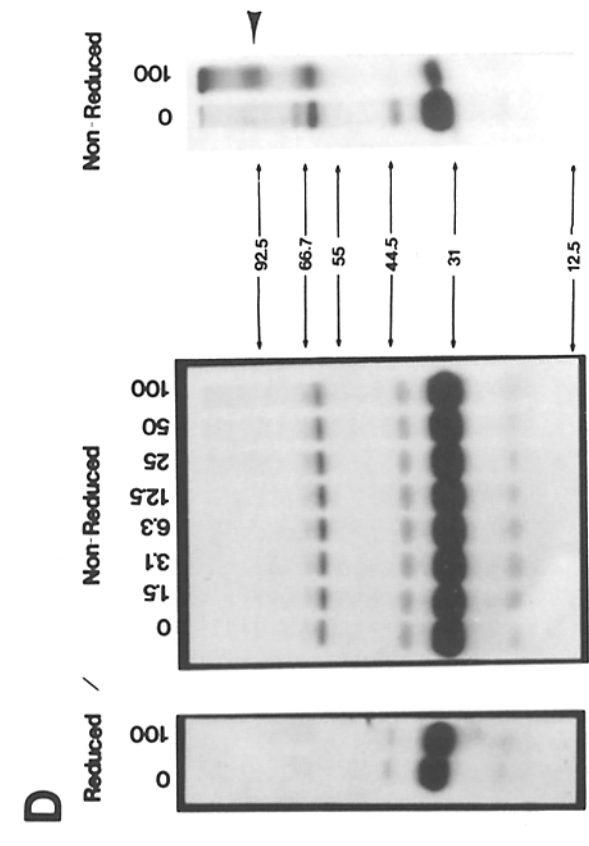
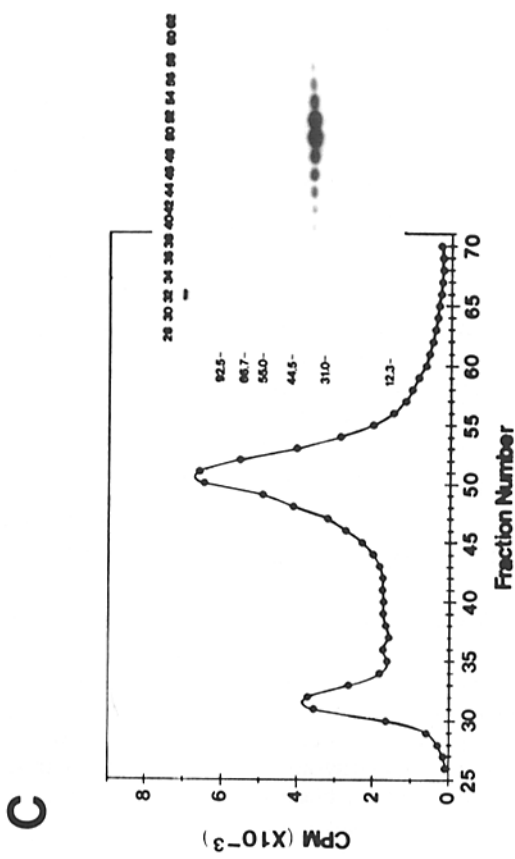
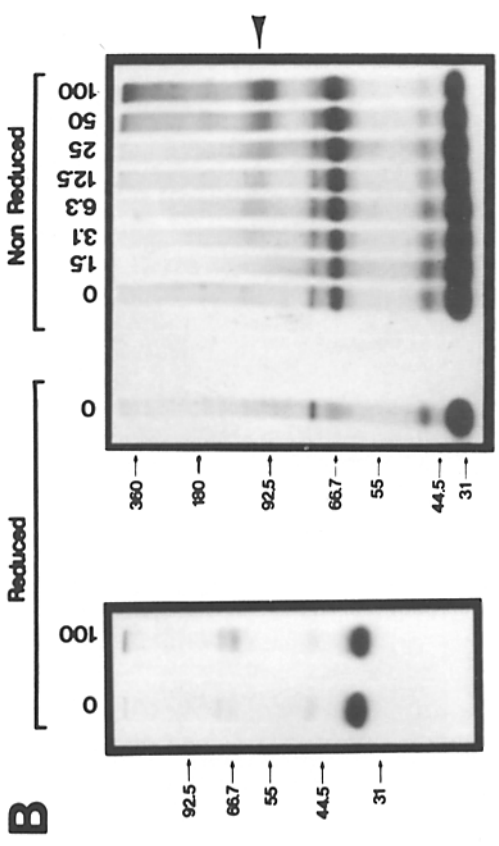
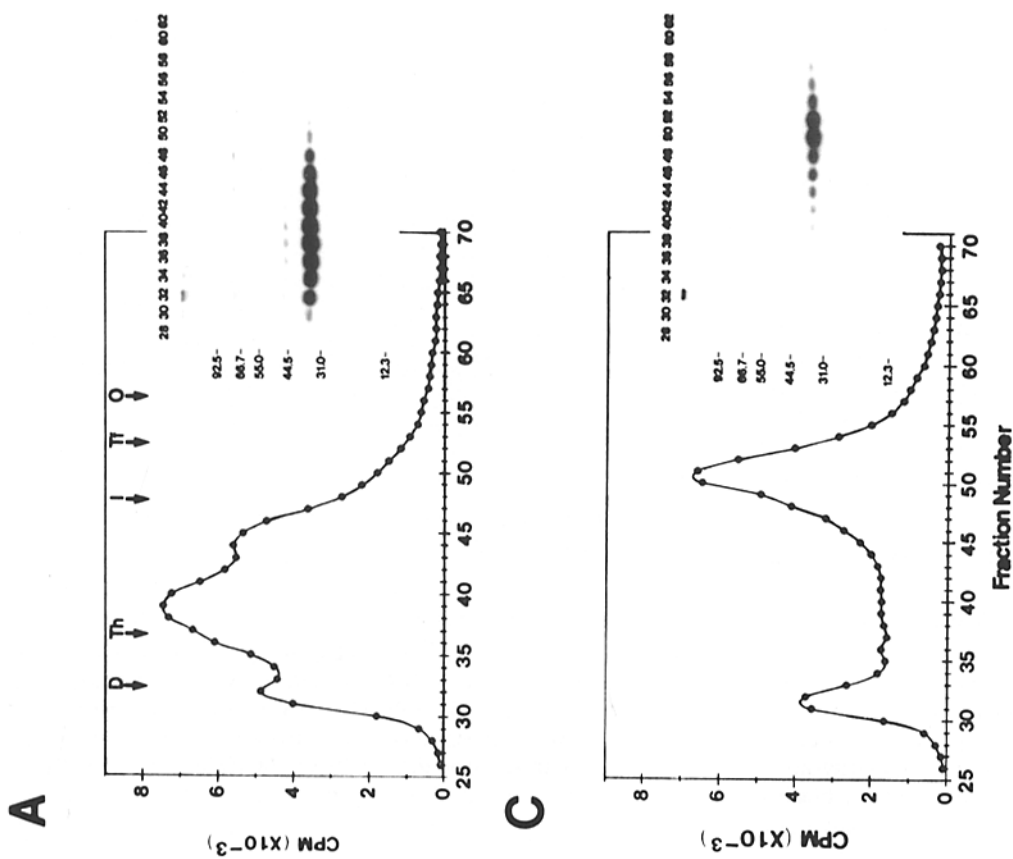
To show that cross-linking was itself not nonspecifically aggregating invariant chain monomers, the [<sup>35</sup>S]met-labeled affinity eluate was denatured by treating at 42°C for 2 h. As shown in Fig. 4 *c*, the 42°C treatment resulted in a shift of the DOC gel filtration peak to  $M_r$  80,000–90,000. When the denatured material was purified in C<sub>12</sub>E<sub>9</sub> and treated with DSP, there were no observable cross-linked products, even upon overexposure of the autoradiograph (Fig. 4 *d*). Nondenatured invariant chain which had been kept at 4°C, treated with DSP, and analyzed coincidentally served as a positive control. Thus, the cross-linked products observed with nondenatured material are preformed complexes that can be dissociated to monomers with an apparent  $M_r$  of 80,000–90,000 in DOC, by treatment at 42°C.

Invariant chain aggregation could also have been induced by the affinity purification conditions. To eliminate this possibility, gel filtration and cross-linking experiments were repeated using unpurified, unlabeled T2 cell extracts. Western blot analysis of DOC gel filtration fractions showed that unpurified invariant chain eluted as a homogeneous peak with an approximate apparent  $M_r$  of 220,000 (Fig. 5 *a*). Western blotting analysis demonstrated that maximally cross-linked unpurified invariant chain migrated with an apparent  $M_r$  of 105,000 by SDS-PAGE (Fig. 5 *b*), again consistent with an invariant chain trimer. No higher order multimers were observed. Thus, invariant chain trimers exist in detergent extracts in the absence of affinity purification.

