The Biogenesis of the MHC Class II Compartment in Human I-Cell Disease B Lymphoblasts

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Abstract. The localization and intracellular transport of major histocompatibility complex (MHC) class II molecules and lysosomal hydrolases were studied in I-Cell Disease (ICD) B lymphoblasts, which possess a mannose 6-phosphate (Man-6-P)-independent targeting pathway for lysosomal enzymes. In the *trans*-Golgi network (TGN), MHC class II-invariant chain complexes colocalized with the lysosomal hydrolase cathepsin D in buds and vesicles that lacked markers of clathrin-coated vesicle-mediated transport. These vesicles fused with the endocytic pathway leading to the formation of "early" MHC class II-rich compartments (MIICs). Similar structures were observed in the TGN of normal β lymphoblasts although they were less abundant. Metabolic labeling and subcellular fractionation experiments indicated that newly synthesized cathepsin D and MHC class II-invariant chain complexes enter a non-clathrin-coated vesicular structure after their passage through the TGN and segregation from the secretory pathway. These vesicles were also devoid of the cation-dependent mannose 6-phosphate (Man-6-P) receptor, a marker of early and late endosomes. These findings suggest that in ICD B lymphoblasts the majority of MHC class II molecules are transported directly from the TGN to "early" MIICs and that acid hydrolases can be incorporated into MIICs simultaneously by a Man-6-P–independent process.

MAJOR histocompatibility complex (MHC)[1] molecules mediate the presentation of antigenic peptides by antigen presenting cells (APCs) to T lymphocytes. Almost all cell types express MHC class I molecules, which display a variety of cytoplasmically derived peptides for presentation to CD8-positive T lymphocytes. MHC class II molecules are found on a restricted set of cell types such as B lymphocytes, macrophages, dendritic cells including epidermal Langerhans cells, and thymic epithelial cells. MHC class II molecules bind peptides generated by the proteolysis of exogenous antigens for presentation to CD4-positive T lymphocytes (reviewed in Cresswell, 1994). The MHC class II complex consists of α and β chains that associate in the endoplasmic reticulum (ER) with the 31–33-kD invariant (I) chain (Marks et al., 1990). It is generally accepted that the association of I-chain with MHC class II prevents the binding of endogenous peptides present in the ER lumen (Teyton et al., 1990; Roche and Cresswell, 1991), and that it is required for the efficient exit of the complex from the ER (Layet and Germain, 1991; Viville et al., 1993; Elliott et al., 1994). After passage through the Golgi complex, the majority of MHC class II-I-chain trimers are believed to be transported directly to an endocytic compartment (reviewed in Neefjes et al., 1991; Harding and Geuze, 1993*b*; Cresswell, 1994). In contrast to most cell surface proteins, MHC class II α and β chains undergo a 2–3-h transport delay in a post-Golgi compartment before arrival at the cell surface (Neefjes et al., 1990). During this period, I-chain is dissociated from the αβ dimer by proteolytic cleavage and is rapidly degraded. A signal in the cytoplasmic tail of I-chain is necessary for the diversion of αβ-I-chain trimers from the constitutive secretory pathway (Claesson-Welsh and Peterson, 1985; Bakke and Dobberstein, 1990; Lotteau et al., 1990; Lamb et al., 1991; Anderson and Miller, 1992; Pieters et al., 1993). However, the pathway by which MHC class II-I-chain complexes exit the *trans*-Golgi network (TGN) and are delivered to the endocytic pathway has not been described.

We have previously reported that in B lymphoblastoid cell lines the majority of intracellular MHC class II molecules reside in a set of compartments, collectively termed the MIIC/MHC class II enriched compartment, which

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1. *Abbreviations used in this paper:* APC, antigen-presenting cells; CD-MPR, cation-dependent Man-6-P receptor; ICD, I-Cell Disease; MHC, major histocompatibility complex; MIIC, MHC class II-rich compartments; I-chain, invariant chain; Man-6-P, mannose 6-phosphate.

share certain characteristics with lysosomes: for instance, they contain acid hydrolases and lysosomal membrane proteins, are acidic, and are positioned relatively late on the endocytic pathway (Peters et al., 1991*a*, 1995; Geuze, 1994; Riberdy et al., 1994). The MIIC was postulated to represent the site at which newly synthesized MHC class II molecules enter the endocytic route and acquire antigenic peptides (Neefjes et al., 1990; Peters et al., 1991*a*; Kleijmeer et al., 1995). Indeed, MIICs have recently been shown to represent the major localization of HLA-DM (Sanderson et al., 1994; Marks et al., 1995; Nijman et al., 1995), a novel MHC class II product that plays a decisive role in peptide loading of the MHC class II molecules (Denzin and Cresswell, 1995; Sherman et al., 1995). Furthermore, a number of studies with macrophages and B lymphocytes have demonstrated that cell fractions with biochemical and morphological characteristics resembling the MIIC mediate both the catabolic production of immunogenic peptides (Harding et al., 1991; West et al., 1994) and their coupling to MHC class II molecules (Harding and Geuze, 1992, 1993*a*; Amigorena et al., 1994; Qiu et al., 1994; Tulp et al., 1994). However since the intracellular MHC class II molecules in some murine B lymphocytes have been recovered in low density compartments positioned early in the endocytic pathway, it is possible that there is functional diversity among the MIICs of different cell types (Amigorena et al., 1994; Barnes and Mitchell, 1995; Mitchell et al., 1995; Mellman et al., 1995).

While considerable information is available concerning the trafficking of newly synthesized MHC class II molecules and the I-chain, little is known about the mechanism whereby acid hydrolases are packaged into the MIICs. The best documented pathway for soluble lysosomal enzyme targeting is that mediated by the mannose 6-phosphate (Man-6-P) recognition system. The N-linked oligosaccharides of newly synthesized lysosomal enzymes acquire Man-6-P residues that serve as recognition signals for binding to either the cation-dependent Man-6-P receptor (CD-MPR) or the Man-6-P/IGF-II receptor in the TGN (reviewed in Kornfeld and Mellman, 1989). Man-6-P receptors with bound enzyme are incorporated into Golgi-derived clathrin-coated vesicles (Geuze et al., 1985; Bleekemolen et al., 1988; Griffiths et al., 1988; Brown, 1990; Klumperman et al., 1993) and transported to acidified endosomal compartments, where the ligands are discharged for subsequent packaging into lysosomes.

However, lysosomal enzymes can also be delivered to lysosomes by a Man-6-P–independent targeting mechanism. Patients with the autosomal recessive disorder termed I-Cell Disease (ICD) have severely reduced levels of the enzyme *N*-acetylglucosamine 1-phosphotransferase, which forms the Man-6-P moiety on newly synthesized lysosomal enzymes (Hasilik et al., 1981; Reitman et al., 1981). Although this disorder results in severe cellular lysosomal enzyme deficiencies in many tissues, certain cell types in ICD patients, such as B lymphocytes, have nearly normal levels of lysosomal enzymes (Little et al., 1987; Okada et al., 1988; Tsuji et al., 1988). The targeting of newly synthesized lysosomal enzymes in an ICD B lymphoblastoid cell line occurs by a direct intracellular route (DiCioccio and Miller, 1991; Glickman and Kornfeld, 1993) and in at least one instance is dependent upon the selective recognition of amino acid sequences present in lysosomal enzymes but absent from related secretory proteins (Glickman and Kornfeld, 1993). Therefore, human B lymphoblasts appear to possess two separate lysosomal enzyme targeting pathways. Since ICD B lymphoblasts lack the Man-6-P–dependent pathway, this cell line allowed us to specifically examine the relationship of the alternate lysosomal enzyme targeting pathway to the formation of the MIIC using both morphological and biochemical techniques. We found that the acid hydrolase cathepsin D and MHC class II-I-chain complexes colocalize in TGN-associated buds and vesicles that lack γ-adaptin, a marker of clathrin-coated vesicles. Metabolic labeling and subcellular fractionation experiments indicate that soon after exiting the TGN, newly synthesized cathepsin D and MHC class II-I-chain complexes enter a vesicular compartment resembling MIICs observed in whole cells. These findings indicate that in ICD B lymphoblasts, acid hydrolases are incorporated into MIICs by a process that is independent of the Man-6-P pathway. Further, these enzymes, together with MHC class II-I-chain complexes, appear to be transported to MIICs via non-clathrin-coated vesicles.

Materials and Methods

Materials

Distilled acrolein was from Polysciences Inc. (Warrington, PA). Protein A and bovine serum albumin (BSA) were conjugated to colloidal gold particles as described (Slot and Geuze, 1985). EXPRE^{35}S^{35}S protein labeling mix was from DuPont-NEN (Boston, MA). [^{3}H]UDP-galactose, ECL system, and Amplify fluorographic reagent were from Amersham (Arlington Heights, IL). Protein A–agarose was from Repligen Corp. (Cambridge, MA). Endoglycosidase H and swainsonine were from Boehringer Mannheim Corp. (Indianapolis, IN). Hygromycin B was from Calbiochem Novabiochem (La Jolla, CA). Percoll was from Pharmacia LKB Nuclear (Gaithersburg, MD). All other chemicals were from Sigma Chemical Co. (St. Louis, MO).

Cells and Antibodies

The human B lymphoblastoid cell line (BLCL) JY was obtained from American Type Culture Collection (ATCC) (Rockville, MD) and was maintained in RPMI 1640, 10% heat-inactivated fetal calf serum (FCS; Hazleton Biologics, Lexena, KS), 100 U/ml penicillin, 100 U/ml streptomycin at a density of 3–5 × 10^5/ml in a humidified 5% CO_2 atmosphere. A B lymphoblastoid cell line derived from an ICD patient (Little et al., 1987) was kindly provided by Dr. A.L. Miller (University of California, San Diego) and was maintained in RPMI 1640, 10% bovine calf serum (Hyclone, Logan, UT), 100 U/ml penicillin, and 100 U/ml streptomycin under the same conditions. ICD BLCL transfected with cDNAs for human pepsinogen or human cathepsin D (Glickman and Kornfeld, 1993) were maintained in the same medium supplemented with 200 U/ml hygromycin B.

Rabbit antisera to human cathepsin D and the Man-6-P/IGF-II receptor have been described previously (Griffiths et al., 1988; Baranski et al., 1990). Rabbit antisera to human pepsinogen were generated by injection of the purified protein (gift of Dr. I. Michael Samloff, UCLA). Rabbit antisera directed against human invariant (I-) chain were generated by injection of KLH-conjugated peptides corresponding to residues 4–20 (IN) or 191–210 (IC) of the I-chain sequence (Strubin et al., 1984). IgG was purified from immune sera using protein A–agarose. Hybridoma cells secreting the anti-MHC class I monoclonal antibody W6/32 (Parham et al., 1979) were obtained from ATCC. Hybridoma cells secreting the anti-MHC class II monoclonal antibodies HK14 (Shipp et al., 1986) and DA6.147 (Guy et al., 1982) were the kind gifts of Dr. M. Nahm (Washington University School of Medicine) and Dr. B. D Schwartz (Monsanto Chem. Corp., St. Louis, MO), respectively. Purified monoclonal antibodies to α-adaptin (AC1-M11; Robinson, 1987), γ-adaptin (100.3; Ahle et al., 1988), clathrin (TD.1; Näthke et al., 1992), and β COP (M3A5; Allan and

Kreis, 1986) were gifts of M. Robinson (University of Cambridge, UK), E. Ungewickell (Washington University, St Louis, MO), F. Brodsky (University of California, San Francisco), and T. Kreis (EMBL, Heidelberg, Germany), respectively.

Other antibodies used for immunoelectron microscopy were raised in rabbits using human cathepsin D (Geuze et al., 1988), human CD63 (Metzelaar et al., 1991), human MHC class I and class II (Peters et al., 1991*a*), the Man-6-P/IGF-II receptor (Geuze et al., 1988), synthetic peptides corresponding to the CD-MPR cytoplasmic domain (Schulze-Garg et al., 1993) and selected COOH-terminal (IC) and NH_2-terminal (IN) peptides of human I-chain (Morton et al., 1995).

Processing for Immuno-Electron Microscopy

Cells in logarithmic growth phase were centrifuged for 10 min at 500 *g*, 4°C, and washed three times with 0.1 M Na phosphate, pH 7.4. Cells were gently resuspended to a density of 2–5 × 10^6/ml in the same buffer and fixed by the addition of an equal volume of 4% paraformaldehyde, 2% acrolein, 0.1 M Na phosphate, pH 7.4 (freshly prepared). After 2 h of constant mixing at room temperature, the fixed cells were washed three times with 0.1 M Na phosphate, pH 7.4, and stored in 2% paraformaldehyde, 0.1 M Na phosphate pH 7.4, until further processing.

BSA–5-nm gold conjugates to be used in endocytic uptake experiments were diluted to an optical density (at 520 nm) of 5 and dialyzed extensively against serum-free RPMI 1640 medium at 4°C. Cells (1 × 10^7) were centrifuged, washed three times in ice-cold serum-free RPMI 1640, and resuspended in dialyzed BSA-gold at a density of ≈5 × 10^6/ml. After 10, 30, or 60 min in a 37°C/5% CO_2 incubator, aliquots of cells were cooled on ice, centifuged 5 min at 500 *g*, 4°C, washed three times in ice-cold serum-free RPMI 1640, and fixed in 2% paraformaldehyde, 1% acrolein as described above.

Cells were embedded in 10% gelatin that was solidified on ice. Gelatin blocks with cells were immersed in 2.3 M sucrose in phosphate buffer for 2 h at 4°C and ultrathin cryosections were collected on a mixture of sucrose and methyl cellulose to improve membrane morphology (Liou and Slot, 1994). Ultrathin cryosections were single and double immunolabeled as described (Slot et al., 1991) with 10- and 15-nm protein A–conjugated colloidal gold probes (Slot and Geuze, 1985).

Quantitation of Immunogold and Endocytosis

Quantitation of immunogold labeling for I-chain and MHC class II in JY and ICD B lymphoblasts was performed at a magnification of 12,000 directly from the screen of the electron microscope as described in the legend to Table I. JY cells were used to quantitate possible colocalization of MHC class I, MHC class II, and the Man-6-P/IGF-II receptor with γ-adaptin in TGN/Golgi regions of 20 random cell profiles. Electron micrographs at a final magnification of 61,000 were taken from the TGN/Golgi regions in sections double-immunogold labeled for MHC class I, MHC class II, or the Man-6-P/IGF-II receptor with 5 nm gold and γ-adaptin with 10 nm gold. Gold particles were allocated to γ-adaptin–positive TGN elements (mostly coated buds and vesicles), γ-adaptin–negative TGN elements, and Golgi cisternae. Membrane measurements were performed by counting intersections of membrane profiles with a lattice of squared lines on a transparent overlay, and revealed that 10% of the TGN membrane was γ-adaptin coated. A total of 710, 677, and 462 gold particles were counted for MHC class I, MHC class II, and the Man-6-P/IGF-II receptor, respectively. The appearance of BSA-gold particles in MIICs was evaluated as described in the legend to Table II. Counting was done directly from the screen in the electron microscope at a magnification of 15,000.

