

Transport and Intracellular Distribution of MHC Class II Molecules and Associated Invariant Chain in Normal and Antigen-processing Mutant Cell Lines

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Abstract. We have compared the intracellular transport and subcellular distribution of MHC class II-invariant chain complexes in a wild-type HLA-DR3 homozygous cell line and a mutant cell line, T2.DR3. The latter has a defect in antigen processing and accumulates HLA-DR3 molecules associated with an invariant chain-derived peptide (CLIP) rather than the normal complement of peptides derived from endocytosed proteins. We find that in the wild-type cells, CLIP is transiently associated with HLA-DR3 molecules, suggesting that the peptide is a normal class II-associated intermediate generated during proteolysis of the invariant chain. In the mutant cell line proteolysis of the invariant chain is less efficient, and HLA-DR3/CLIP complexes are generated much more

slowly. Examination of the mutant cell line by immunoelectronmicroscopy shows that class II-invariant chain complexes accumulate intracellularly in large acidic vesicles which contain lysosomal markers, including β -hexosaminidase, cathepsin D, and the lysosomal membrane protein CD63. The markers in these vesicles are identical to those seen in the class II-containing vesicles (MIICs) seen in the wild-type cells but the morphology is drastically different. The vesicles in the mutant cells are endocytic, as measured by the internalization of BSA-gold conjugates. The implication of these findings for antigen processing in general and the nature of the mutation in particular are discussed.

MAJOR histocompatibility complex (MHC)¹ molecules present antigenic peptides to T cells during an immune response. Class I molecules present peptides from cytosolic proteins to CD8⁺ T cells (Townsend and Bodmer, 1989), while class II molecules predominantly present peptides from exogenous proteins to CD4⁺ T cells (Unanue, 1984). Intracellularly, class II molecules associate with a third protein, the invariant (I) chain (Jones et al., 1979; Machamer and Cresswell, 1982). I chain associates with class II heterodimers in the endoplasmic reticulum (ER) in a nine-chain complex consisting of a core I chain trimer with three associated heterodimers (Marks et al., 1990; Roche et al., 1991). This complex contains no detectable associated antigenic peptides (Roche and Cresswell, 1990;

Newcomb and Cresswell, 1993; Roche et al., 1992). Once assembled, the $\alpha\beta$ I complex traverses the Golgi stacks and is sorted to a compartment that is both acidic and endocytic (Lotteau et al., 1990; Lamb et al., 1991; Bakke and Dobberstein, 1990). The key signal for this sorting event resides in the cytoplasmic domain of the I chain (Bakke and Dobberstein, 1990). In Epstein Barr virus (EBV)-transformed human B-cell lines this compartment has been extensively characterized by electron microscopy. It shares features characteristic of lysosomes, and has been designated the MHC-class II-containing compartment (MIIC) (Peters et al., 1991b). Biosynthetic studies of class II molecules *in vivo* have shown that I chain is proteolytically degraded, probably in this endocytic compartment (Blum and Cresswell, 1988; Nguyen et al., 1989), releasing $\alpha\beta$ dimers which are competent to bind antigenic peptides (Roche and Cresswell, 1991; Newcomb and Cresswell, 1993). Thus, the efficient degradation and complete dissociation of I chain is critical for the class II complex to fulfill its function of presenting antigenic peptides to T cells.

Maturation of class II molecules *in vivo*, dissociation of I chain, and peptide binding correlates with the acquisition of an SDS-stable phenotype for class II $\alpha\beta$ dimers (Germain and Hendrix, 1991). HLA-DR3 molecules from cell lines

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1. *Abbreviations used in this paper:* DAMP, 3-(2,4-dinitroanilino)-3-amino-N-methylidipropylamine; EBV, Epstein Barr virus; ER, endoplasmic reticulum; MHC, major histocompatibility complex; MIIC, class II-containing vesicles; MPR, mannose-6-receptors.

defective in class II-restricted antigen processing, have been shown to be SDS-unstable (Mellins et al., 1990; Riberdy and Cresswell, 1992). Further, the class II molecules purified from two such mutant cell lines expressing HLA-DR3 lack a wild-type repertoire of endogenously bound peptides and are instead primarily associated with high levels of a nested set of invariant chain-derived peptides (class II-associated invariant chain-derived peptides or CLIP [Riberdy et al., 1992; Sette et al., 1992]). Peptides generated from the same region of I chain have also been isolated from wild-type class II molecules indicating that such peptides are not solely generated in mutant cell lines (Chicz et al., 1992; Rudensky et al., 1991; Hunt et al., 1992). Here we show that in wild-type cells HLA-DR3 $\alpha\beta$ dimers are transiently associated with CLIP during intracellular transport. In contrast, the mutant cell line T2.DR3 accumulates $\alpha\beta$ CLIP complexes during biosynthesis and ultimately expresses these complexes at the cell surface. Electron microscopy reveals an accumulation of class II molecules in T2.DR3 cells in large vesicular compartments. These contain many of the markers characteristic of the class II-containing compartment seen in wild-type B-cell lines, but differ drastically in morphology. We propose that the defect in antigen processing in the mutant cells induces the accumulation of class II molecules in this compartment, and that this accumulation induces its distention.

Materials and Methods

Cell Lines

The cell lines T1.DR3, T2.DR3, and Pala have been previously described (Riberdy and Cresswell, 1992) and were cultured in Iscove's Dulbecco's modified medium (IMDM, GIBCO BRL, Gaithersburg, MD) with 5% calf serum (CS, Gibco) at 37°C.