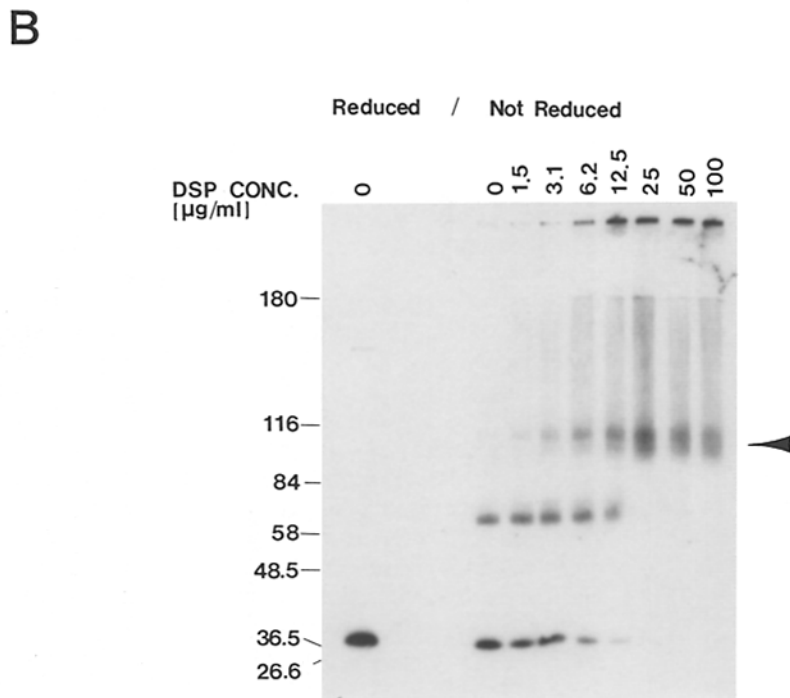
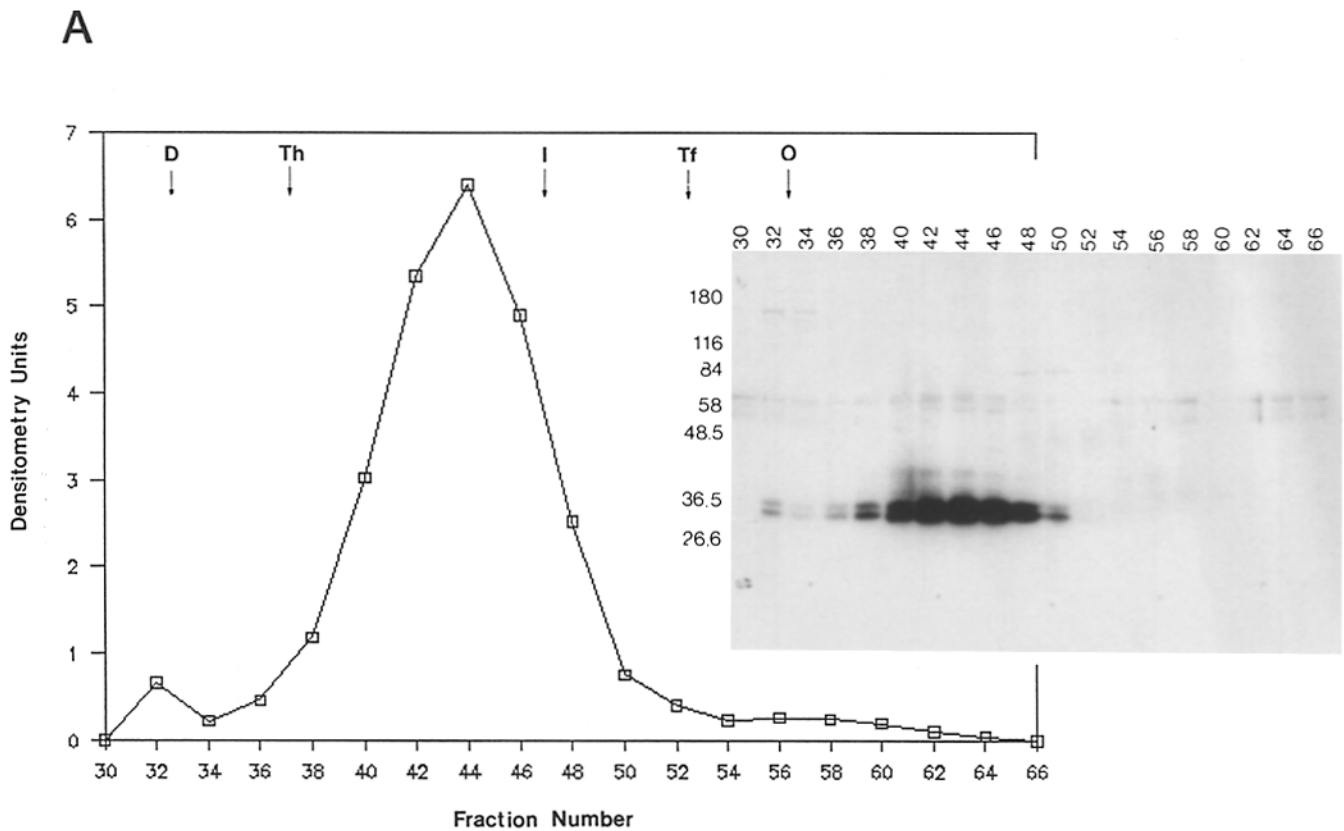
To determine whether invariant chain multimers exist *in vivo*, T2 cell membranes were cross-linked in the absence of detergent. Total cellular membranes were treated with increasing concentrations of disuccinimidyl suberimidate (DSS), a nonreducible analogue of DSP. Western blot analysis of solubilized membranes with an anti-invariant chain peptide antibody, EQLP, revealed a shift of the 33,000  $M_r$  invariant chain band to a primary 105,000  $M_r$  species with increasing amounts of DSS (Fig. 6), again consistent with a trimer. Higher order multimers were also observed, but to a lesser extent than the trimers. Probing a similar blot with an antibody to a resident RER protein, protein disulfide isomerase, revealed no observable cross-linked products (not shown), demonstrating that the invariant chain multimers observed were specific. Thus, invariant chain is primarily trimeric in the RER membrane, although some higher multimers may exist.

### ***Invariant Chain Multimerization Is Mediated by the Luminal Region***

Invariant chain could potentially self-associate by interactions within the cytoplasmic, transmembrane, or luminal domains. Proteolytic fragments of the invariant chain, lacking the transmembrane region and cytoplasmic domain, were previously shown to result from proteinase K treatment of HLA-DR  $\alpha\beta$ -invariant chain complexes. These fragments remained associated with the HLA-DR  $\alpha\beta$  dimer (41). As shown in Fig. 7 *a*, fragments identical in two-dimensional PAGE mobility to the 21,500 (K1), 19,500 (K2), and 18,000 D

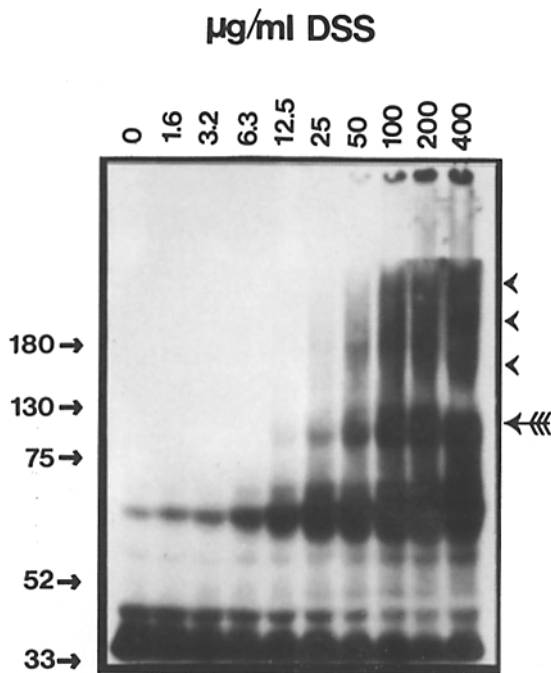


**Figure 4.** Stoichiometry of T2 invariant chain: gel filtration in sodium deoxycholate and cross-linking in  $C_{12}E_9$ . Affinity purified invariant chain from 8-h  $[^{35}S]$ met-labeled T2 cells was analyzed either directly (*A* and *B*) or after a 2-h treatment at  $42^\circ C$  in 1%  $C_{12}E_9$  (*C* and *D*). (*A* and *C*) Analysis was by gel filtration on Sephacryl S300 in 0.5% DOC followed by liquid scintillation counting (*graph*) and SDS-PAGE (*inset*). Numbers above insets indicate fraction numbers; numbers to the left of insets indicate mobility of molecular weight markers (kilodaltons). (*B* and *D*) Material was separated by gel filtration using Sephacryl S300 in 0.1%  $C_{12}E_9$ . Peak fractions were pooled and either mock treated or treated with increasing concentrations of DSP (marked above lanes of gels in micrograms per milliliter). Ethanol precipitated samples were analyzed by SDS-PAGE on 10% (*B*, left; *D*) or 7% (*B*, right) acrylamide separating gels. Arrowheads indicate the position of the invariant chain trimer. The two lanes at the extreme lower right show nondenatured invariant chain mock-treated or treated with DSP and separated on a 10% acrylamide gel under nonreducing conditions.