Metabolic Labeling and Subcellular Fractionation

Cells (1–2 × 10^8) were washed twice in methionine-free, glutamine-free RPMI 1640, 10% dialyzed FCS, 25 mM Hepes-NaOH, pH 7.4, 100 U/ml penicillin, 100 U/ml streptomycin, and resuspended in this media at ≈1–2 × 10^7 cells/ml. EXPRE^{35}S^{35}S was added to 0.5 mCi/ml, and the cells were incubated for 10 min at 37°C and chased in the presence of 5 mM methionine for 15–20 min at 37°C. The cells were incubated for 1 h in a 20°C water bath, and then for various times in a 37°C/5% CO_2 incubator as described in Results. Samples harvested at each time point were chilled on ice, and cells were pelleted and washed twice with ice-cold PBS and once with ice-cold vesicle homogenization buffer (VB: 0.14 M sucrose, 75 mM K acetate, 10 mM MES-KOH, 1 mM EGTA, 0.5 mM Mg acetate, pH 6.6). All further operations were carried out at 4°C. Cells were resuspended in 1.0 ml VB containing 1 mM PMSF, 0.25 TIU/ml aprotinin, and 1× of a protease inhibitor cocktail containing 2 μg/ml (final) leupeptin, pepstatin A, chymostatin, and antipain and homogenized with 12 passes through a ball bearing homogenizer with a 0.0011″ clearance (Balch et al., 1984; manufactured by Stanford University Medical School machine shop). The homogenate was centrifuged for 10 min at 500 *g* in a Beckman J-6B centrifuge, the postnuclear supernatant was decanted, and the pellet was resuspended in 500 μl VB for a second round of homogenization. The pooled PNS fraction was spun for 20 s in a microfuge to pellet any residual cells and nuclei and layered on a 2.5-ml cushion of 0.25 M sucrose in VB and centrifuged for 2 h at 120,000 *g* in a Beckman SW 55 rotor. The supernatant was carefully aspirated, the walls of the tube blotted dry, and the pellet resuspended in 300–400 μl VB plus protease inhibitors. In some experiments, membranes were collected on a 500-μl pad of 2.5 M sucrose and harvested in a minimum volume of the above buffer. Aliquots of this fraction were loaded on a 10–90% D_2O gradient prepared in a 13 × 51 mm Ultra Clear tube by layering 500 μl each of 90, 75, 60, 45, 30, 20, and 10% D_2O in VB plus protease inhibitors. Gradients were centrifuged for 30 min at 45,000 *g* in an SW55 rotor. 13 320-μl fractions were collected from the top (1 = Top), and pelleted material was solubilized in 320 μl VB plus 0.5% TX100 (fraction 14). Fractions were used for immunoprecipitation, enzyme assay or immunoblotting as described elsewhere in Materials and Methods. D_2O concentration in fractions was determined by refractometry and comparison to a standard curve.

Immunoprecipitation

All operations were performed at 4°C. Subcellular fractions were diluted in 0.1 M Tris-HCl, 0.1 M NaCl, 1% Triton X-100, 1 mM PMSF, 0.1 TI U/ml aprotinin, 0.2% BSA, pH 8.0, briefly sonicated, and precleared by 1-h incubation with protein A–agarose with constant agitation. The supernatant from this incubation was collected, appropriate antiserum or hybridoma culture supernatant was added, and the samples were incubated for 4–12 h with constant agitation. For immunoprecipitation with anti-cathepsin D and anti-pepsinogen antisera, samples were adjusted to 0.5% Na deoxycholate and 0.2% SDS. 25 μl of protein A–agarose was added, and the sample was incubated for an additional 60 min with constant agitation. Agarose beads were pelleted and washed four times with 0.1 M Tris-HCl, 0.1 M NaCl, 1% Triton X-100, pH 8.0, and twice with 0.1 M Tris-HCl, pH 8.0. The supernatants were decanted and cleared with a second round of protein A–agarose for additional rounds of immunoprecipitation. Beads were eluted with nonreducing (anti-cathepsin D and anti-pepsinogen) or reducing SDS sample buffer by heating at 100°C and separated on 10 or 12% SDS–polyacrylamide gels (Laemmli, 1970). Gels were fixed, impregnated with Amplify, dried, and fluorographed using Kodak XAR film. Radioactive bands were quantitated by excision and scintillation counting as described previously (Glickman and Kornfeld, 1993) or by densitometric scanning with a Molecular Dynamics Personal Densitometer (Sunnyvale, CA). Both methods gave similar results.

Immunolabeling of Subcellular Fractions

Velocity gradient fractions (typically fractions 2 through 8) were pooled, diluted two- to threefold with the appropriate homogenization buffer plus protease inhibitors and layered on a 1 ml pad of 2.5 M sucrose, 25 mM Hepes-KOH, pH 7.2, in a 14 × 89-mm Ultra Clear tube. This sample was centrifuged for 3 h at 150,000 *g*, 4°C, and membranes collecting on the pad were harvested in a minimum volume and fixed in 2% paraformaldehyde, 1% acrolein, 0.1 M Na phosphate, pH 7.4, at room temperature. The fixed sample was stored in microfuge tubes precoated with 1% BSA until further processing. For double-immunogold labeling, fixed samples were concentrated at 12,000 *g* for 5 min. Samples of 2 μl were put on grids and were allowed to adhere to the supporting film on the grids for 15 min in a moistened environment to prevent drying. Immunolabeling was performed as described above.

Assays and Miscellaneous Methods

β-Hexosaminidase, α-glucosidase II (Little et al., 1987), and mannosidase II were assayed using 1 mM of the appropriate 4-methylumbelliferyl substrate at pH 4.5, 7.0, and 6.0, respectively, in the presence of 0.1% Triton X-100. Fluorescence was measured on an Aminco fluorimeter. Mannosidase II activity was calculated as the amount of substrate hydrolysis inhibited by 10 μM swainsonine (Bischoff and Kornfeld, 1984). Galactosyltransferase activity was assayed as described in Goldberg and Kornfeld

(1983). Protein was assayed with the Bio-Rad (Bio-Rad Labs., Richmond, CA) dye reagent using bovine serum albumin as a standard.

For Western blotting, proteins were precipitated with chloroform/methanol (Pohl, 1990), resuspended in reducing SDS sample buffer, electrophoresed on SDS-PAGE minigels (Laemmli, 1970), and blotted onto nitrocellulose using a Bio Rad miniblotter. Blots were blocked overnight in 20 mM Tris-HCl, 150 mM NaCl, 0.1% Tween 20 (TBST) plus 5% dry milk, and incubated for 2 h with the appropriate dilution of primary antibody in TBST, 0.5% dry milk. The blots were washed, incubated with appropriate HRP-conjugated secondary antibodies, washed, and developed with chemiluminescent reagents (Amersham) according to the manufacturer's instructions.

Results

ICD B Lymphoblasts Have Typical MIICs

When the distribution of MHC class II molecules in a human B lymphoblastoid cell line (JY) was examined in immunogold labeled cryosections, the molecules were predominantly located at the plasma membrane (64% of the total) and in MIICs (32% of the total; Table I). By contrast, I-chain was mostly located in the ER (43% of the total), the Golgi complex including the TGN (28%) and in MIICs (27%) and was undetectable at the cell surface. Thus, the distribution of MHC class II and I-chain molecules showed a major overlap in the TGN and in MIICs, confirming that I-chain is a good marker for the biosynthetic pathway taken by MHC class II molecules en route to the plasma membrane (Pieters et al., 1991; Neefjes and Ploegh, 1992).

As shown in most of the figures and especially in Figs. 1 *A*, and 2, *B* and *C*, ICD B lymphoblasts also exhibited numerous MIICs with internal membrane sheets and vesicles. The distribution of I-chain and MHC class II in ICD B cells was almost identical to that found in JY cells (Table I). As previously observed in JY cells (Peters et al., 1991*a*), the MIICs in ICD B cells contained the lysosomal membrane protein CD63, were devoid of both of the Man-6-P receptors, and accumulated the weak base DAMP, indicating that their content is acidic (not shown). The MIICs that contained I-chain were small, irregularly shaped and were usually located close to the Golgi complex (Figs. 1 *A*, 2 *B*, and see Fig. 10, *A* and *B*). These MIICs can be recognized by their morphology often showing an empty space surrounded by a cisternae with internal vesicles (Figs. 2 *B* and 10 *B*). These structures likely represent forming MIICs. MIICs lacking I-chain were larger and often exhibited membrane sheets in their lumens (Figs. 1 *A* and 2 *B*). Mixed multivesicular and multilaminar MIICs were also present (Fig. 2 *B*). Classical lysosomes, which had amorphous contents and did not label for MHC class II molecules, were also present (Fig. 1 *A*; see also Harding and Geuze, 1993*a*). Endosomes lacked MHC class II- and I-chain labeling and were scarce in both JY and ICD B lymphoblasts. Since I-chain is rapidly degraded after passage through the Golgi complex (Neefjes and Ploegh, 1992; Riberdy et al., 1994; Morton et al., 1995), we conclude that the small, irregularly shaped MIICs with detectable I-chain labeling represent newly formed, "early" MIICs and that the larger, more peripheral structures lacking I-chain are more mature, "late" MIICs.

Table I. Distribution of Invariant Chain and MHC Class II in Normal (JY) and ICD B Lymphoblasts

	JY		ICD	
	I-chain	MHC class II	I-chain	MHC class II
	%		%	
ER	43	–	45	4
Golgi	9	–	9	–
TGN	19	3	15	2
MIIC	27	32	24	20
Vesicles	2	1	7	–
PM	–	64	–	74

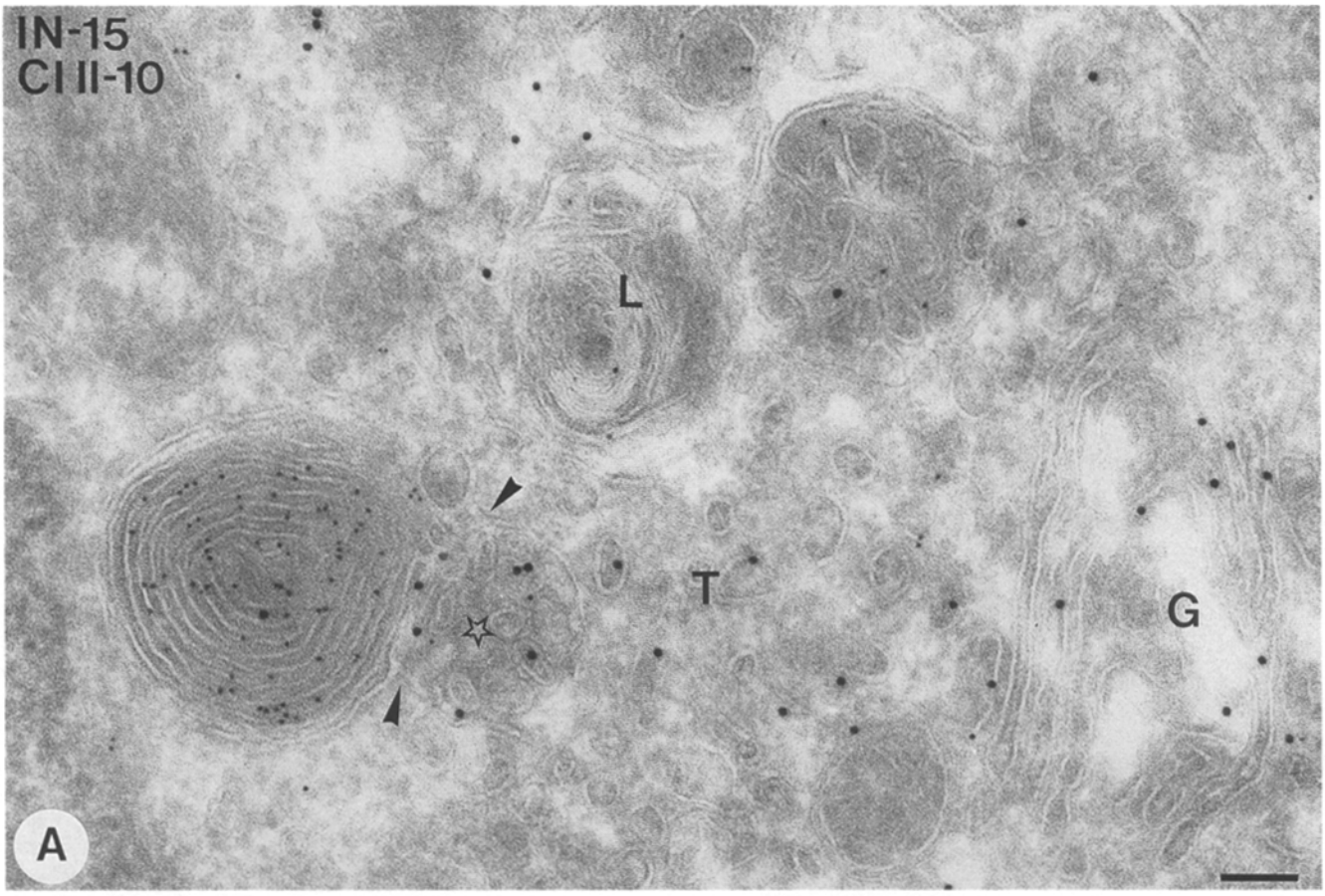
Cryosections of JY cells and ICD B lymphoblasts were single-labeled with an antibody against the NH_2 terminus of I-chain or with anti-MHC class II and 10-nm protein A-gold. Gold particles were counted in the electron microscope at a magnification of 12,000. 20 random cell profiles of each cell type were evaluated. The total numbers of gold particles counted for I-chain and MHC class II were 1656 and 6062 in JY cells and 909 and 5141 in ICD B cells, respectively. Background labeling on companion sections labeled with anti-amylase antibodies amounted to 3% of this total and was subtracted for each compartment. The values represent percentages of total gold particles counted. *ER*, endoplasmic reticulum; *PM*, plasma membrane; *TGN*, *trans*-Golgi network.