**Figure 5.** Western blotting analysis of T2 invariant chain: gel filtration and cross-linking profiles. Unlabeled T2 cell extracts were separated by gel filtration on Sephacryl S300 in DOC (A) or  $C_{12}E_9$  (B). (A) Fractions were analyzed by SDS-PAGE followed by Western blotting using Matilda anti-invariant chain serum. Shown is an autoradiograph of the blot and the profile from densitometric scanning of the 33-kD (invariant chain) band. (B) Peak fractions were pooled and treated with DSP at the indicated concentrations. Samples were precipitated with ethanol, separated by SDS-PAGE, transferred to nitrocellulose and invariant chain detected with Matilda and  $^{125}I$ -LORGI. The arrowhead indicates position of the invariant chain trimer.





**Figure 6.** Cross-linking of T2 invariant chain in cell membrane homogenates. Membranes from unlabeled T2 cells were resuspended without detergent and either mock treated or treated with increasing concentrations of DSS. After solubilization in Triton X-100, samples were precipitated with ethanol and fractionated on SDS-PAGE using a 7% acrylamide separating gel. After transferring to nitrocellulose invariant chain was detected using affinity purified anti-EQLP antibody and  $^{125}\text{I}$ -LORGI as secondary reagent. Arrow indicates position of presumed trimer. Arrowheads indicate positions of higher order multimers.

(K3) proteolytic products of class II-associated invariant chain (41) can also be generated from free invariant chain. To determine whether the luminal domain was responsible for oligomerization, the smallest fragment, K3, was purified by gel filtration and subjected to cross-linking. SDS-PAGE analysis (Fig. 7 *b*) shows that with increasing amounts of DSP, the 18,000 *M<sub>r</sub>* K3 band is cross-linked to a 55,000-D form, consistent with the expected size of a trimer of 18-kD monomers. A dimer form is observed as an intermediate between monomer and trimer forms, but is not observed in the absence of cross-linker. This is consistent with the loss of the cytoplasmic domain (and the single cysteine residue) by proteolysis. Thus, invariant chain trimerization can be accounted for by interactions of the extracytoplasmic regions.

#### **Structure and Localization of Excess Invariant Chain in HLA Class II-positive Cells**

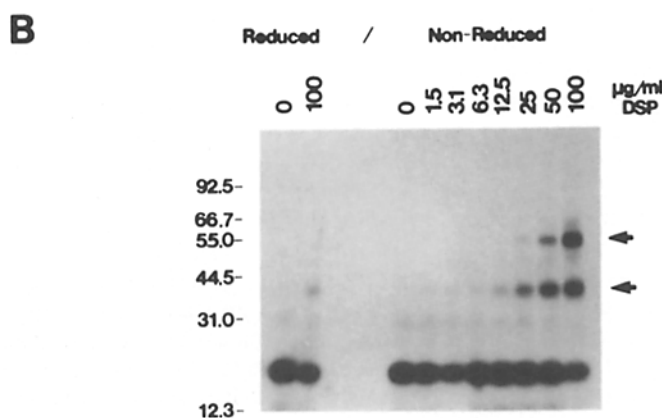
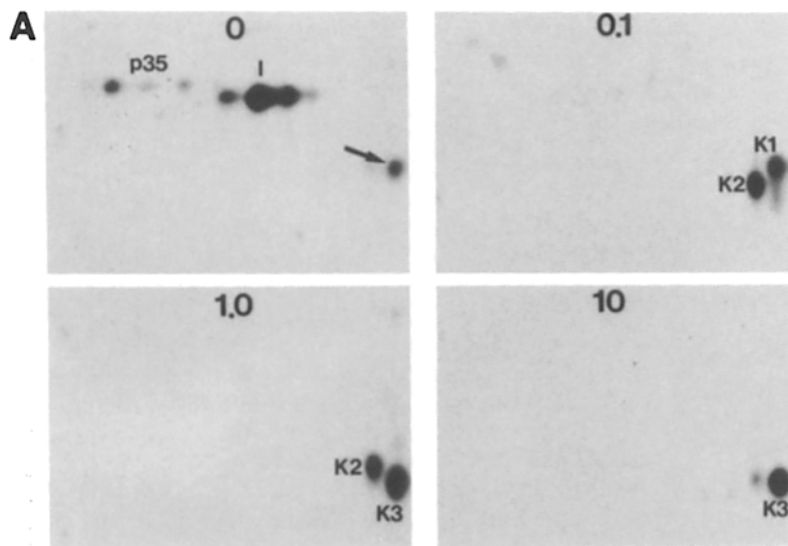
The results described above suggest that in the absence of HLA class II antigens, the invariant chain remains sequestered in the RER and is predominantly trimeric. Cells with an invariant chain-positive, class II-negative phenotype are, at best, uncommon *in vivo* (36, 45). However, it is well documented that the invariant chain is present in a large excess over HLA class II  $\alpha$  and  $\beta$  chains in class II-positive cells (38, 39). This excess invariant chain could conceivably possess similar biosynthetic and structural characteristics to

invariant chain in class II-negative cells. To determine this we analyzed the properties of the excess invariant chain in a number of cell lines expressing HLA class II glycoproteins.

The intracellular transport of invariant chain in the class II-negative T2 cell line was compared by pulse-chase analysis with that of the excess free invariant chain in T1, the class II-positive parent cell from which T2 was derived. Cell extracts from each chase period were cleared of HLA class II glycoproteins and associated molecules by immunoprecipitation with two anti-class II mAbs. The cleared extracts were then immunoprecipitated with an anti-invariant chain rabbit antiserum, Matilda<sub>CEM</sub>. Two-dimensional PAGE analysis of each immunoprecipitate (Fig. 8) shows that most of the excess invariant chain in T1 cells remains in its unprocessed form. Furthermore, the excess invariant chain present in class II-depleted cell extracts of two class II-positive B-LCL, Swei and Jijoye, was as sensitive to endo H as to endo F (Fig. 8 *B*). In addition, the excess invariant chain has a longer half-life than that associated with class II molecules in T1 (compare Fig. 8 with Fig. 1, *left*). Although this is somewhat shorter than the invariant chain half-life in T2, it has been shown that excess invariant chain can associate with newly synthesized  $\alpha$  and  $\beta$  chains (31, 39). This would deplete the excess pool and thereby shorten the apparent half-life. These results suggest that excess invariant chain in class II-positive cell lines has a similar biosynthetic profile to that of free invariant chain in class II-negative mutants, and are consistent with localization proximal to the medial Golgi.

The localization of invariant chain in class II-positive cells was also analyzed by indirect intracellular immunofluorescence. Fixed, permeabilized cells were stained with either a rabbit anti-class II antiserum or a rabbit anti-invariant chain serum. Shown in Fig. 9 are the results using the class II-positive B-LCL Swei, the class II-negative, invariant chain-negative T-LCL CEM, and the class II-positive/class II-negative parent/mutant pairs T0/T2, Jijoye/P3HR-1, and T5-1/6.1.6. CEM, as expected, does not stain with either antiserum. T2, P3HR-1, and 6.1.6 are negative with the anti class II antiserum, and exhibit a bright, perinuclear staining pattern with the anti invariant chain antiserum. In the class II-positive cells, class II molecules appear in multiple cytoplasmic vesicles. An identical pattern was observed with the monoclonal anti class II antibody XD5.A11 (not shown), and the pattern is similar to that observed with antibodies to cathepsin D and galactosyl transferase, markers for lysosomes/endosomes and Golgi, respectively (e.g., see Fig. 3). Staining of class II-positive cells with the anti-invariant chain antibody resulted in a pattern that overlapped the class II-negative cell invariant chain pattern and the class II pattern, with both vesicular and perinuclear staining. Both the class II and invariant chain patterns were consistent among eight different class II-positive cell lines (not shown). The results suggest that there are two pools of invariant chain in class II-positive cells, one that overlaps with HLA class II antigens in an intracellular vesicular compartment, and one that does not overlap. The latter pool of "excess" invariant chain appears to be present in the RER since its staining pattern was identical to that observed with an antibody to the RER marker, protein disulfide isomerase (see Fig. 3 and data not shown).

To determine whether the excess invariant chain from class II-positive cells exhibited an oligomeric structure, extracts



**Figure 7.** A luminal invariant chain fragment remains trimeric. (A) [<sup>35</sup>S]Met-labeled invariant chain, purified from 8-h [<sup>35</sup>S]met-labeled T2 cells by affinity chromatography on POP.I4.3-Biogel in C<sub>12</sub>E<sub>9</sub>, was either mock treated (0) or treated with proteinase K at 0.1, 1, or 10 µg/ml as indicated. Products were precipitated with ethanol and analyzed by two-dimensional PAGE. Arrow in the first panel marks presence of the invariant chain proteolytic product with mobility similar to that of K1. (B) Purified [<sup>35</sup>S]met-labeled invariant chain was treated with 10 µg/ml proteinase K for 90 min at 0°C. Aliquots were then applied to Sephacryl S300 in C<sub>12</sub>E<sub>9</sub>, and peak fractions were analyzed by cross-linking with DSP. Arrows indicate positions of the dimeric and trimeric K3 fragments.

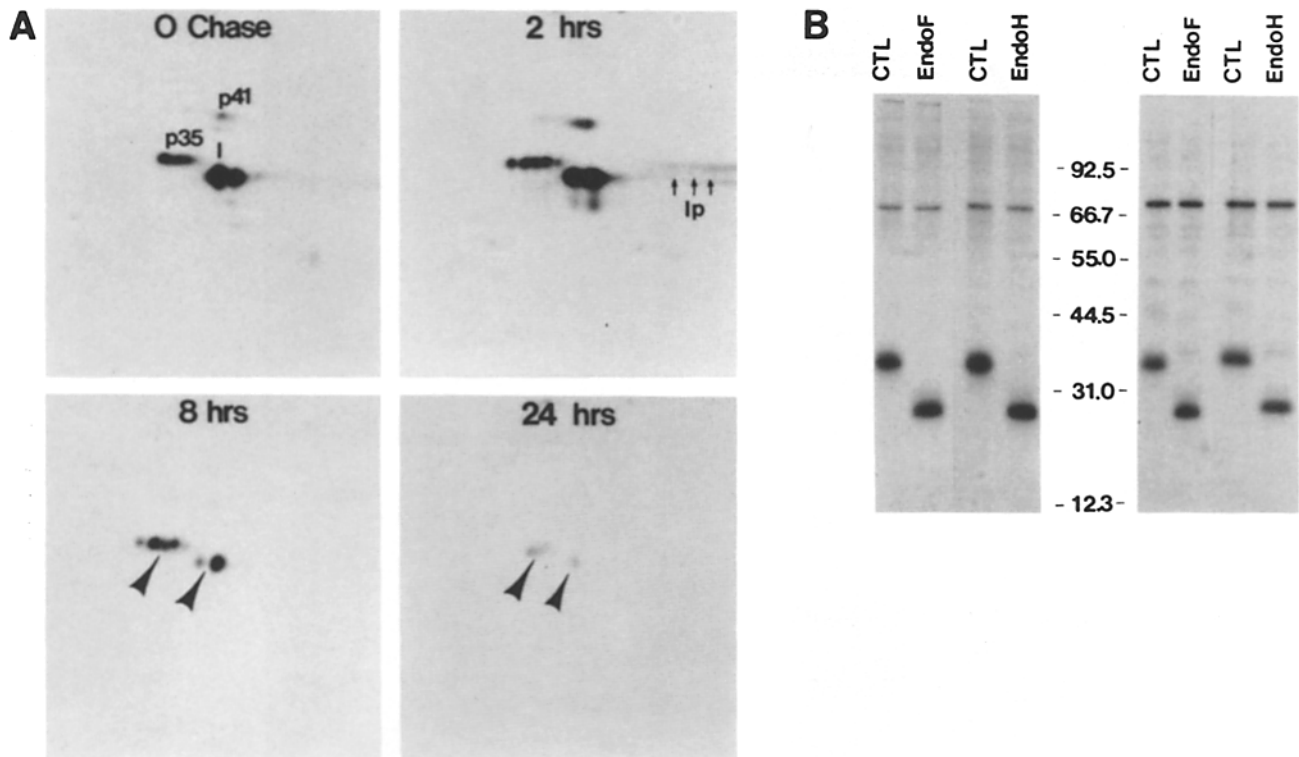
from 8-h [<sup>35</sup>S]met-labeled Swei B-LCL were cleared of class II antigens and the remaining invariant chain was purified by affinity chromatography. The eluate was analyzed by gel filtration and by cross-linking with DSP. The invariant chain was found to elute with an  $M_r$  of 220,000 by gel filtration in DOC (Fig. 10, *a* and *b*) and treatment with increasing amounts of DSP yielded an end product that migrated with an  $M_r$  of 100,000 by SDS-PAGE (Fig. 10 *c*), both results consistent with a homotrimeric structure. An identical pattern was observed for the excess invariant chain in the Jijoye B-LCL (results not shown). Thus, as in class II-negative mutant cell lines, the excess, class II-unassociated invariant chain in class II-positive cells self-associates into a trimeric structure.

## Discussion

One hypothesized function of the invariant chain is as a transport and/or retention protein for HLA class II antigens to a post-Golgi intracellular compartment. Such a hypothesis would be supported if it could be shown that invariant chain localized to a similar compartment in the absence of HLA class II antigens. The data presented here, however, show that invariant chain from class II-negative mutants localizes instead to the RER. The N- and O-linked oligosaccharides

of invariant chain remained unprocessed in class II-negative cells (Figs. 1 and 2), and identical immunofluorescence patterns were obtained using reagents directed against invariant chain and a RER marker, protein disulfide isomerase (Fig. 3). Similarly, RER-localized invariant chain was found even in HLA class II-positive cells (Figs. 8 and 9), corresponding to the previously described excess pool (31, 37, 38). Thus, efficient invariant chain transport out of the RER appears to require association with HLA class II antigens. Consistent results have been obtained in the mouse using a fibroblast transfection system (50). However, these data do not a priori rule out a role for invariant chain in class II transport or post-Golgi targeting once it is complexed with the  $\alpha$  and  $\beta$  chains.

Note that some invariant chain "escapes" the RER in the absence of class II antigens; a very small amount of processing was observed by pulse-chase analysis (Figs. 1 and 8 *a*), and punctate staining was observed with anti-invariant chain reagents in some T2 and P3HR-1 cells by immunofluorescence (see Figs. 3 and 9). In addition, certain monoclonal and antipeptide antibodies detect small amounts of invariant chain on the surface of B cell lines, and we have recently been able to detect low amounts on the surface of T2 by immunofluorescence using POP.I4.3 (Davis, J. E., and P. Cresswell, unpublished results). Furthermore, processed invariant chain can be isolated from T2 cell extracts using *Lens culin-*



**Figure 8.** Pulse-chase and endoglycosidase analysis of excess invariant chain in class II-positive cells. (A) T1 cells were pulse labeled with  $^{35}\text{S}$ -met for 10 min and chased for the times indicated with an excess of cold methionine. Cell extracts were precleared of HLA class II antigens and associated molecules by immunoprecipitation first with DA6.147 and then with XD5.A11, and cleared extracts were immunoprecipitated with Matilda<sub>CEM</sub>. Matilda<sub>CEM</sub> precipitates were analyzed by two-dimensional PAGE. Arrowheads indicate presence of p35 and p33 invariant chains after long periods of chase. (B) Extracts from 8-h [ $^{35}\text{S}$ ]met-labeled Swei or Jijoye B-LCL were cleared of HLA class II antigens by passage through L243-, DA6.147-, and XD5.A11-Biogel, and then remaining invariant chain was affinity purified on POP.I4.3-Biogel in  $\text{C}_{12}\text{E}_9$ . POP.I4.3 eluates were further purified by gel filtration on Sephacryl S300 in  $\text{C}_{12}\text{E}_9$ . Peak fractions were pooled, precipitated with ethanol, and either mock treated or treated with endo H or endo F. Products were analyzed by one-dimensional SDS-PAGE.

aris hemagglutinin, which binds N-linked oligosaccharides that have been fucosylated by the medial Golgi fucosyl transferase (29). However,  $\sim 10$ -fold less invariant chain was precipitable with this lectin that with rabbit anti-invariant chain serum, (results not shown), suggesting that only a small proportion passes the medial Golgi. In addition, only a small amount of invariant chain is converted to the chondroitin sulfate proteoglycan form in class II-negative, invariant chain-positive cells (data not shown), as observed in the mouse (50).