MHC Class II Localization in the TGN of ICD B Lymphoblasts

To further investigate the structural relationship between the early MIICs and the TGN, we processed cryosections according to a modified procedure (Liou and Slot, 1994) that results in a sharper visualization of membranes than was previously possible. Fig. 1 *B* shows small, irregularly shaped MIIC profiles located amidst membranes labeled for the Golgi clathrin-coated vesicle protein γ-adaptin (Ahle et al., 1988). Membrane-associated γ-adaptin is found almost exclusively in the TGN (Robinson, 1987; Ahle et al., 1988; Geuze et al., 1992; Robinson and Kreis, 1992), and consequently is a marker for a portion of the TGN. Some of the MIIC profiles are closely associated with γ-adaptin–labeled tubules (Fig. 1 *B*, *arrowheads*), indicating that these structures are related to the TGN rather than endosomes or some other compartment. γ-adaptin was rarely detectable on the MHC class II-positive structures themselves, but was often present on buds and vesicles adjacent to them (Figs. 1 *B* and 3). The small I-chain–positive multivesicular MIICs near the Golgi often appeared to be fusing with each other and with multilaminar MIICs that lack I-chain (Fig. 1 *A*).

Quantitation of double-labeled cryosections of JY cells revealed a similar segregation between MHC class II mol-

Figure 1. Localization of MHC class II in ICD B lymphoblasts. (*A*) Golgi area with a stack of Golgi cisternae (*G*) and TGN (*T*) showing many I-chain–positive vesicles and a possible fusion profile (*arrowheads*) between an early MIIC with MHC class II labeling and abundant I-chain labeling and a later MIIC with only MHC class II labeling. *L*, lysosome. (*B*) Golgi complex (*G*) and extensive *trans* Golgi area showing several MIICs (*asterisks*), some of which are connected to TGN elements (*arrowheads*) that are labeled for γ-adaptin. Abbreviations used throughout the figures are: *Ad*, γ-adaptin; *CD*, cathepsin D; *Cl II*, MHC class II; *Clat*, clathrin; *IC*, COOH-terminal (lumenal) epitope of invariant chain; *IN*, NH_2-terminal (cytoplasmic) epitope of invariant chain; *MPR*, cation-dependent mannose 6-phosphate receptor. The numbers following each abbreviation refer to the size of the gold particle used, in nm. Bars, 0.1 μm.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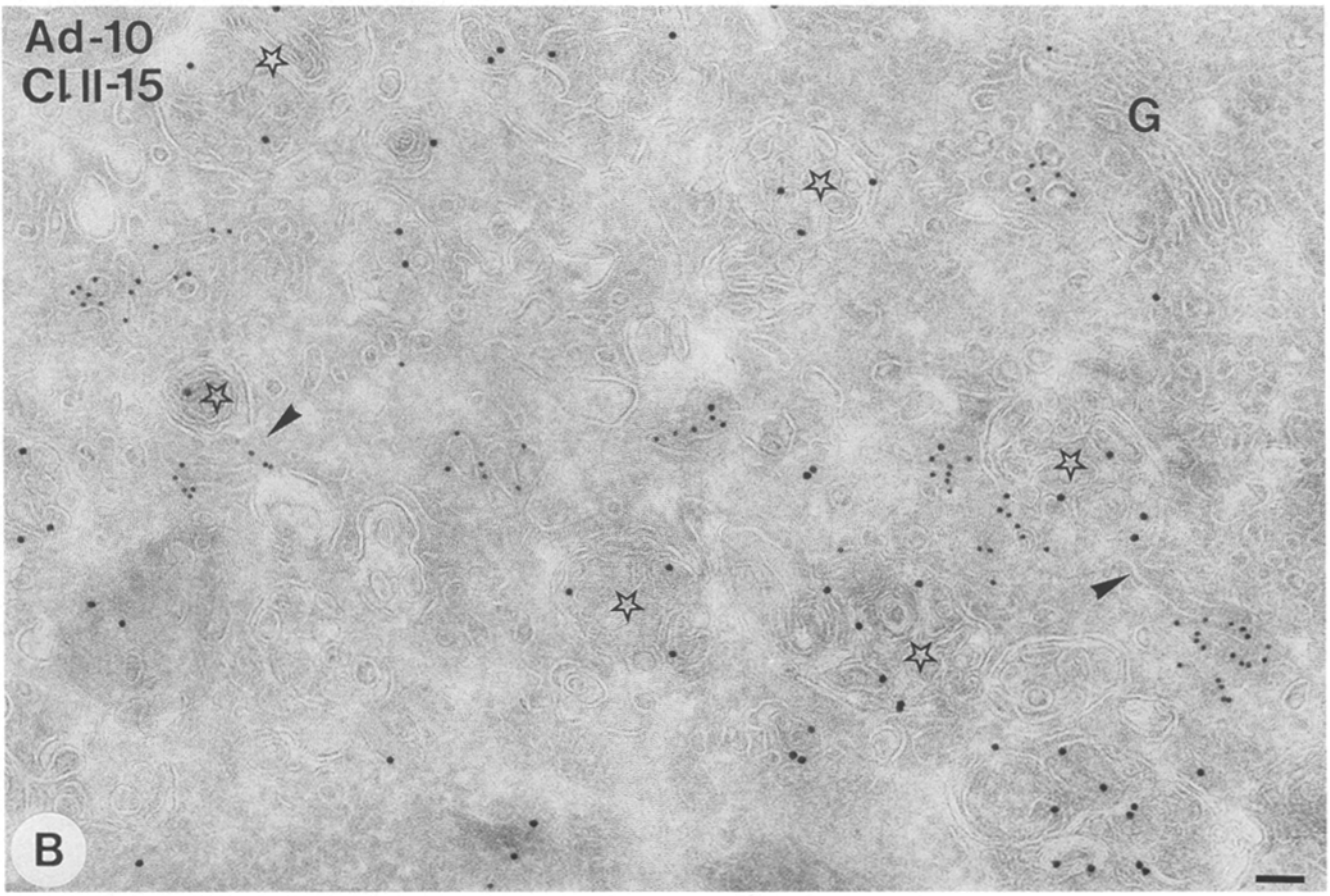
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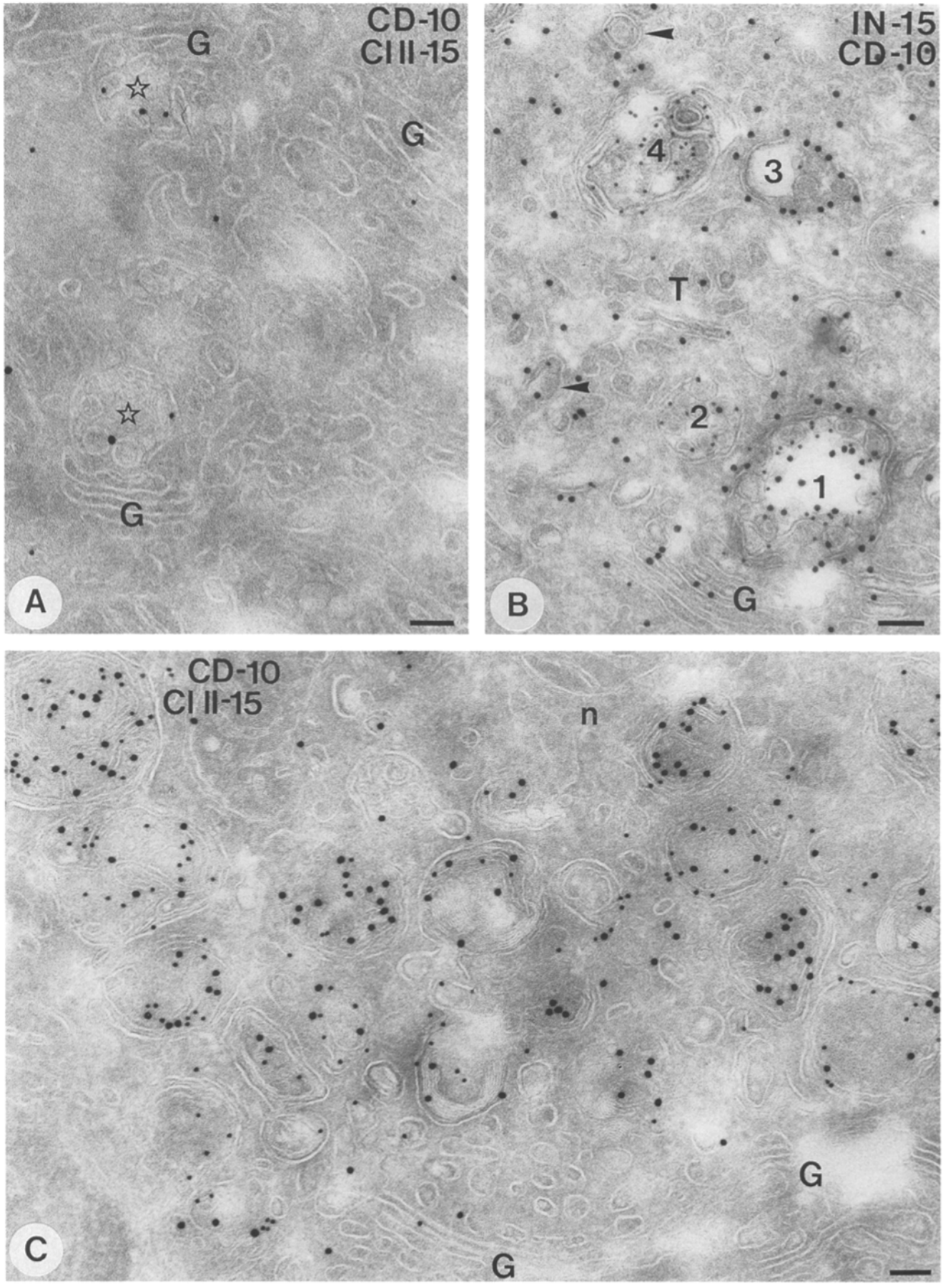
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ecules and Golgi clathrin-coated vesicles. Thus, only 2% of the anti-MHC class II particles in the Golgi regions were localized to γ-adaptin–positive TGN membranes, whereas 84% were present in γ-adaptin–negative TGN membranes and 14% were in the Golgi stack. The corresponding values for MHC class I, which is transported to the cell surface by the constitutive secretory pathway and is segregated from MHC class II molecules in the TGN (Neefjes et al., 1990; Peters et al., 1991*a*), were 2, 48, and 50%, respectively. By contrast, 13% of the total Golgi-associated Man-6-P/IGF-II receptor molecules were present on γ-adaptin–positive TGN membranes (mostly coated buds and vesicles), 76% occurred over γ-adaptin–negative TGN, and 11% were found in the Golgi stack. These results indicate that in B lymphoblasts, the Man-6-P/IGF-II receptor is concentrated relative to other proteins in γ-adaptin–positive TGN coated buds, as found previously in hepatoma cells (Klumperman et al., 1993), while MHC class II molecules are excluded from these structures. In normal cells, therefore, newly synthesized lysosomal enzymes bound to the Man-6-P/IGF-II receptor should not cosegregate with MHC class II molecules in the TGN.

Cathepsin D Colocalization with MHC Class II

In preliminary experiments, we found that the level of endogenous cathepsin D in B lymphoblasts was too low to allow reliable localization of this marker in the TGN, although late MIICs and classical lysosomes contained detectable amounts of this acid hydrolase. Consequently, to study the localization of cathepsin D in the TGN and in early MIICs, we used ICD B lymphoblasts that transfected with the human cathepsin D cDNA. These transfected cells sort cathepsin D to lysosomes with about the same efficiency as nontransfected cells (Glickman and Kornfeld, 1993). Fig. 2 shows that early MIIC profiles associated with the TGN contain cathepsin D together with MHC class II (Fig. 2, *A* and *C*) and I-chain (Fig. 2 *B*). As was observed for MHC class II, cathepsin D did not colocalize with γ-adaptin in the TGN (Fig. 3, *A* and *B*). Thus, I-chain and MHC class II-positive structures appear to acquire cathepsin D while still associated with the TGN or shortly thereafter.

Identification of Intracellular Vesicles Bearing Cathepsin D and MHC Class II-Invariant Chain Complexes

To document the cotransport of MHC class II molecules and cathepsin D further, we attempted to isolate post-Golgi vesicles containing one or both of the newly synthesized proteins. To do this, cathepsin D-transfected ICD lymphoblasts were labeled with ^{35}S-methionine for 10 min, chased for 15 min at 37°C, and incubated at 20°C for 1 h to accumulate labeled proteins in the Golgi. The lymphoblast cultures were then incubated for various times at 37°C, and the cells were collected and homogenized. Total cellular membranes were fractionated on 10–90% D_2O velocity gradients, which separate membranes on the basis of sedimentation coefficient and have been used to resolve small vesicles and larger organelle fragments (Pearse, 1982; Woodman and Warren, 1991). The velocity gradient fractions were then immunoprecipitated with anti-cathepsin D antisera. When the lymphoblasts were held at 20°C, the great majority of radiolabeled procathepsin D pelleted to the bottom of the gradient and very little secretion of cathepsin D occurred (Fig. 4 *A*). After a 30-min incubation at 37°C, a significant proportion (34 ± 2% over three separate experiments; see Fig. 6) of cellular procathepsin D was recovered in fractions 2 through 8, in the upper portion of the gradient (Fig. 4 *B*). Cathepsin D secretion could now be detected (lane *M*), indicating that exit from the TGN via the constitutive secretory pathway was occurring. The procathepsin D molecules in these slowly sedimenting fractions had an electrophoretic mobility identical to secreted procathepsin D, indicating that they were sialylated and were either in or had passed beyond the TGN (see Glickman and Kornfeld, 1993). The proportion of radiolabeled cathepsin D associated with fractions 2 through 8 was maximal at 90 min after the shift to 37°C and declined thereafter (Fig. 4, *C* and *D* and Fig. 6). For the duration of the experiment, sialylated procathepsin D was the only cathepsin D species associated with the slowly sedimenting fractions. Nonsialylated procathepsin D located in the ER and Golgi stack and the mature and heavy chain forms of cathepsin D that are generated in endosomal or lysosomal compartments (Gieselmann et al., 1983) were found almost entirely in the pellets of these gradients (Fig. 4, *C* and *D*). All forms of cathepsin D analyzed on velocity gradients were resistant to proteinase K digestion in the absence of detergent (results not shown), indicating that they had been isolated inside sealed membrane compartments. These results indicate that after exiting the TGN but before arriving at the lysosome, cathepsin D passes transiently through an intermediate compartment that is detectable by velocity gradient centrifugation.