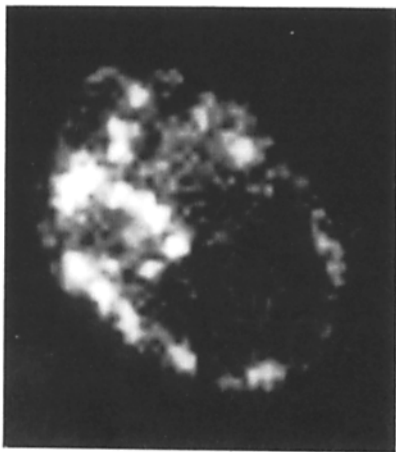
Misfolded proteins or isolated members of incomplete protein complexes are often retained in the RER (46). Some are bound by BiP or BiP-like resident RER proteins (2), which are themselves retained by possession of an anchor signal, such as the KDEL sequence found in a number of soluble resident RER proteins (43). Invariant chain that is not associated with class II antigens does not appear to bind to any such molecules. In T2, no invariant chain-associated proteins were observed by co-immunoprecipitation (Fig. 1) or by affinity chromatography and gel filtration after labeling with [ $^{35}\text{S}$ ]met, [ $^3\text{H}$ ]leucine, or silver staining (Fig. 4 and results not shown). Similar results were obtained in a second class II-negative cell line, P3HR-1 (results not shown), and with the excess invariant chain from class II-positive cell

lines (Figs. 8 and 10, results not shown). Furthermore, invariant chain was not observed in anti-BiP immunoprecipitates from T2 (Lamb C., and P. Cresswell, unpublished). Thus, it appears that association with resident RER proteins is an unlikely explanation for invariant chain retention.

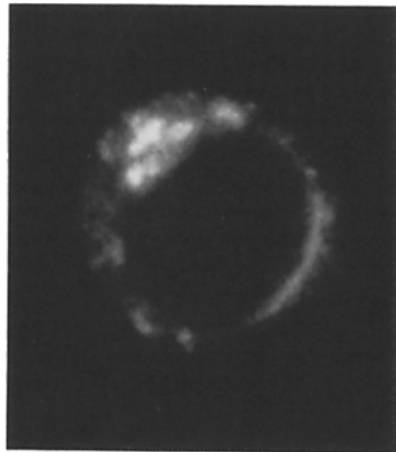
Misfolded proteins that are not transported are often degraded early after synthesis in a RER-associated proteolytic compartment (5, 34). Invariant chain, however, has a longer half-life in the absence than in the presence of associated class II antigens, as determined by pulse-chase analysis (Figs. 1 and 8). Thus, degradation cannot be responsible for the lack of transport.

A number of misfolded proteins that are retained within the RER have an unusual stoichiometry compared with their correctly folded counterparts (46). For example, VSV-G and flu-HA normally associate into trimers before exit from the RER. Mutants of these proteins that remain as monomers or form large aggregates are retained in the RER (3, 9, 10, 16, 30). Invariant chain in the absence of MHC class II antigens is oligomeric, predominantly existing as a trimer even in intact membranes (Figs. 4-6 and 10). Because this result was obtained both after affinity purification and by Western blotting of unpurified cell extracts, using two different anti-invariant chain reagents, all detectable invariant chain in the cell ap-

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SWEI



MAT

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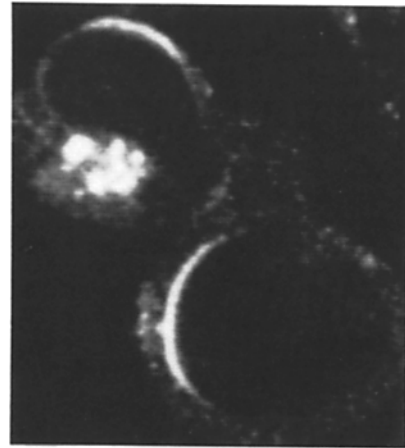
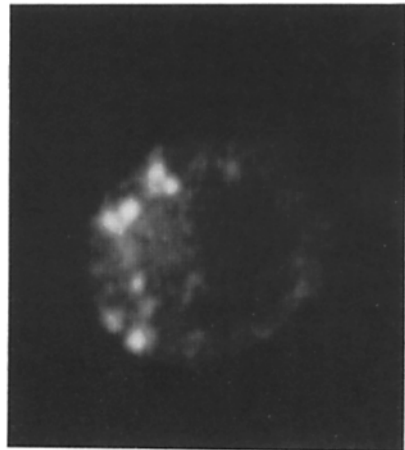


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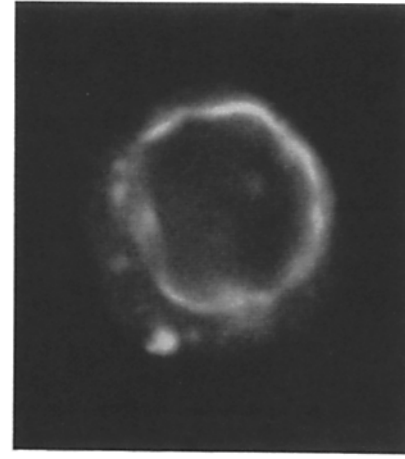
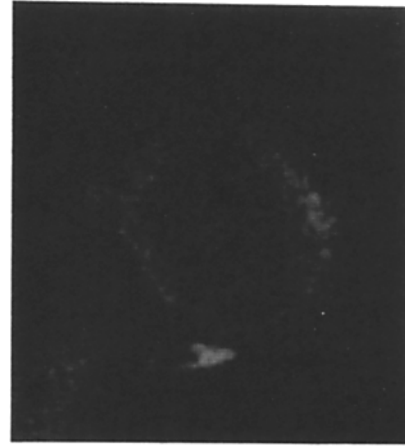


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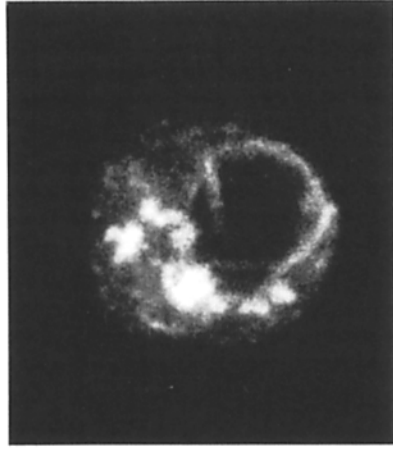
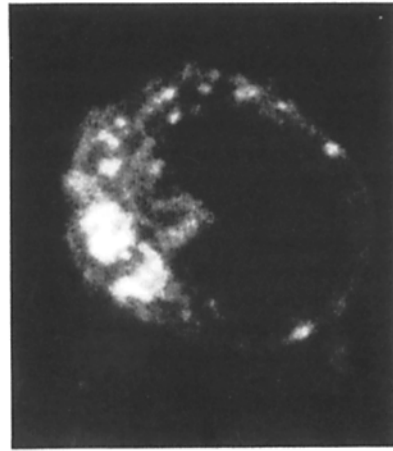
T0



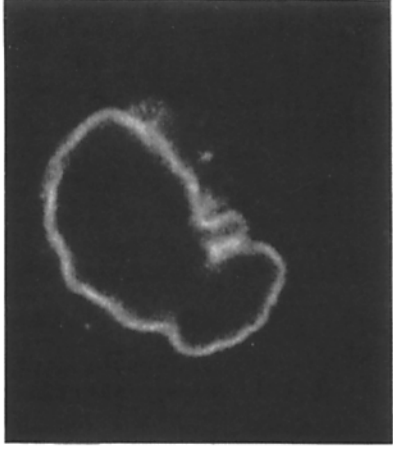
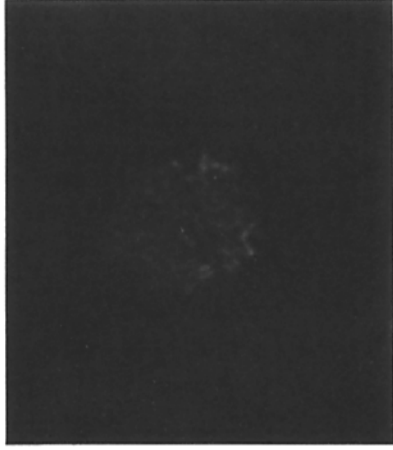
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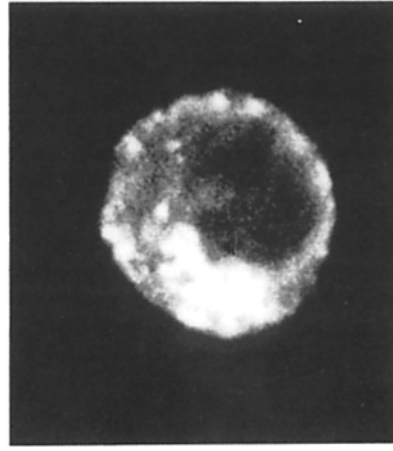
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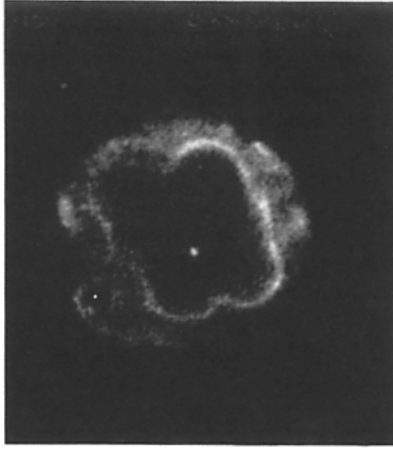
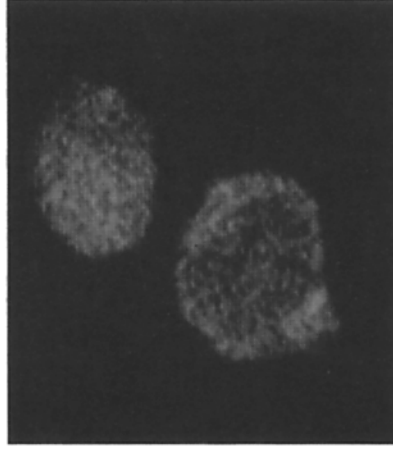
P3HR-1



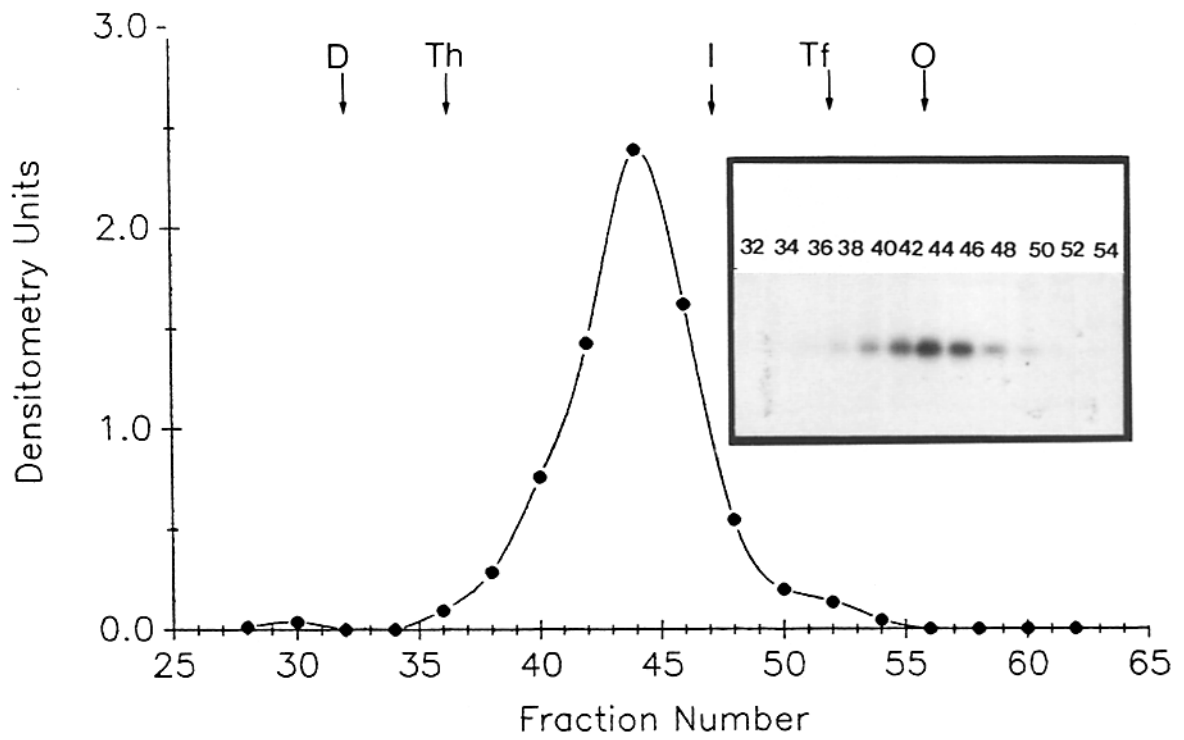
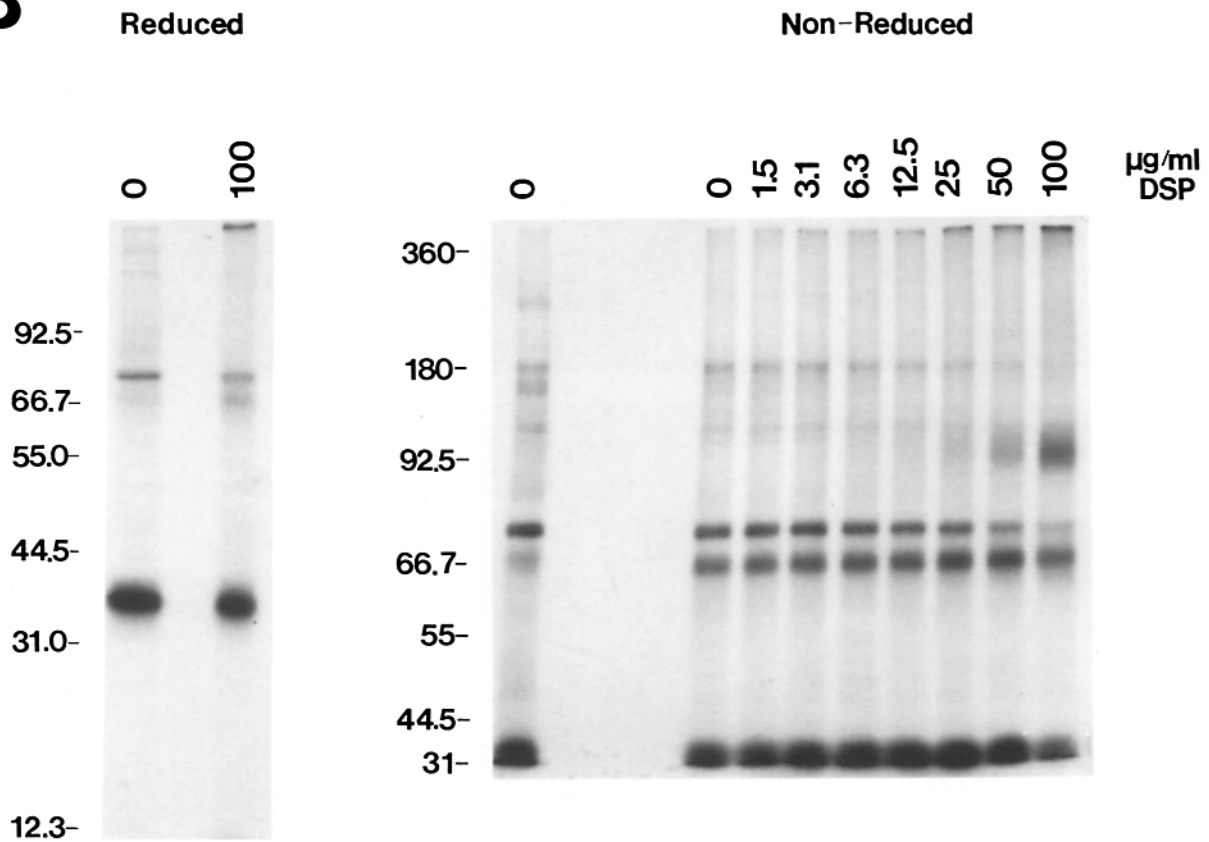
T5-1



6.1.6



**Figure 9** Immunofluorescence localization of invariant chain in class II-positive cell lines. Log phase class II-positive cell lines Swei, T0, Jijoye, and T5-1 (*left*) as well as class II-negative, invariant chain-positive cell lines T2, P3HR-1, and 6.1.6 and the class-II negative, invariant chain-negative CEM (*right*) were analyzed by indirect intracellular immuno-fluorescence, as described in Materials and Methods, using 247<sub>Hsb</sub> (anti-HLA-DR; 247) or Matidac<sub>CEM</sub> (anti-invariant chain; MAT) as primary antibody.

**A****B**

pears to be oligomeric. Experiments using pulse-labeled affinity-purified invariant chain further suggest that trimerization is an early event, occurring within 10 min after synthesis (results not shown). Such kinetics are similar to those observed for trimerization of the VSV-G protein and flu-HA (3, 9). Thus, invariant chain stoichiometry is more like that observed for transport-competent forms of the viral glycoproteins than for transport-incompetent forms. Since the excess invariant chain trimers in class II-positive cells are capable of associating with newly synthesized MHC class II antigens (31, 38), this suggests that gross misfolding of free invariant chain complexes does not occur.

Trimerization of invariant chain is mediated by luminal interactions (Fig. 7), indeed by the same region of the molecule that mediates binding to HLA-DR antigens (41). Any aggregation of trimers must occur via regions outside the limits of the soluble K3 fragment digestion product, most likely in the transmembrane or cytoplasmic regions, since only trimers of K3 were observed upon cross-linking with DSP under conditions in which higher order multimers of intact invariant chain were observed (compare Fig. 4 B with Fig. 7).