When we analyzed the distribution of MHC class II species in D_2O velocity gradient experiments similar to those described above, we found that, as for procathepsin D, MHC class II-I-chain complexes appeared primarily in fractions 2 through 8 only after removal of the 20°C block (Fig. 5, *A* and *B*). The reduced intensity of the I-chain band relative to the MHC class II α chain band in these fractions compared to that present in the total membrane fraction (Fig. 5 *B*, lane *T*) suggests that some dissociation of I-chain from MHC class II had occurred. The association of MHC class II molecules with these fractions was

Figure 2. Localization of cathepsin D in transfected ICD B lymphoblasts. (*A*) Two MIIC-like structures, closely associated with the *trans*-Golgi and labeled for both cathepsin D and MHC class II. *G*, Golgi complex. (*B*) *trans*-Golgi area showing several MIIC-like compartments, three of which are of the multivesicular type (1, 2, 3) and contain labeling of both cathepsin D and I-chain NH_2 terminus. MIICs 1 and 3 located closest to the TGN (*T*) show the most intense labeling for I-chain, are irregularly shaped and represent the early type of MIIC. The mixed multivesicular and multilaminar MIIC (4) only shows cathepsin D and is a late MIIC. Arrowheads point to small double-labeled vesicles that may represent the immediate post-TGN transport intermediate in MHC class II and cathepsin D transport. (*G*) Golgi stack. (*C*) Survey of a *trans*-Golgi area with numerous MIICs labeled for cathepsin D and MHC class II. Note that the smaller and irregularly shaped MIICs are located closest to the Golgi stack (*G*). Bars, 0.1 μm.

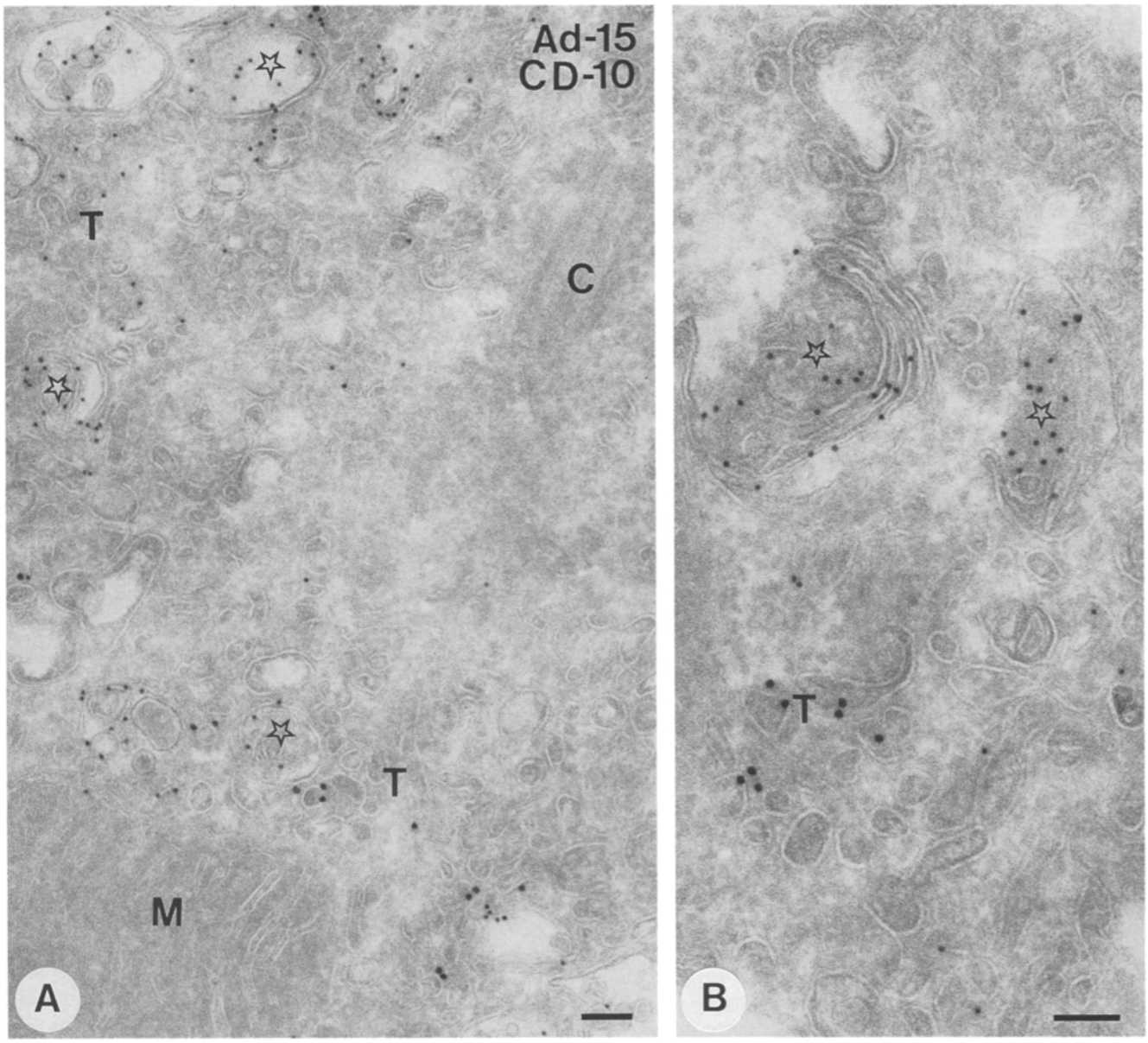

Figure 3. Cathepsin D-transfected ICD B lymphoblasts. (*A*) Survey of Golgi area with TGN (*T*) and centrioles (*C*). The small MIICs (*asterisks*) contain labeling for cathepsin D. There is no overlap of cathepsin D and γ-adaptin labeling. *M*, mitochondrion. (*B*) Higher magnification of a similar area. The irregular MIICs (*asterisks*) only show cathepsin D while γ-adaptin is localized to TGN tubules (*T*). Note the morphology of MIICs is suggestive of membrane folding. Bars, 0.1 μm.

maximal after 60 min incubation at 37°C (Fig. 6) but persisted over several hours and was marked by more complete dissociation from invariant chain (Fig. 5 *C*). Thus, the slowly sedimenting gradient fractions also contain a post-Golgi intermediate compartment(s) in MHC class II-I-chain transport.

The position of a variety of marker enzymes and proteins in the D_2O velocity gradient is shown in Fig. 7. The fractions most strongly positive for procathepsin D (Fig. 4 *C*, fractions 3–7) contained 12% of the total protein, 13% of the Golgi marker galactosyltransferase, and 4% of the lysosomal marker β-hexosaminidase recovered from the gradient (Fig. 7 *F*). Immunoblots for clathrin (Fig. 7 *A*), the Golgi clathrin-coated vesicle protein γ-adaptin (Fig. 7 *B*) and the plasma membrane coated vesicle protein α-adaptin (not shown) demonstrated that the distribution of these proteins paralleled that of sialylated cathepsin D (compare Fig. 7 *B* with 4 *C*). A sample of purified bovine brain-coated vesicles sedimented to an equivalent position on a parallel gradient (results not shown). The remaining immunoreactivity for these proteins was found at the bottom of the gradient and presumably represents clathrin coats assembled on Golgi and plasma membranes (Robinson, 1987; Ahle et al., 1988; Robinson and Kreis, 1992) or aggregates of clathrin-coated vesicles. A small fraction of the total cellular Man-6-P/IGF-II receptor also cofractionated with the clathrin coat proteins (Fig. 7 *C*). The remaining receptor was recovered in the bottom of the gradient, most likely associated with the TGN, endosomes, and the plasma membrane. We also examined the distribution of

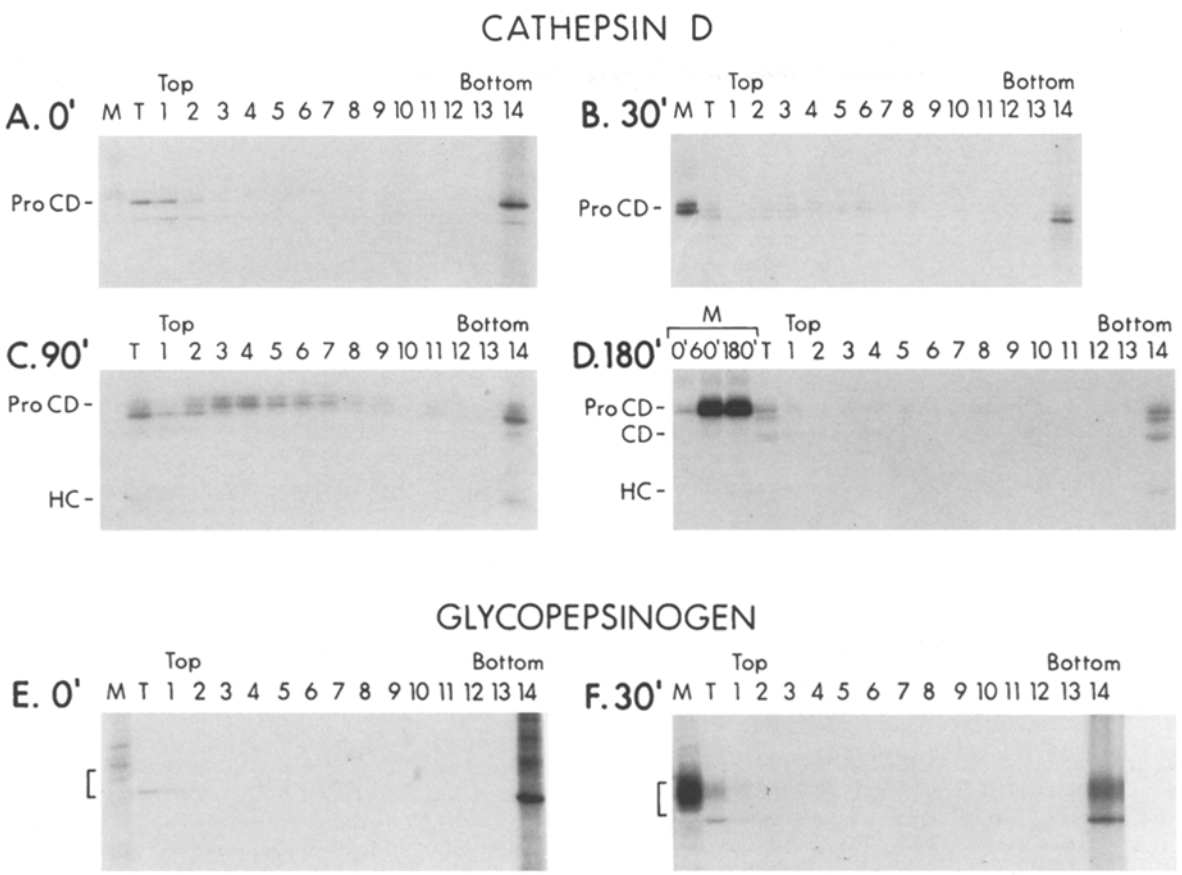

Figure 4. Velocity gradient analysis of cathepsin D and glycopepsinogen transport in transfected ICD B lymphoblasts. Membranes from cathepsin D (*A–D*) or glycopepsinogen (*E–F*) transfected cells labeled as described in the Results section were analyzed on D_2O velocity gradients. Equal portions of all velocity gradient fractions were immunoprecipitated with antisera to cathepsin D or pepsinogen, as appropriate, and analyzed by SDS-PAGE and fluorography. Aliquots of culture media (lanes *M*) and of total membranes (lanes *T*) from each time point were immunoprecipitated in parallel to the velocity gradient fractions. Positions of procathepsin D (*proCD*), mature cathepsin D (*CD*), and cathepsin D heavy chain (*HC*) are indicated. (*A*) Cathepsin D, 20°C block (i.e., 0′ at 37°C); (*B*) 30 min at 37°C; (*C*) 90 min at 37°C; (*D*) 180 min at 37°C; (*E*) Glycopepsinogen, 20°C block (i.e., 0′ at 37°C); (*F*) 30 min at 37°C.

β-COP, a coat protein present primarily in pre-Golgi transport vesicles (Duden et al., 1991; Serafini et al., 1991). In contrast to the clathrin-coated vesicle proteins, the great majority of β-COP immunoreactivity was found at the bottom of the gradient (Fig. 7 *E*). The small amount of β-COP found near the top of the gradient cofractionated with galactosyltransferase activity (Fig. 7 *F*) and was clearly distinct from the distribution of the other markers. Only a small fraction of total cellular HLA DRα chain and I-chain, as assessed by immunoblotting, was found in fractions 2 through 8 of the gradient (Fig. 7 *D* and results not shown). Therefore, the sedimentation properties of the procathepsin D- and MHC class II-containing intermediate(s) resemble those of clathrin-coated vesicles but do not correspond to those of any other organelle marker tested.

To determine the relationship of the MHC class II intermediates with clathrin-coated vesicles, the membranes isolated from the pooled velocity gradient fractions were analyzed by immunoelectron microscopy. To avoid clustering of vesicles, diluted aliquots of fixed membranes were placed on grids and double-labeled with antibodies directed against the cytoplasmic domains of I-chain and the CD-MPR and against clathrin and γ-adaptin. As shown in Fig. 8, the I-chain labeling was found in vesicular structures that were devoid of γ-adaptin, CD-MPR or clathrin labeling (Fig. 8, *A–C*, respectively). The γ-adaptin and clathrin were present only on small vesicles bearing a morphologic coat while the CD-MPR was found on these structures as well as on larger, vesicular structures. In an attempt to localize cathepsin D in the content of the vesicles, we immunolabeled ultrathin cryosections of the fractions but were unable to detect cathepsin D because of technical difficulties.