Klausner has recently proposed three mechanisms whereby proteins are specifically targeted to or retained within compartments of the secretory pathway (28). The first two, possession of positive transport signals and positive retention signals within the primary or tertiary structure of a polypeptide or glycoprotein, are active mechanisms of localization, requiring receptors to recognize and act upon the protein. The third "signal," referred to as transport incompetence, dictates that some inherent property within the structure of the glycoprotein or peptide chain, such as tendency to aggregate, hydrophobicity, or some other property, would prevent it from achieving a conformation that would allow transport to the next step along the secretory pathway. Transport incompetence by means of aggregation is an attractive hypothesis to explain the RER localization of free invariant chain. The higher order multimers of invariant chain observed when intact membranes were cross-linked (Fig. 6) may be indicative of a tendency for trimers to aggregate *in vivo*. As aggregation is most likely dependent on a number of environmental factors, such as protein concentration, pH, and ionic strength, that could vary within subdomains of the RER, the small amount of leakage of invariant chain to the Golgi could be explained by slow localized disaggregation into trimers. In class II-positive cells, the class II glycoproteins themselves may prevent or reverse aggregation of the invariant chain, thus allowing transport. Clearly, this hypothesis remains to be tested.

The presence of excess trimeric invariant chain in the RER of class II-positive cells has implications for the structure of the intracellular class II  $\alpha\beta$ -invariant chain complex. It is

known that the three chains associate within the RER (31, 37). It is conceivable that trimeric invariant chain may form a core for the complex, with  $\alpha\beta$  dimers binding to each individual chain of the trimer. The predicted complex would be consistent with the high apparent  $M_r$  ( $\sim 270,000$ ) previously obtained on gel filtration of purified, accumulated  $\alpha\beta$ -invariant chain complexes, at the time ascribed to association with the proteoglycan form of the invariant chain (27). Alternatively, binding of  $\alpha$  and  $\beta$  chains to an individual invariant chain molecule could disrupt the trimer, resulting in heterotrimers of  $\alpha$ ,  $\beta$ , and invariant chains. The stoichiometry of intracellular class II complexes is currently under investigation.

The presence of excess invariant chain in the RER of class II-positive cells emphasizes the importance of a very early association of HLA class II antigens with invariant chain. This interaction is important enough for class II expressing cells to dedicate a significant amount of energy to making a large excess of a protein that is destined, in the absence of such an association, to merely occupy the lumen of the RER. Such an adaptation suggests that either (a) class II antigen function would be severely compromised in the absence of an association with invariant chain, or (b) the presence of the class II antigens in the RER or Golgi would be detrimental to the cell or the organism. Both possibilities would be consistent with a role for the invariant chain in facilitating the association of class II proteins with foreign antigenic peptides (37). This role could be to direct the transport of newly synthesized class II antigens to a subcellular compartment in which they might encounter foreign peptides or to retain class II antigens within this compartment. We have recently shown that invariant chain association inhibits the binding of peptides to class II molecules, which may be a mechanism to ensure that the peptide binding site is not accessible until such a compartment is reached (45a). The lack of an observable effect of invariant chain on class II antigen biosynthesis in murine fibroblast transfectants (50) would tend to argue against a role in transport, but further study of the role on invariant chain in class II antigen transport and peptide interactions is clearly required.

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**Figure 10.** Gel filtration and cross-linking analysis of the free pool of invariant chain in Swei B-LCL. [ $^{35}$ S]Met-labeled Swei cell extracts were depleted of class II antigens and associated molecules using L243, DA6.147, and XD5.A11 affinity columns, and remaining free invariant chain was purified on POP.I4.3-Biogel in  $C_{12}E_9$ . Eluates were applied to Sephacryl S300 in either DOC (A) or  $C_{12}E_9$  (B). Fractions were analyzed by SDS-PAGE (A, inset) and subsequent densitometry of the 33kd invariant chain band. Fraction numbers are indicated above the inset. Pooled fractions were analyzed by cross-linking with DSP (B). The mobility of molecular weight markers (kilodaltons) is indicated.