To determine whether constitutively secreted proteins also moved through the same structures, we performed similar gradient fractionation experiments with glycopepsinogen-transfected ICD lymphoblasts. After a 60-min incubation at 20°C, no secretion of glycopepsinogen had occurred and 95% of the [^{35}S]methionine-labeled enzyme pelleted to the bottom of the D_2O velocity gradient (Fig. 4 *E*). At this time point, ~60% of cellular glycopepsinogen was Endo H sensitive (not shown) and therefore had not reached the TGN. During the 30 min following warming of the culture to 37°C, 90% of the cellular glycopepsinogen acquired Endo H resistance and was secreted (Fig. 4 *F*). In contrast to cathepsin D, glycopepsinogen did not accumulate in the slowly sedimenting fraction during this time (Fig. 6). Therefore, glycopepsinogen, which traverses the secretory pathway, is secreted without appearing in the

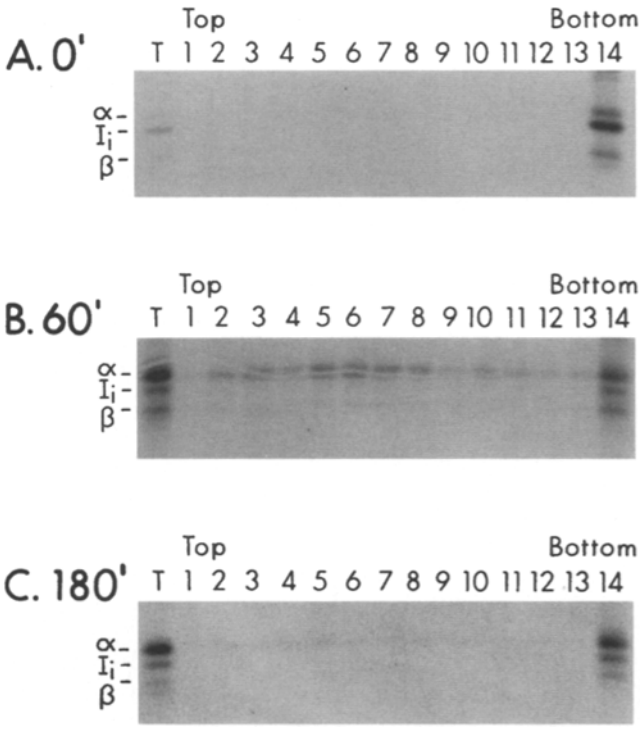

Figure 5. Velocity gradient analysis of MHC class II transport in ICD B lymphoblasts. Experimental details were similar to those in Fig. 4, except that gradient fractions were immunoprecipitated with the anti-HLA DR monoclonal antibody HK14. Positions of MHC class II α and β chains and I-chain are indicated. (*A*) 0 min at 37°C; (*B*) 60 min at 37°C; (*C*) 180 min at 37°C.

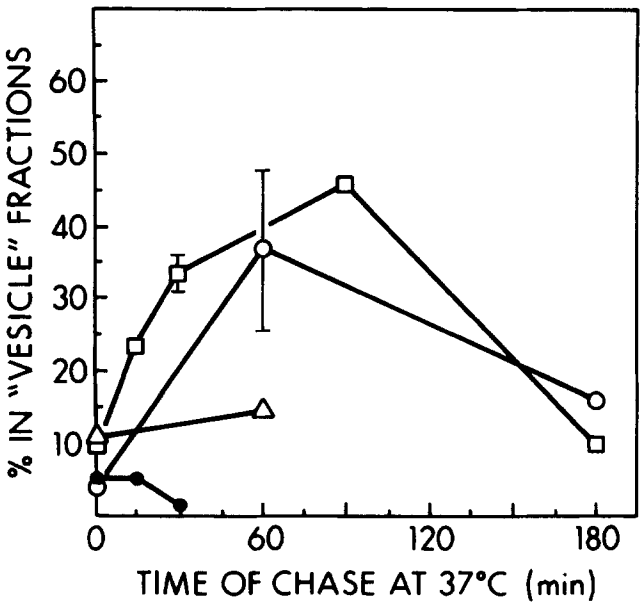

Figure 6. Quantitation of velocity gradient results, including those presented in Figs. 4 and 5. *Open squares,* procathepsin D; *closed circles,* glycopepsinogen; *open circles,* HLA DR α chain. Distribution of MHC class I heavy chain molecules (*open triangles*) was determined by serial immunoprecipitation of velocity gradient fractions with the monoclonal antibody W6/32. Radioactivity in bands was determined by excision and scintillation counting of gel slices, or by densitometric scanning of autoradiographs. The proportion of radiolabeled protein recovered in velocity gradient fractions 3 through 8 is plotted as a percentage of the total recovered from the gradient. Where error bars are given, values represent the mean ± SD of three independent experiments. All other values are single determinations.

gradient fractions that transiently accumulate procathepsin D and MHC class II (Fig. 6). Similarly, newly synthesized MHC class I molecules, another marker of the constitutive secretory pathway, did not accumulate in the velocity gradient fractions above their steady-state level (Fig. 6).

MHC Class II Localization in Normal B Lymphoblasts

As in ICD B lymphoblasts, JY cells showed close associations of MHC class II-positive structures and the TGN. A favorable section through a Golgi area is depicted in Fig. 9 showing three instances of membrane connections between TGN tubules and MHC class II-positive buds and vesicles. Labeling for γ-adaptin is absent from these profiles, but is present at other TGN subdomains. There is a close similarity between the TGN-associated MHC class II-positive structures present in the JY lymphoblasts and those in ICD B lymphoblasts (compare Fig. 9 with Figs. 2 *B* and 10, *A* and *B*). γ-Adaptin–coated vesicles at the *trans*-Golgi side were negative for MHC class II (Fig. 9, *inset*). These observations suggest that in normal B lymphoblasts, MHC class II can exit from the TGN in a similar fashion as in ICD B lymphoblasts. However, in ICD B lymphoblasts, MHC class II-positive buds were much more frequent.

The Endocytic Tracer BSA-Gold Enters Early MIIC

Previous studies (Peters et al., 1991*a*; Harding and Geuze, 1992, 1993*a*) have shown that MIICs are positioned late in the endocytic pathway, being labeled with endocytic tracers after 30 min or more of uptake. To identify the site where the biosynthetic pathway of MHC class II molecules and the endocytic route intersect, we allowed untransfected ICD B lymphoblasts and JY cells to internalize the fluid phase endocytic marker BSA-gold for 10, 30, and 60 min. The distribution of this tracer was then examined in cryosections labeled for I-chain and MHC class II. After 10 min of uptake, BSA-gold was found at the plasma membrane of each cell type and in small endosomal vesicles that did not label for I-chain (results not shown and Table II). Quantitation of immunolabeled sections revealed that only 5% of BSA-gold–positive structures contained MHC class II at this time. After 30 min of uptake, endocytic tracer had reached the MIICs. Strikingly, in both JY and ICD B lymphoblasts, endocytic tracer was found preferentially in irregular I-chain–positive MIICs at this time point (Fig. 10, *A* and *B*). Quantitation of double-immunolabeled cryosections (Table II) showed that whereas BSA-gold reached these I-chain–positive early MIICs between 10 and 30 min of uptake, significant amounts of tracer reached I-chain–negative MIICs only after 60 min. These results indicate that breakdown of I-chain occurs after MHC class II molecules have entered the endocytic pathway, and that "mature" I-chain–negative MIICs are positioned later on the endocytic pathway than are I-chain–

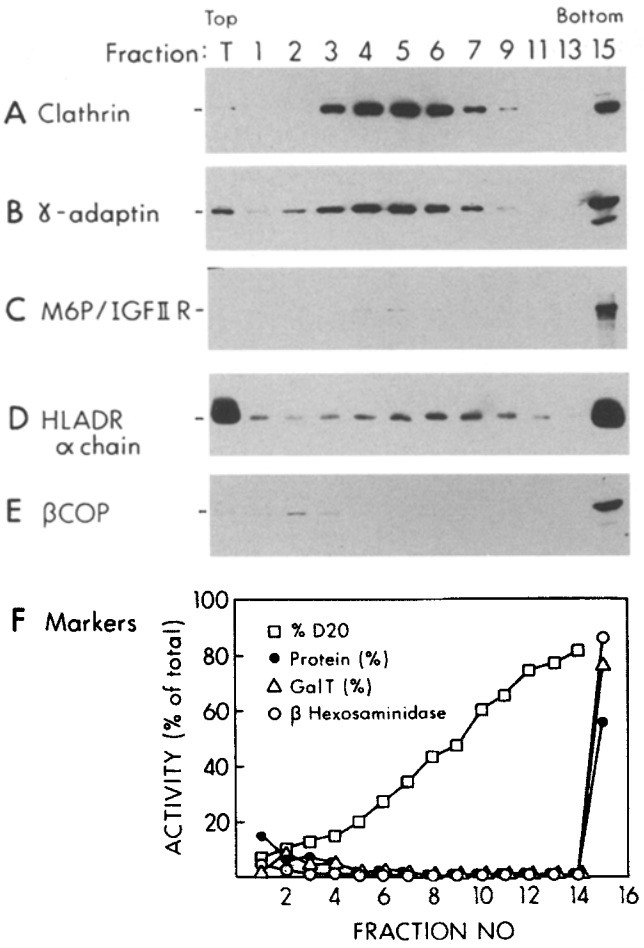

Figure 7. Characterization of velocity gradient fractions. (*A–E*) Equal portions of the indicated fractions were separated by SDS-PAGE, blotted onto nitrocellulose, and probed with antibodies against the indicated proteins: (*A*) anti-clathrin mAb TD.1; (*B*) anti-γ adaptin mAb100.3; (*C*) affinity-purified rabbit anti–Man-6-P/IGF-II receptor; (*D*) anti HLA DR α chain mAb DA6.147; (*E*) anti β-COP mAb M3A5. Bound antibody was detected with the appropriate horseradish peroxidase-conjugated secondary antibodies and chemiluminescence. Similar results for each marker were obtained in two to five independently run gradients. (*F*) distribution of marker enzymes. Results are typical of at least three determinations for each assay. *GalT*, galactosyltransferase. D_2O percentage in each fraction should be read on the scale to the left of the graph.

positive MIICs. In accordance with previous observation in normal B lymphoblasts (Peters et al., 1995), these results suggest that in ICD B lymphoblasts the pathways traveled by biosynthetic MHC class-II and endocytosed material intersect at a site in close proximity to the TGN, probably the irregularly shaped multivesicular early MIIC.

Discussion

In this study, the trafficking and localization of MHC class II, I-chain and cathepsin D were examined in a B lymphoblastoid cell line derived from a patient with I-cell disease. While these cells are defective in the synthesis of the Man-6-P recognition marker on soluble acid hydrolases, they are still able to selectively target these enzymes to lysosomes by a Man-6-P–independent mechanism (DiCioccio and Miller, 1991; Glickman and Kornfeld, 1993). Consequently the ICD B lymphoblasts provided an opportunity to examine whether acid hydrolases are also delivered to the MIICs by an alternate targeting pathway.

Table II. Appearance of BSA-Gold Particles in MIIC's of JY and ICD B Lymphoblasts

Minutes of uptake	JY		ICD	
	+I-chain	−I-chain	+I-chain	−I-chain
10	2	–	–	–
30	53	1	40	6
60	57	14	48	28

After the indicated times of continuous uptake of 5 nm BSA-gold particles, cells were harvested and fixed, and cryosections were double-immunogold labeled for I-chain and MHC class II with 10-nm and 15-nm gold particles, respectively. For each time-point, MIIC's with BSA-gold tracer in 50 random cell profiles were scored for labeling of I-chain or for MHC class II only. The values represent numbers of MIIC's containing tracer which were positive for MHC class II and I-chain (+I-chain) or for MHC class II only (−I-chain).

Immunoelectron microscopy of ultrathin cryosections revealed that ICD B lymphoblasts possess MIICs similar to those previously observed in normal B lymphoblasts (Peters et al., 1991*a*, 1995; Geuze, 1994), and that the overall distribution of MHC class II and I-chain was nearly identical in the two cell types. The Golgi region of ICD B lymphoblasts contained numerous MHC class II- and I-chain–positive profiles, some of which appeared to be in close association with membranes of the TGN as defined by immunolabeling for γ-adaptin, but were themselves negative for γ-adaptin. It is proposed that these vesicles represent transport vehicles for newly synthesized MHC class II. In the *trans*-Golgi region, MIICs were observed that labeled with antibodies against the I-chain lumenal and cytoplasmic domains and could be labeled with endocytic tracers. These findings indicate that the biosynthetic pathway of MHC class II and the endocytic pathway intersect immediately after Golgi passage of MHC class-II in newly formed MIICs. These early MIICs then undergo a maturation process during which the I-chain is destroyed and more internal membranes are formed by infolding and internal budding of limiting membrane. Certainly other mechanisms may exist for MHC class II- and I-chain to exit the TGN. Nevertheless, if this interpretation is correct, it implies that the MHC class II- and I-chain can leave the TGN by a process that does not require clathrin-coated vesicles. While similar profiles were observed in a normal B lymphoblast cell line (JY), they were much less abundant. This suggests that the budding process that we describe here may be amplified in the ICD B lymphoblasts and perhaps represents a less developed pathway in normal human B lymphoblasts.

In cathepsin D-transfected cells, cathepsin D labeling was abundant in MHC class II- and I-chain–positive buds and vesicles, and like MHC class II, was largely absent from γ-adaptin–positive regions of the TGN. However the MHC class II–positive structures labeled only weakly for glycopepsinogen (results not shown), a protein related to cathepsin D that is largely secreted from transfected ICD B lymphoblasts (Glickman and Kornfeld, 1993). Although Man-6-P–dependent transport presumably plays a role in the endosomal and lysosomal sorting of acid hydrolases in

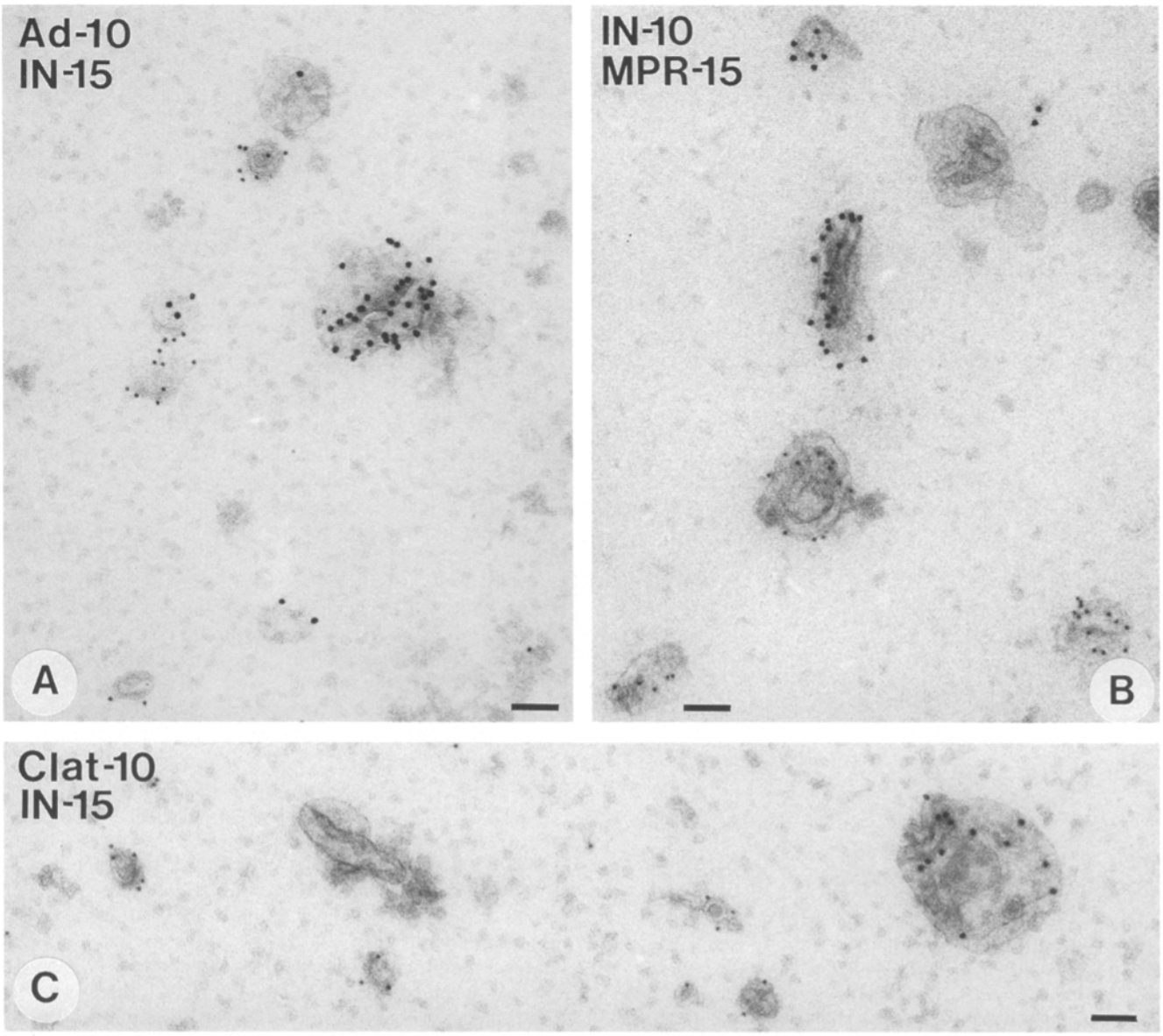

Figure 8. Morphology of velocity gradient fractions. Pooled D_2O velocity gradient fractions (fractions 3 through 8; see Figs. 4, 5, and 7) from 10^9 ICD B lymphoblasts were concentrated and fixed as described in Materials and Methods and immunogold labeled with antibodies against γ-adaptin, clathrin, and the cytoplasmic domains of I-chain (*IN*) and the cation-dependent Man-6-P receptor (*MPR*). I-chain did not colocalize with γ-adaptin (*A*), MPR (*B*), or clathrin (*C*). Bars, 0.1 μm.

normal B cells, the efficient and selective packaging of cathepsin D into MIICs in ICD B lymphoblasts shows that this process can occur independently of the Man-6-P targeting system.

Since the above results raised the possibility that MHC class II and cathepsin D may exit the TGN and reach MIICs by a clathrin-independent transport process, we designed kinetic experiments to identify vesicular intermediates in the transport of these molecules. These experiments showed that newly synthesized procathepsin D and MHC class II transiently associate with membrane vesicles distinguishable by their slow sedimentation on velocity gradients. Several findings support the contention that these vesicles derive from the Golgi and carry endosomally or lysosomally targeted procathepsin D and MHC class II. First, sialylated procathepsin D is the only form of cathepsin D associated with this fraction, indicating that the cathepsin D has passed through the *trans*-Golgi, where sialylation occurs, but has not reached the lysosome, where the prosegment is cleaved (Gieselmann et al., 1983). In addition, the MHC class II heterodimers found in these fractions also contain complex-type oligosaccharides (results not shown) and are only partially complexed with I-chain, arguing for a similar post-Golgi localization. Second, the procathepsin D and MHC class II molecules only enter the vesicles after removal of the 20°C block, which prevents proteins from exiting the Golgi. Third, we found that the fractions transiently positive for procathepsin D are significantly depleted of markers for the endoplasmic reticulum, Golgi, and lysosomes, and account for only a small fraction of cellular protein and [^{35}S]methionine-labeled protein.

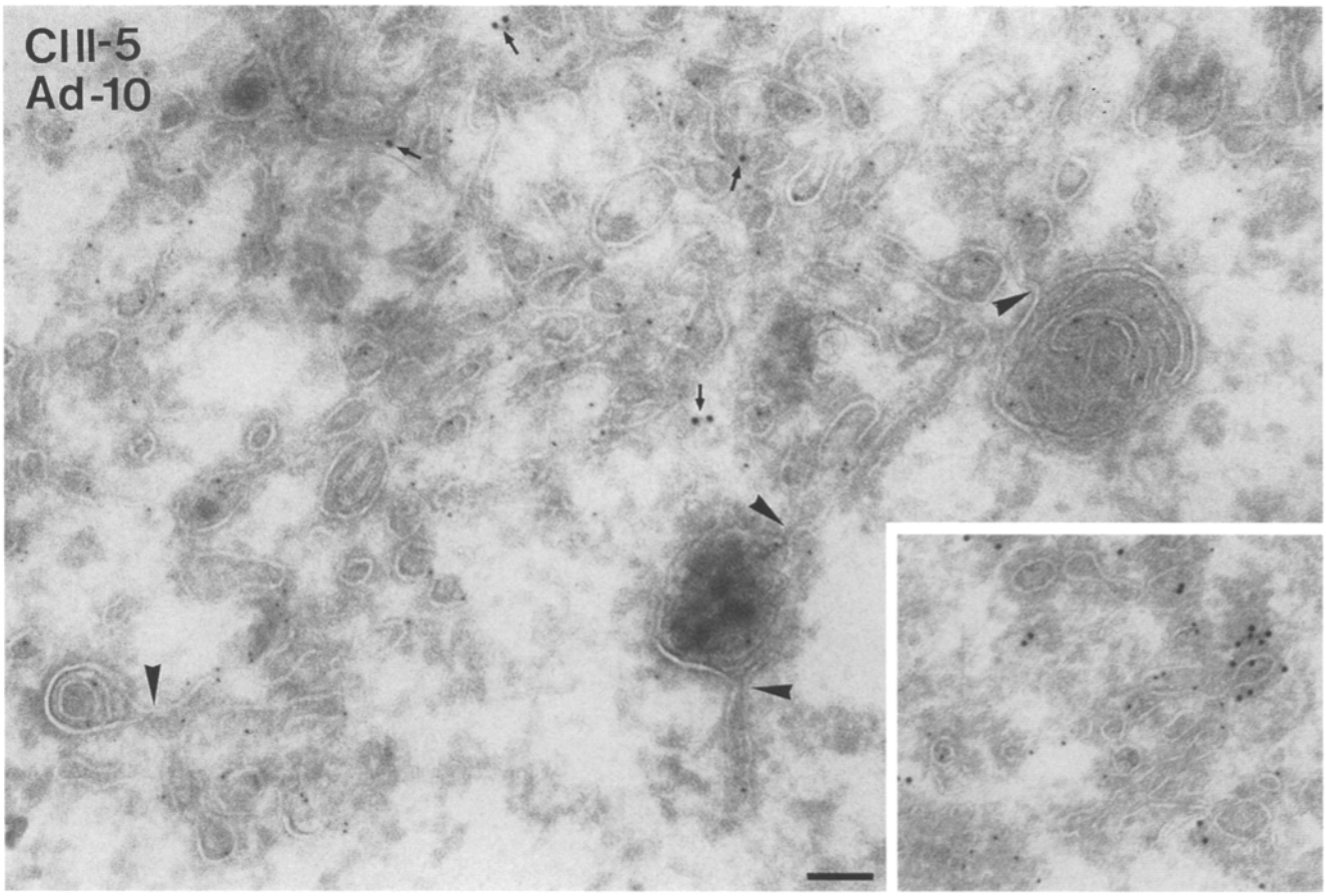

Figure 9. *trans*-Golgi area in normal B lymphocytes (*JY*). Network of TGN tubules showing scarce labeling for γ-adaptin (*arrows*) and abundant MHC class II labeling. The arrowheads indicate connections between the tubules and small MIIC-like profiles with internal membranes. (*Inset*) Another TGN subdomain showing distinct labeling for MHC class II and γ-adaptin. Bars, 0.1 μm.

Even though the procathepsin D and MHC class II molecules are only transiently associated with the vesicles, this fraction can account for as much as 45% of labeled procathepsin D and 37% of radiolabeled MHC class II, indicating that this intermediate lies on a major post-Golgi transport pathway. Finally, these fractions do not accumulate glycopepsinogen or MHC class I molecules during their packaging in secretory vesicles, which are short-lived and difficult to detect except in cell-free assays (de Curtis and Simons, 1989; Tooze and Huttner, 1990).

Immunoelectron microscopy of these fractions revealed the presence of vesicular structures that contained I-chain, but lacked the markers of clathrin-coated vesicles. Further, the I-chain–positive vesicles were distinct from vesicles containing the CD-MPR. Since the CD-MPR is a marker of both early and late endosomes (Klumperman et al., 1993), this finding indicates that the vesicles containing I-chain are not typical endosomes. These results are consistent with the suggestion that in ICD B lymphoblasts a significant portion of newly synthesized MHC class II and I-chain exits the TGN in non-clathrin-coated vesicles that fuse with and/or mature into MIICs without passing through Man-6-P receptor positive early and late endosomal structures. While it is likely that the MHC class II and I-chain positive vesicles also contain newly synthesized procathepsin D, it was not possible to detect the latter in these fractions by immunolabeling for technical reasons.

The proper sorting of MHC class II molecules to endocytic compartments involves cytoplasmic tail sequences in the associated I-chain (Bakke and Dobberstein, 1990; Lotteau et al., 1990; Pieters et al., 1993). Interestingly, one I-chain cytoplasmic tail sequence implicated in its targeting, an LI dipeptide, is related to the dileucine-based sorting signal found in the Man-6-P/IGF-II receptor and other proteins (Letourneur and Klausner, 1992; Johnson and Kornfeld, 1992; Chen et al., 1993). Although this sequence appears to be necessary for efficient entry of the Man-6-P/IGF-II receptor into Golgi-derived clathrin-coated vesicles, our results suggest that the invariant chain signal directs sorting by a clathrin-independent mechanism. Since MHC class II–negative cells were generally used to define the putative I-chain–targeting signals, the manner in which I-chain directs endosomal targeting may differ when it is complexed with MHC class II molecules in antigen-presenting cells.

Calafat et al. (1994) have shown that the expression of wild-type MHC class II/I-chain complexes in human embryonal kidney 293 cells is sufficient to induce the formation of multivesicular endocytic structures resembling MIIC. These structures are enriched in lysosomal membrane proteins and cathepsin D but devoid of Man-6-P receptors. The formation of the MIIC-like compartment was dependent on the transmembrane and cytoplasmic regions of the class II molecules. The ability of class II molecules

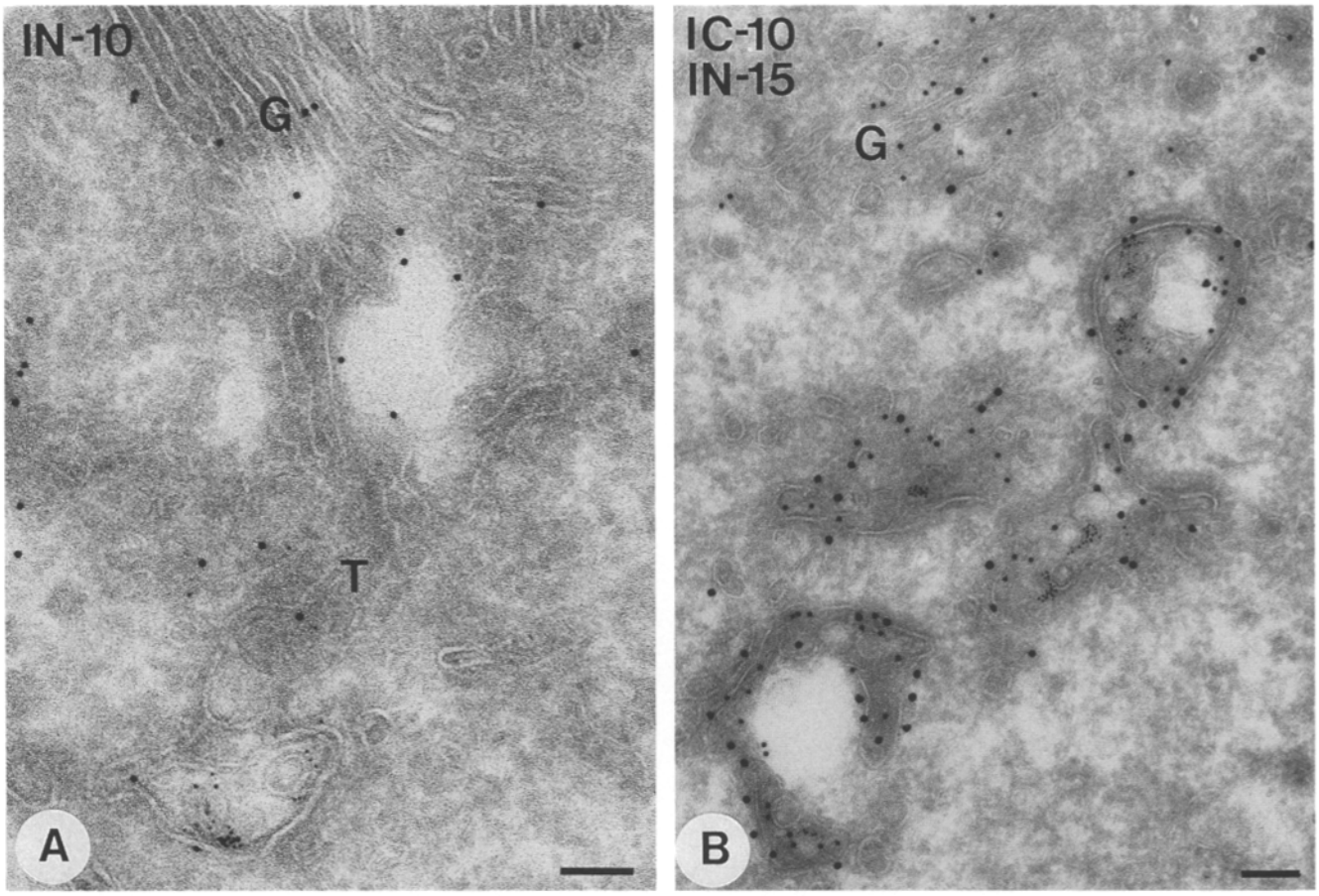

Figure 10. BSA-gold internalization into ICD B lymphoblasts. Cells were allowed to internalize BSA coupled to 5-nm gold particles for 30 min at 37°C. (*A*) An irregularly shaped MIIC with tracer in its content is associated with the TGN (*T*). I-chain is present in the Golgi stack, TGN, and in the MIIC. (*B*) Several irregularly shaped MIICs located close to the Golgi complex (*G*) labeled for both the lumenal and cytoplasmic domains of I-chain and containing BSA-gold tracer. Bars, 0.1 μm.

to induce the formation of MIIC, possibly via aggregation involving the transmembrane and cytoplasmic domains, provides a possible mechanism for the formation of MHC class II enriched buds in the TGN.