## References

- Blum, J. S., and P. Cresswell. 1988. Role for intracellular proteases in the processing and transport of class II HLA antigens. *Proc. Natl. Acad. Sci. USA*. 85:3975-3979.
- Bole, D. G., L. M. Hendershot, and J. F. Kearney. 1986. Post-translational association of immunoglobulin heavy chain binding protein with nascent heavy chains in non secreting and secreting hybridomas. *J. Cell Biol.* 102:1558-1566.
- Boulay, F., R. W. Doms, R. G. Webster, and A. Helenius. 1988. Post-translational oligomerization and cooperative acid activation of mixed influenza hemagglutinin trimers. *J. Cell Biol.* 106:629-639.
- Burkhardt, J. K., S. Hester, and Y. Argon. 1989. The glycoprotein of VSV accumulates in a distal Golgi compartment in the presence of CCCP. *J. Cell Sci.* 92:643-654.
- Chen, C., J. S. Bonifacino, L. C. Yuan, and R. D. Klausner. 1988. Selective degradation of T cell antigen receptor chains retained in a pre-Golgi compartment. *J. Cell Biol.* 107:2149-2161.
- Claesson, L., D. Larhammar, L. Rask, and P. A. Peterson. 1983. cDNA clone for the human invariant gamma chain of class II histocompatibility antigens and its implications for the protein structure. *Proc. Natl. Acad. Sci. USA*. 80:7395-7399.
- Claesson-Welsh, L., P. E. Barker, D. Larhammar, L. Rask, F. H. Ruddle, and P. A. Peterson. 1984. The gene encoding the human class II antigen-associated  $\tau$  chain is located on chromosome 5. *Immunogenetics*. 20:89-93.
- Claesson-Welsh, L., and P. A. Peterson. 1985. Implications of the invariant gamma-chain on the intracellular transport of class II histocompatibility antigens. *J. Immunol.* 135:3551-3557.
- Copeland, C., R. W. Doms, E. M. Bolzau, R. G. Webster, and A. Helenius. 1986. Assembly of influenza hemagglutinin trimers and its role in intracellular transport. *J. Cell Biol.* 103:1179-1191.
- Copeland, C. S., K.-P. Zimmer, K. R. Wagner, G. A. Healey, I. Mellman, and A. Helenius. 1988. Folding, trimerization, and transport are sequential events in the biogenesis of influenza virus hemagglutinin. *Cell*. 53:197-209.
- Cresswell, P. 1985. Intracellular class II HLA antigens are accessible to transferrin-neuraminidase conjugates internalized by receptor-mediated endocytosis. *Proc. Natl. Acad. Sci. USA*. 82:8188-8192.
- Cresswell, P., and J. S. Blum. 1988. Intracellular transport of class II HLA antigens. In *Processing and Presentation of Antigens*. B. Pernis, S. C. Silverstein, and H. J. Vogel, editors. Academic Press, San Diego, CA. 43-51.
- Cresswell, P., J. S. Blum, D. N. Kelner, and M. S. Marks. 1987. Biosynthesis and processing of class II histocompatibility antigens. *CRC Crit. Rev. Immunol.* 7:31-53.
- Doms, R. W., A. Ruusala, C. Machamer, J. Helenius, A. Helenius, and J. K. Rose. 1988. Differential effects of mutations in three domains on folding, quaternary structure, and intracellular transport of vesicular stomatitis virus G protein. *J. Cell Biol.* 107:89-99.
- Elder, J. H., and S. Alexander. 1982. Endo- $\beta$ -N-acetylglucosaminidase F: endoglycosidase from *Flavobacterium meningosepticum* that cleaves both high mannose and complex glycoproteins. *Proc. Natl. Acad. Sci. USA*. 79:4540-4544.
- Gething, M. J., K. McCammon, and J. Sanbrook. 1986. Expression of wild-type and mutant forms of influenza hemagglutinin: the role of folding and intracellular transport. *Cell*. 46:939-950.
- Giacoletto, K. S., A. J. Sant, C. Bono, J. Gorka, D. M. O'Sullivan, V. Quaranta, and B. D. Schwartz. 1986. The human invariant chain is the core protein of the human class II-associated proteoglycan. *J. Exp. Med.* 164:1422-1439.
- Gladstone, P., and D. Pious. 1978. Stable variants affecting B cell alloantigens in human lymphoid cells. *Nature (Lond.)*. 271:459-461.
- Grey, H. M., and R. Chestnut. 1985. Antigen processing and presentation to T cells. *Immunol. Today*. 6:101-106.
- Guagliardi, L. E., B. Koppelman, J. S. Blum, M. S. Marks, P. Cresswell, and F. M. Brodsky. 1990. Molecules involved in antigen processing and presentation colocalize in an early endocytic compartment. *Nature (Lond.)*. 343:133-139.
- Guy, K., V. van Heyningen, B. B. Cohen, D. L. Deane, and C. M. Steel. 1982. Differential expression and serologically distinct subpopulations of human Ia antigens detected with monoclonal antibodies to Ia alpha and beta chains. *Eur. J. Immunol.* 12:942-948.
- Hasilik, A., R. Pohlmann, and K. von Figura. 1983. Inhibition of cyanate of the processing of lysosomal enzymes. *Biochem. J.* 210:795-802.
- Hinuma, Y., M. Konn, J. Yamaguchi, D. J. Wudarski, J. R. Blakeslee, Jr., and J. T. Grace. 1967. Immunofluorescence and herpes-type virus particles in the P3HR-1 Burkitt lymphoma cell line. *J. Virol.* 1:1045-1051.
- Hurtley, S. M., D. G. Bole, H. Hoover-Litty, A. Helenius, and C. S. Copeland. 1989. Interactions of misfolded influenza virus hemagglutinin with binding protein (BiP). *J. Cell Biol.* 108:2117-2126.
- Kaetzel, C. S., C. K. Rao, and M. E. Lamm. 1987. Protein disulfide-isomerase from human placenta and rat liver: purification and immunological characterization with monoclonal antibodies. *Biochem. J.* 241:39-47.
- Kappes, D., and J. L. Strominger. 1988. Human class II major histocompatibility complex genes and proteins. *Annu. Rev. Biochem.* 57:991-1028.
- Kelner, D., and P. Cresswell. 1986. Biosynthetic intermediates of HLA class II antigens from B-lymphoblastoid cell lines. *J. Immunol.* 137:2632-2639.
- Klausner, R. D. 1989. Sorting and traffic in the central vacuolar system. *Cell*. 57:703-706.
- 28a. Koppelman, B., and P. Cresswell. 1990. Rapid non-lysosomal degradation of assembled HLA Class II glycoproteins incorporating a mutant DR  $\alpha$ -chain. *J. Immunol.* In press.
- Kornfeld, K., M. L. Reitman, and R. Kornfeld. 1981. The carbohydrate-binding specificity of pea and lentil lectins: fucose is an important determinant. *J. Biol. Chem.* 256:6633-6640.
- Kreis, T. E., and H. F. Lodish. 1986. Oligomerization is essential for transport of VSV viral glycoprotein to the cell surface. *Cell*. 46:929-937.
- Kvist, S., K. Wiman, L. Claesson, P. A. Peterson, and B. Dobberstein. 1982. Membrane insertion and oligomeric assembly of HLA-DR histocompatibility antigens. *Cell*. 29:61-69.
- Lampson, L. A., and R. Levy. 1980. Two populations of Ia-like molecules on a human B cell line. *J. Immunol.* 125:293-299.
- Lipp, J., and B. Dobberstein. 1986. Signal recognition particle-dependent membrane insertion of mouse invariant chain: A membrane-spanning protein with a cytoplasmically exposed amino terminus. *J. Cell Biol.* 102:2169-2175.
- Lippincott-Schwartz, J., J. S. Bonifacino, L. C. Yuan, and R. D. Klausner. 1988. Degradation from the endoplasmic reticulum: Disposing of newly synthesized proteins. *Cell*. 54:209-220.
- Lodish, H. F. 1988. Transport of secretory and membrane glycoproteins from the rough endoplasmic reticulum to the Golgi: A rate limiting step in protein maturation and secretion. *J. Biol. Chem.* 263:2107-2110.
- Long, E. O. 1985. In search of a function for the invariant chain associated with Ia antigens. *Surv. Immunol. Res.* 4:27-34.
- Long, E. O. 1989. Intracellular traffic and antigen processing. *Immunol. Today*. 10:232-234.
- Machamer, C. E., and P. Cresswell. 1982. Biosynthesis and glycosylation of the invariant chain associated with HLA-DR antigens. *J. Immunol.* 129:2564-2569.
- Machamer, C. E., and P. Cresswell. 1984. Monensin prevents terminal glycosylation of the N- and O-linked oligosaccharides of the HLA-DR-associated invariant chain and inhibits its dissociation from the  $\alpha$ - $\beta$  chain complex. *Proc. Natl. Acad. Sci. USA*. 81:1287-1291.
- Markert, M. L., and P. Cresswell. 1982. Human B cell alloantigens:  $\alpha$  subunit variability. *J. Immunol.* 128:1999-2003.
- Marks, M. S., and P. Cresswell. 1986. Invariant chain associates with HLA class II antigens via its extra-cytoplasmic region. *J. Immunol.* 136:2519-2525.
- Minami, Y., A. M. Weissman, L. E. Samelson, and R. D. Klausner. 1987. Building a multichain receptor: synthesis, degradation and assembly of the T cell antigen receptor. *Proc. Natl. Acad. Sci. USA*. 84:2688-2692.
- Munro, S., and H. R. B. Pelham. 1987. C-terminal signal prevents secretion of luminal ER proteins. *Cell*. 48:899-907.
- Ozato, K., N. Mayer, and D. Sachs. 1980. Hybridoma cell lines secreting monoclonal antibodies to mouse H-2 and Ia antigens. *J. Immunol.* 124:533-540.
- Quaranta, V., O. Majdic, G. Stingl, K. Liszka, H. Honigsman, and W. Knapp. 1984. A human Ia cytoplasmic determinant located on multiple forms of invariant chain ( $\tau$ 1,  $\tau$ 2,  $\tau$ 3). *J. Immunol.* 132:1900-1905.
- Roche, P. A., and P. Cresswell. 1990. Invariant chain-associated HLA-DR molecules do not bind an immunologic peptide. *Nature (Lond.)*. 345:615-618.
- Rose, J. K., and R. W. Doms. 1988. Regulation of protein export from the endoplasmic reticulum. *Annu. Rev. Cell Biol.* 4:257-288.
- Roth, J., and E. G. Berger. 1982. Immunocytochemical localization of galactosyl transferase in HeLa cells: Codistribution with thiamine pyrophosphatase in trans Golgi cisternae. *J. Cell Biol.* 92:223-229.
- Salter, R. D., D. N. Howell, and P. Cresswell. 1985. Genes regulating HLA class I antigen expression in T-B lymphoblast hybrids. *Immunogenetics*. 21:235-246.
- Sekaly, R. P., C. Tonnel, M. Strubin, B. Mach, and E. O. Long. 1986. Cell surface expression of class II histocompatibility antigens occurs in the absence of the invariant chain. *J. Exp. Med.* 164:1490-1504.
- Simonis, S., J. Miller, and S. E. Cullen. 1989. The role of the Ia-Invariant chain complex in the posttranslational processing and transport of Ia and Invariant chain glycoproteins. *J. Immunol.* 143:3619-3625.
- Spiro, R. C., and V. Quaranta. 1989. The invariant chain is a phosphorylated subunit of class II molecules. *J. Immunol.* 143:2589-2594.
- Spiro, R. C., T. Sairenji, and R. E. Humphreys. 1984. Enhanced Ia expression after n-butylate treatment of a P3HR-1 Burkitt's lymphoma subline which does not express HLA-D. *Hematol. Oncol.* 2:239-247.
- Stockinger, B., U. Pessara, R. H. Lin, J. Habicht, M. Grez, and N. Koch. 1989. A role of Ia-associated invariant chains in antigen processing and presentation. *Cell*. 56:683-689.
- Strubin, M., B. Mach, and E. O. Long. 1984. The complete sequence of



- the mRNA for the HLA-DR-associated invariant chain reveals a polypeptide with an unusual transmembrane polarity. *EMBO (Eur. Mol. Biol. Organ.) J.* 3:869-872.
55. Strubin, M., C. Berte, and B. Mach. 1986. Alternative splicing and alternative initiation of translation explain the four forms of the Ia antigen-associated invariant chain. *EMBO (Eur. Mol. Biol. Organ.) J.* 5: 3483-3488.
56. Strubin, M., E. O. Long, and B. Mach. 1986. Two forms of the Ia antigen-associated invariant chain result from alternative initiations at two in-phase AUGs. *Cell.* 47:619-625.
57. Tarentino, A. L., T. H. Plummer, and F. Maley. 1974. The release of intact oligosaccharides from specific glycoproteins by endo- $\beta$ -N-acetylglucosaminidase H. *J. Biol. Chem.* 249:818-824.
58. Towbin, H., T. Staechelin, and J. Gordon. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets. *Proc. Natl. Acad. Sci. USA.* 76:4350-4354.
59. Unanue, E. R. 1984. Antigen presenting function of the macrophage. *Annu. Rev. Immunol.* 2:395-428.
60. Virtanen, I., P. Ekblom, and P. Laurila. 1980. Subcellular compartmentalization of saccharide moieties in cultured normal and malignant cells. *J. Cell Biol.* 85:429-434.