Recent studies have suggested that MHC class II-enriched vesicles may represent a heterogeneous group of compartments (Barnes and Mitchell, 1995; Mitchell et al., 1995; Mellman et al., 1995; Peters et al., 1995). Thus Amigorena et al. (1994) have described in murine B lymphoblasts the "class II vesicles" or CIIVs that are enriched in MHC class II, but devoid of lysosomal membrane markers. The CIIVs also contain low levels of transferrin and Man-6-P receptors, markers of the recycling endosomal pathway that are absent in MIIC. The CIIV may represent the early MIIC subclass (Peters et al., 1995). On the other hand, if the MHC class II-enriched compartments prove to be truly heterogeneous, then it would not be unexpected that a variety of delivery pathways are used to transport newly synthesized MHC class II to these structures. In fact, several studies have demonstrated the presence of small amounts of I-chain and MHC class II-I-chain complexes on the cell surface, and it has been shown that these molecules are rapidly internalized (Wraight et al., 1990; Roche et al., 1993). The molecules may have reached the cell surface by direct delivery from the TGN or indirectly following targeting to early endosomes and recycling to the cell surface. Alternatively, these surface molecules may be derived from MIICs that fuse with the plasma membrane. Exocytotic fusion of early MIICs with the plasma membrane has recently been demonstrated in B cells (Raposo et al., 1995). It is difficult to estimate the fraction of newly synthesized MHC class II-I-chain complexes that traverse the cell surface. Studies with an I-chain/transferrin receptor chimera containing the I-chain cytoplasmic tail and transmembrane region indicated less than 20% of these molecules traffic via the cell surface (Odorizzi et al., 1994). It seems likely that MHC class II-I-chain complexes are targeted to the MIICs by both routes, with the intracellular pathway being the predominant one. Castellino and Germain (1995) have presented evidence that in splenic B lymphocytes at least some of the newly synthesized MHC class II is initially delivered to low density, early endosomal compartments whereas Xu et al. (1995) and Benaroch et al. (1995) using different cell types have suggested that the MHC class II molecules are transported from the TGN directly to the MIIC. These latter findings are consistent with the results obtained with the ICD B lymphoblasts.

The observations concerning cathepsin D trafficking in the ICD B lymphoblasts provide insight into the Man-6-P–

independent targeting pathway. In normal B lymphoblasts, the newly synthesized acid hydrolases acquire Man-6-P residues that allow binding to the Man-6-P receptors and packaging in clathrin-coated vesicles in the TGN. The receptor-ligand complexes are transported to endosomal compartments where the low pH induces the release of the acid hydrolases (Kornfeld and Mellman, 1989; Ludwig et al., 1991; Klumperman et al., 1993). The soluble acid hydrolases are then transported to lysosomes and presumably to MIICs whereas the Man-6-P receptors recycle to the TGN for additional rounds of transport or to the cell surface. As shown in this study, cathepsin D and probably other acid hydrolases transported by the Man-6-P–independent pathway appear to reach the endocytic pathway in non-clathrin-coated vesicles that also contain MHC class II and I-chain. As in the case of the transport of the MHC class II molecules, we cannot exclude the possibility that a portion of the acid hydrolases are packaged into clathrin-coated vesicles in the TGN. The "lysosomal" localization of soluble acid hydrolases transported by the Man-6-P–independent pathway may reflect their passive flow from MIICs into lysosomes, or it may result from the further maturation of MIICs as they lose MHC class II molecules. MHC class II molecules in this compartment acquire antigenic peptides after the dissociation of I-chain and are transported to the cell surface (Raposo et al., 1995).

In defining the site where exogenous antigen is processed into peptides that can be loaded onto nascent MHC class II molecules, the early MIICs are of particular interest. Early MIICs contain cathepsin D and probably other soluble acid hydrolases, have variable amounts of I-chain COOH-terminal epitope (Peters et al., 1995; this study) indicating I-chain breakdown, and are the first MIICs type that can be labeled with an endocytosed tracer. Thus, breakdown of endocytosed antigen and of I-chain may occur simultaneously during maturation of MIICs, so that as soon as the binding grooves of the MHC class II molecules are freed from I-chain, properly sized peptides can bind.

Although our studies have been confined to B lymphocytes, a number of observations suggest that other cell types may possess similar acid hydrolase-targeting mechanisms. For instance, the cytolytic granules of cytotoxic T lymphocytes share many characteristics with MIICs and lysosomes, including membrane sheets and vesicles in their lumens, acidic lumenal pH, presence of acid hydrolases and lysosomal membrane proteins, absence of Man-6-P receptors, and late position in the endocytic pathway (Peters et al., 1991*b*). Recently, studies on human cytotoxic T cells have shown that two cell type–specific proteases, granzymes A and B, acquire Man-6-P residues but can be sorted to cytolytic granules by a Man-6-P–independent mechanism in ICD T cells (Griffiths and Isaaz, 1993). Further studies should reveal whether the cytolytic granules and MIICs share a similar pathway of formation, and whether T and B lymphocytes possess a common Man-6-P–independent targeting system for acid hydrolases.

We thank W. Gregory for providing antisera to human cathepsin D and pepsinogen and to the Man-6-P/IGF-II receptor; E. Ungewickell, M. Robinson, T. Kreis, F. Brodsky, M. Nahm, H. Ploegh, and B. Schwartz for gifts of antibodies; Cliff Harding for advice concerning subcellular fractionation; G. Griffiths and A. Lanzavecchia for sharing unpublished results; and members of the Kornfeld laboratory for helpful suggestions and critical reading of the manuscript.

This research was supported by United States Public Health Service grant CA08759 (S. Kornfeld) and the Life and Medical Research Fund Scholarship (J.N. Glickman), and by program grant 900-23-094 from Nederlandse Organisatie voor Wetenschappelijk Onderzoek (Dutch Soc. for Scientific Research), the Netherlands.

Received for publication 28 September 1995 and in revised form 21 December 1995.

References

Ahle, S., A. Mann, U. Eichelsberger, and E. Ungewickell. 1988. Structural relationships between clathrin assembly proteins from the Golgi and the plasma membrane. *EMBO (Eur. Mol. Biol. Organ.) J.* 4:919–929.

Allan, V.J., and T.E. Kreis. 1986. A microtubule-binding protein associated with membranes of the Golgi apparatus. *J. Cell Biol.* 103:2229–2239.

Amigorena, S., J.R. Drake, P. Webster, and I. Mellman. 1994. Transient accumulation of new class II MHC molecules in a novel endocytic compartment in B lymphoctes. *Nature (Lond.).* 369:113–120.

Anderson, M.S., and J. Miller. 1992. Invariant chain can function as a chaperon protein for class II major histocompatibility complex molecules. *Proc. Natl. Acad. Sci. USA.* 89:2282–2286.

Balch, W.E., W.G. Dunphy, W.A. Braell, and J.E. Rothman. 1984. Reconstitution of the transport of protein between successive compartments of the Golgi measured by the coupled incorporation of N-acetylglucosamine. *Cell.* 39:405–416.

Bakke, O., and B. Dobberstein. 1990. MHC class II-associated invariant chain contains a sorting signal for endosomal compartments. *Cell.* 63:707–716.

Baranski, T.J., P.L. Faust, and S. Kornfeld. 1990. Generation of a lysosomal targeting signal in the secretory protein pepsinogen. *Cell.* 63:281–291.

Barnes, K.A., and R.N. Mitchell. 1995. Detection of functional class II-associated antigen: role of a low density endosomal compartment in antigen processing. *J. Exp. Med.* 181:1715–1727.

Benaroch, P., M. Yilla, G. Raposo, K. Ito, K. Miwa, H.J. Geuze, and H.L. Pleogh. 1995. How MHC class II molecules reach the endocytic pathway. *EMBO (Eur. Mol. Biol. Organ.) J.* 14:37–49.

Bischoff, J., and R. Kornfeld. 1984. The effect of 1-deoxymannojirimycin on rat liver α-mannosidases. *Biochem. Biophys. Res. Commun.* 125:324–331.

Bleekemolen, J.E., M. Stein, K. von Figura, J.W. Slot, and H.J. Geuze. 1988. The two mannose 6-phosphate receptors have almost identical subcellular distributions in U937 monocytes. *Eur. J. Cell Biol.* 47:366–372.

Brown, W.J. 1990. Cation-independent mannose 6-phosphate receptors are concentrated in trans Golgi elements in normal and I-cell disease fibroblasts. *Eur. J. Cell Biol.* 51:201–218.

Calafat, J., M. Nijenhuis, H. Janssen, A. Tulp, S. Dusseljee, R. Wubbolts, and J. Neefjes. 1994. Major histocompatibility complex class II molecules induce the formation of endocytic MIIC-like structures. *J. Cell Biol.* 126:967–977.

Castellino, F., and R.N. Germain. 1995. Extensive trafficking of MHC class II-invariant chain complexes in the endocytic pathway and appearance of peptide-loaded class II in multiple compartments. *Immunity.* 2:73–88.

Chen, H.J., J. Remmler, J.C. Delaney, D.J. Messner, and P. Lobel. 1993. Mutational analysis of the cation-independent mannose 6-phosphate/insulin-like growth factor II receptor. *J. Biol. Chem.* 268:22338–22346.

Claesson-Welsh, L., and P.A. Peterson. 1985. Implication of the invariant chain γ-chain on the intracellular transport of class II histocompatibility antigens. *J. Immunol.* 135:3551–3558.

Cresswell, P. 1994. Assembly, transport, and function of MHC class II molecules. *Annu. Rev. Immunol.* 12:259–293.

de Curtis, I., and K. Simons. 1989. Isolation of exocytic carrier vesicles from BHK cells. *Cell.* 58:719–727.

Denzin, L.K., and P. Cresswell. 1995. HLA-DM induces CLIP dissociation from MHC class II αβ dimers and facilitates peptide loading. *Cell.* 82:155–165.

DiCioccio, R., and A.L. Miller. 1991. Biosynthesis, processing, and secretion of α-L-fucosidase in lymphoid cells from patients with I-cell disease and pseudo-Hurler polydystrophy. *Glycobiology.* 1:595–604.

Duden, R., G. Griffiths, R. Frank, P. Argos, and T.E. Kreis. 1991. β-COP, a 110 kd protein associated with non-clathrin-coated vesicles and the Golgi complex, shows homology to β-adaptin. *Cell.* 64:649–665.

Elliott, E.A., J.R. Drake, S. Amigorena, J. Elsemore, P. Webster, I. Mellman, and R.A. Flavell. 1994. The invariant chain is required for intracellular transport and function of major histocompatibility complex class II molecules. *J. Exp. Med.* 179:681–694.

Geuze, H.J. 1994. A novel lysosomal compartment engaged in antigen presentation. *Eur. J. Cell Biol.* 64:3–6.

Geuze, H.J., J.W. Slot, G.J. Strous, A. Hasilik, and K. von Figura. 1985. Possible pathways for lysosomal enzyme delivery. *J. Cell Biol.* 101:2253–2262.

Geuze, H.J., W. Stoorvogel, G.J. Strous, J.W. Slot, J.E. Bleekemolen, and I. Mellman. 1988. Sorting of mannose 6-phosphate receptors and lysosomal membrane proteins in endocytic vesicles. *J. Cell Biol.* 107:2491–2501.

Geuze, H.J., J.W. Slot, and A.L. Schwartz. 1992. The trans-Golgi exits of MPR. *In* Hepatic Endocytosis of Lipids and Proteins. E. Windler and H. Greten, editors. Zuckschwerdt Verlag, NY. 37–43.

Gieselmann, V., R. Pohlmann, A. Hasilik, and K. von Figura. 1983. Biosynthesis and transport of cathepsin D in cultured human fibroblasts. *J. Cell Biol.* 97:1–5.

Glickman, J.N., and S. Kornfeld. 1993. Mannose 6-phosphate-independent targeting of lysosomal enzymes in I-Cell Disease B lymphoblasts. *J. Cell Biol.* 123:99–108.

Goldberg, D.E., and S. Kornfeld. 1983. Evidence for extensive subcellular organization of asparagine-linked oligosaccharide processing and lysosomal enzyme phosphorylation. *J. Biol. Chem.* 258:3159–3165.

Griffiths, G.M., and S. Isaaz. 1993. Granzymes A and B are targeted to the lytic granules of lymphocytes by the mannose 6-phosphate receptor. *J. Cell Biol.* 120:885–896.

Griffiths, G., B. Hoflack, K. Simons, I. Mellman, and S. Kornfeld. 1988. The mannose 6-phosphate receptor and the biogenesis of lysosomes. *Cell.* 52: 329–341.

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 the Ia alpha and beta chains. *Eur. J. Immunol.* 12:942–948.

Harding, C.V., and H.J. Geuze. 1992. Class II MHC molecules are present in macrophage lysosomes and phagolysosomes that function in the phagocytic processing of Listeria monocytogenes for presentation to T cells. *J. Cell Biol.* 119:531–542.

Harding, C.V., and H.J. Geuze, 1993*a*. Immunogenic peptides bind to class II MHC molecules in an early lysosomal compartment. *J. Immunol.* 151:3988–3998.

Harding, C.V., and H.J. Geuze. 1993*b*. Antigen processing and intracellular traffic of antigens and MHC molecules. *Curr. Opin. Cell Biol.* 5:596–605.

Harding, C.V., D.S. Collins, J.W. Slot, H.J. Geuze, and E.R. Unanue. 1991. Liposome-encapsulated antigens are processed in lysosomes, recycled and presented to T cells. *Cell.* 64:393–401.

Hasilik, A., A. Waheed, and K. von Figura. 1981. Enzymatic phosphorylation of lysosomal enzymes in the presence of UDP N-acetylglucosamine: absence of the activity in I-Cell fibroblasts. *Biochem. Biophys. Res. Commun.* 98:761–767.

Johnson, K.F., and S. Kornfeld. 1992. The cytoplasmic tail of the mannose 6-phosphate/insulin-like growth factor II receptor has two signals for lysosomal enzyme sorting in the Golgi. *J. Cell Biol.* 119:249–257.

Kleijmeer, M.J., M.A. Ossevoort, C.J.H. Van Veen, J.J. van Hellemond, J.J. Neefjes, W.M., Kast, C.J.M. Melief, and H.J. Geuze. 1995. MHC class II compartments and the kinetics of antigen presentation in activated spleen dendritic cells. *J. Immunol.* 154(II):5715–5724.

Klumperman, J., A. Hille, T. Veenendaal, V. Oorschot, W. Stoorvogel, K. von Figura, and H.J. Geuze. 1993. Differences in the endosomal distributions of the two mannose 6-phosphate receptors. *J. Cell Biol.* 121:997–1010.

Kornfeld, S., and I. Mellman. 1989. The biogenesis of lysosomes. *Annu. Rev. Cell Biol.* 5:483–525.

Laemmli, U.K. 1970. Cleavage of structural protein during the assembly of the head of bacteriophage T4. *Nature (Lond.).* 227:680–685.

Lamb, C.A., J.W. Yewdell, J.R. Benninck, and P. Cresswell. 1991. Invariant chain targets HLA class II molecules to acidic endosomes containing internalized influenza virus. *Proc. Natl. Acad. Sci. USA.* 88:5998–6002.

Layet, C., and R.N. Germain. 1991. Invariant chain promotes egress of poorly expressed, haplotype-mismatched class II major histocompatibility complex Aa-Ab dimer from the endoplasmic reticulum/cis-Golgi compartment. *Proc. Natl. Acad. Sci. USA.* 88:2346–2350.

Letourneur, F., and R.D. Klausner. 1992. A novel di-leucine motif and a tyrosine-based motif independently mediate lysosomal enzyme targeting and endocytosis of CD3 chains. *Cell.* 69:1143–1157.

Liou, W., and J.W. Slot. 1994. Improved fine structure in immunolabeled cryosections after modifying the sectioning and pick-up conditions. *Proc. Int. Conf. Electron Microsc.* pp. 13.

Little, L.E., M. Alcouloumre, A. Drotar, S. Herman, R. Robertson, R.Y. Yeh, and A.L. Miller. 1987. Properties of N-acetylglucosamine 1-phosphotransferase from human lymphoblasts. *Biochem. J.* 248:151–159.

Lotteau, V., L. Teyton, A. Peleraux, T. Nilsson, L. Karlson, S.L. Schmid, V. Quaranta, and P.A. Peterson. 1990. Intracellular transport of class II MHC molecules directed by invariant chain. *Nature (Lond.).* 348:600–605.

Ludwig, T., G. Griffiths, and B. Hoflack. 1991. Distribution of newly synthesized lysosomal enzymes in the endocytic pathway of normal rat kidney cells. *J. Cell Biol.* 115:1561–1572.

Marks, M.S., J.S. Blum, and P. Cresswell. 1990. Invariant chain trimers are sequestered in the rough endoplasmic reticulum in the absence of association with HLA class II antigens. *J. Cell Biol.* 111:839–855.

Marks, M.S., P.A. Roche, E. VanDonselaar, L. Woodruff, P. Peters, and J.S. Bonifacino. 1995. A lysosomal targeting signal in the cytoplasmic tail of the β chain directs HLA-DM to MHC class II compartments. *J. Cell Biol.* 131: 351–369.

Mellman, I., P. Pierre, and S. Amigorena. 1995. Lonely MHC molecules seeking immunogenic peptides for meaningful relationship. *Curr. Opin. Cell Biol.* 7: 564-572.

Metzelaar, M.J., P.L.J. Wijngaard, P.J. Peters, J.J. Sixma, H.K. Nieuwenhuis, and H.C. Clevers. 1991. CD63 antigen: a novel lysosomal membrane glycoprotein, cloned by a screening method for intracellular antigens in eukaryotic cells. *J. Biol. Chem.* 266:3239–3245.

Mitchell, R.N., K.A. Barnes, S.A. Grupp, M. Sanchez, Z. Misulovin, M.C. Nussenzweig, and A.K. Abbas. 1995. Intracellular targeting of antigens internalized by membrane immunoglobulin in B lymphocytes. *J. Exp. Med.* 181: 1709–1714.

Morton, P.A., M.L. Zacheis, K.S. Giacoletto, J.A. Manning, and B.D. Schwartz. 1995. Delivery of nascent MHC class II-invariant chain complexes to lysosomal compartments and proteolysis of invariant chain by cysteine proteases precedes peptide binding in B-lymphoblastoid cells. *J. Immunol.* 154:137–150.

Näthke, I., J. Heuser, A. Lupas, J. Stock, C.W. Turck, and F.M. Brodsky. 1992. Folding and trimerization of clathrin subunits at the triskelion hub. *Cell.* 68: 899–910.

Neefjes, J.J., and H.L. Ploegh. 1992. Inhibition of endosomal proteolytic activity by leupeptin blocks surface expression of MHC class II molecules and their conversion to SDS resistant αβ heterodimers in endosomes. *EMBO (Eur. Mol. Biol. Organ.) J.* 11:411–416.

Neefjes, J.J., V. Stollorz, P.J. Peters, H.J. Geuze, and H.L. Ploegh. 1990. The biosynthetic pathway of MHC class II but not MHC class I molecules intersects the endocytic route. *Cell.* 61:171–183.

Neefjes, J.J., T.N.M. Schumacher, and H.L. Ploegh. 1991. Assembly and intracellular transport of major histocompatibility complex molecules. *Curr. Opin. Cell Biol.* 3:601–609.

Nijman, H.W., M.J. Kleijmeer, M.A. Ossevoort, V.M.J. Dorschot, M.P.M. Vierboom, M. van der Keur, P. Kenemans, W.M. Kast, H.J. Geuze, and C.J.M. Melief. 1995. Antigen capture and major histocompatibility class II compartments of freshly isolated and cultured human blood dendritic cells. *J. Exp. Med.* 182:163–174.

Odorizzi, C.G., I.S. Trowbridge, L. Xue, C.R. Hopkins, C.D. Davis, and J.F. Collawn. 1994. Sorting signals in the MHC class II invariant chain cytoplasmic tail and transmembrane region determine trafficking to an endocytic processing compartment. *J. Cell Biol.* 126:317–330.

Okada, S., M. Handa, T. Hashimoto, J. Nishimoto, K. Inui, M. Furuyama, J. Furuyama, H. Yabuachi, M. Tate, S. Gasa, and A. Makita. 1988. Biochemical studies on lymphoblastoid cells with inherited N-acetylglucosamine 1-phosphotransferase deficiency (I-Cell Disease). *Biochem. Int.* 17:375–383.

Parham, P., C.J. Barnstable, and W.F. Bodmer. 1979. Use of monoclonal antibody (W6/32) in structural studies of HLA-A,B,C antigens. *J. Immunol.* 123: 342–349.

Pearse, B.M.F. 1982. Coated vesicles from human placenta carry ferritin, transferrin, and immunoglobulin G. *Proc. Natl. Acad. Sci. USA.* 79:451–455.

Peters, P.J., J.J. Neefjes, V. Oorschot, H.L. Ploegh, and H.J. Geuze. 1991*a*. Segregation of MHC class II molecules from MHC class I molecules in the Golgi complex for transport to lysosomal compartments. *Nature (Lond.).* 349:669–676.

Peters, P.J., J. Borst, V. Oorschot, M. Fukuda, O. Krahenbuhl, J. Tschopp, J.W. Slot, and H.J. Geuze. 1991*b*. Cytolytic T lymphocyte granules are secretory lysosomes, containing both perforin and granzymes. *J. Exp. Med.* 173:1099–1109.

Peters, P.J., G. Raposo, J.J. Neefjes, V. Oorschot, R.L. Leyendekker, H.J. Geuze, and H.L. Ploegh. 1995. Major histocompatibility complex class II compartments in human B lymphoblastoid cells are distinct from early endosomes. *J. Exp. Med.* 182:325–334.

Pieters, J., H. Horstmann, O. Bakke, G. Griffiths, and J. Lipp. 1991. Intracellular transport and localization of major histocompatibility complex class II molecules and associated invariant chain. *J. Cell Biol.* 115:1213–1223.

Pieters, J.H., O. Bakke, and B. Dobberstein. 1993. The MHC class II-associated invariant chain contains two endosomal targeting signals within its cytoplasmic tail. *J. Cell Sci.* 106:831–846.

Pohl, T. 1990. Concentration of proteins and removal of solutes. *Methods Enzymol.* 182:68–83.

Qiu, Y., X. Wu, A. Wandinger-Ness, D.P. Dalke, and S.K. Pierce. 1994. Separation of subcellular compartments containing distinct functional forms of MHC class II. 1994. *J. Cell Biol.* 125:595–605.

Raposo, G., H.W. Nijman, W. Stoorvogel, R. Leyendekker, C.V. Harding, C.J.N. Meliet, and H.J. Geuze. 1995. B-lymphocytes secrete antigen presenting vesicles. *J. Exp. Med.* In press.

Reitman, M.L., A.P. Varki, and S. Kornfeld. 1981. Fibroblasts from patients with I-cell disease and pseudo-Hurler polydystrophy are deficient in UDP-N-acetylglucosamine: glycoprotein N-acetylglucosamine-1-phosphotransferase. *J. Clin. Invest.* 67:1574–1579.

Riberdy, J.M., R.R. Avva, H.J. Geuze, and P. Cresswell. 1994. Transport and intracellular distribution of MHC class II molecules and associated invariant chain in normal and antigen-processing mutant cell lines. *J. Cell Biol.* 125(6): 1225–1237.

Robinson, M.S. 1987. 100-kD coated vesicle proteins: molecular heterogeneity and intracellular distribution studied with monoclonal antibodies. *J. Cell Biol.* 104:887–895.

Robinson, M.S., and T.E. Kreis. 1992. Recruitment of coat proteins onto Golgi membranes in intact and permeabilized cells: effects of brefeldin A and G protein activators. *Cell.* 69:129–138.

Roche, P.A., and P. Cresswell. 1991. Proteolysis of the class II-associated invariant chain generates a peptide binding site in intracellular HLA-DR mol-

ecules. *Proc. Natl. Acad. Sci. USA.* 88:3150–3154.

Roche, P.A., C.L. Teletski, E. Stang, O. Bakke, and E.O. Long. 1993. Cell surface HLA-DR-invariant chain complexes are targeted to endosomes by rapid internalization. *Proc. Natl. Acad. Sci. USA.* 90:8581–8583.

Sanderson, F., M.J. Kleijmeer, A. Kelly, D. Verwoerd, A. Tulp, J.J. Neefjes, H.J. Geuze, and J. Trowsdale. 1994. Accumulation of HLA-DM, a regulator of antigen presentation in MHC class II compartments. *Science (Wash. DC).* 266:1566–1569.

Schulze-Garg, C., C. Boker, S. Kumar Nadimpalli, K. von Figura, and A. Hille-Rehfeld. 1993. Tail-specific antibodies that block return of 46,000 M_r mannose 6-phosphate receptor to the *trans*-Golgi network. *J. Cell Biol.* 122:541–551.

Serafini, T., G. Steinbeck, A. Brecht, F. Lottspeich, L. Orci, J.E. Rothman, and F.T. Wieland. 1991. A coat subunit of Golgi-derived non-clathrin-coated vesicles with homology to the clathrin-coated vesicle coat protein β-adaptin. *Nature (Lond.).* 349:215–220.

Sherman, M.A., D.A. Weber, and P.E. Jensen. 1995. DM enhances peptide binding to class II MHC by release of invariant chain-derived peptide. *Immunity.* 3:197–205.

Shipp, M.A., P. Ahmed, C.C. Kannapell, J.C. Ford, D. McCourt, J.F. Leykam, M. Zacheis, C. Bono, J.M. Davie, E. Mustain, et al. 1986. A new ploymorphic determinant on HLA-DQ molecules. *Hum. Immunol.* 16:24–37.

Slot, J.W., and H.J. Geuze. 1985. A new method of preparing gold probes for multiple-labeling cytochemistry. *Eur. J. Cell Biol.* 38:87–93.

Slot, J.W., H.J. Geuze, S. Gigengack, G.E. Lienhard, and D.E. James. 1991. Immuno-localization of the insulin regulatable glucose transporter in brown adipose tissue of the rat. *J. Cell Biol.* 113:123–135.

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.

Teyton, L., C. O'Sullivan, P.W. Dickson, V. Lotteau, A. Sette, P. Fink, and P.A. Peterson. 1990. Invariant chain distinguishes between the exogenous and endogenous presentation pathways. *Nature (Lond.).* 348:39–44.

Tooze, S.A., and W.B. Huttner. 1990. Cell-free protein sorting to the regulated and constitutive secretory pathways. *Cell.* 60:837–847.

Tsuji, A., K. Omura, and Y. Suzuki. 1988. I-Cell disease: evidence for a mannose 6-phosphate independent pathway for translocation of lysosomal enzymes in lymphoblastoid cells. *Clin. Chim. Acta.* 176:115–122.

Tulp, A., D. Verwoerd, B. Dobberstein, H.L. Ploegh, and J. Pieters. 1994. Isolation and characterization of the intracellular MHC class II compartment. *Nature (Lond.).* 369:120–126.

Viville, S., J. Neefjes, V. Lotteau, A. Dierich, M. Lemeur, H. Ploegh, C. Benoist, and D. Mathis. 1993. Mice lacking the MHC class II-associated invariant chain. *Cell.* 72:635.

West, M.A., J.M. Lucocq, and C. Watts. 1994. Antigen processing and class II MHC peptide-loading compartments in human B lymphoblastoid cells. *Nature (Lond.).* 369:147–151.

Woodman, P., and G. Warren. 1991. Isolation of functional, coated, endocytic vesicles. *J. Cell Biol.* 112:1133–1141.

Wraight, C.J., P. van Endert, P. Miller, J. Lipp, N.R. Ling, I.C.M. MacLennan, N. Koch, and G. Moldenhauer. 1990. Human major histocompatibility complex class II invariant chain is expressed at the cell surface. *J. Biol. Chem.* 265:5787–5792.

Xu, X., W. Song, H. Cho, Y. Qiu, and S.K. Pierce. 1995. Intracellular transport of invariant chain—MHC class II complexes to the peptide-loading compartment. *J. Immunol.* 155(6):2984–2992.