Radiolabeling

Continuous metabolic labels were performed as described (Machamer and Cresswell, 1982). Cells were preincubated in methionine-free DME, supplemented with L-glutamine, 3% dialyzed FCS, and 0.5% gentamicin (all from Gibco) for 1 h at 37°C. Cells were then resuspended in methionine-free medium described above and labeled at 37°C with [³⁵S]methionine trans-label (ICN, K & K laboratories, Inc., Plainview, NJ). For pulse-chase experiments, cells were preincubated as described above, labeled with [³⁵S]methionine trans-label (ICN) and chased in IMDM (Gibco) supplemented with 5% calf serum, 0.5% gentamicin, and a 15-fold excess of cold methionine (Sigma Chem. Co., St. Louis, MO).

Immunoprecipitations

Metabolically labeled cells were extracted in 1% Triton X-100 (TX-100, Sigma Chem. Co.), 0.1 mM tosyllysine chloromethyl ketone (TLCK, Sigma Chemical Co.) and 0.5 mM PMSF (Sigma Chem. Co.) in Tris-saline, pH 7.4 (TS, pH 7.4, 10 mM Tris and 0.15 M NaCl, both from Sigma Chem. Co.). Extraction was performed on ice at 2×10^6 cells per ml for 30 min with occasional agitation. Nuclei were pelleted at 1,000 g for 10 min at 4°C. The lysate was clarified by centrifugation for 10 min at 14,000 rpm in a microcentrifuge. Supernatants were precleared with 2 μ l rabbit anti-human immunoglobulin and 30 μ l protein A-Sepharose CL-4B (both from Sigma Chem. Co.) at 4°C for 1 h. Lysates were then incubated with specific antibody for 1 h at 4°C and subsequently incubated for an additional hour with 30 μ l protein A-Sepharose at 4°C. The beads were then washed four times with 0.05% TX-100 in TS, pH 7.4.

One-Dimensional Gel Analysis

SDS-PAGE was performed as described (Laemmli, 1970). All gels con-

tained a final concentration of 10.5% acrylamide and were 1.5-mm thick. Gels were fixed in 7.5% acetic acid and 5% MeOH for at least 30 min, and then immersed for 30 min in 100 ml of Enlightning (Dupont, New England Nuclear, Boston, MA) for fluorography, before drying.

Affinity Purification

Metabolically labeled cells were lysed in 2% polyoxyethylene 9 lauryl ether (C₁₂E₉, Sigma Chem. Co.), 0.1 mM TLCK, 0.5 mM PMSF, 5 mM iodoacetamide, 0.02% NaN₃ (all from Sigma Chem. Co.) in TS, pH 7.4 for 45 min on ice, with occasional agitation. Extraction concentration was 10⁶ cells per ml of lysis buffer. Nuclei were pelleted at 1,000 g, 4°C, and removed. The extract was further clarified by ultracentrifugation at 100,000 g for 1 h, 4°C. HLA-DR3 molecules were purified by immunoaffinity chromatography, using a mouse immunoglobulin precolumn connected in series to a L243 (HLA-DR specific mAb) affinity column as previously described (Riberdy et al., 1992). The specific columns were then washed with 200 ml of 0.5% deoxycholate (DOC) and 0.02% NaN₃ in TS, pH 8.0, reequilibrated in 1% N-octylglucoside (Boehringer Mannheim, Indianapolis, IN), 0.02% NaN₃ in TS, pH 8.0, and purified molecules were eluted with 0.1 M Tris, pH 11.5, 1% N-octylglucoside and 0.02% NaN₃. Immediately after elution the fractions were neutralized by addition of 1 M acetic acid (Fischer Co., Pittsburgh, PA) and the peak radioactive fractions were pooled and stored at 4°C until use. For preparations to be analyzed by reversed-phase HPLC, 0.1% C₁₂E₉ was used in the reequilibration and elution buffers instead of 1% N-octylglucoside.

Separation of Bound Peptides from Purified Class II Complexes

Class II molecules purified by affinity chromatography were denatured by addition of acetic acid to a final concentration of 10%. The peptides were immediately separated by centrifugation through a Centricon 10 filtration unit (Amicon, Danvers, MA) at 7,000 rpm for 50 min at room temperature. The α - and β -chains were retained above the filter.

HPLC Analysis of Peptides

Endogenous peptides purified as described above were analyzed by HPLC using a Delta-Pak analytical C18 column (Waters Chromatography Division, Milford, MA). Peptide samples were adjusted to a final concentration of 0.1% hydrochloric acid (Pierce Chem. Co., Rockford, IL) and subjected to chromatography with the following gradient: 0%–15% acetonitrile from 0–20 min and 15–45% acetonitrile from 20–120 min at a flow rate of 0.5 ml/min.

Tris-tricine Gel Electrophoresis

Electrophoresis conditions were performed as described by Shagger and von Jagow (1987). The stock acrylamide solution used in these experiments was designated by Shagger and von Jagow as 49.5%T, 3%C (48% acrylamide, 1.5% N,N-methylenebisacrylamide, Bio-rad Labs., Hercules, CA). 1.5-cm stacker gel was polymerized on top of 1.5-cm spacer gel and ~6.5-cm separating gel (using the separating gel recipe designated as 16.5%T, 3%C). Both the spacer and separating gels were copolymerized. Samples were run into the stacker gel at 30 V, and then run to the bottom of the gel at 60 V. Upon completion, gels were immediately placed in 100 ml of 10% MeOH, 10% acetic acid, 5% glycerol and rocked for 30 min at room temperature. Gels were briefly rinsed with H₂O and placed in 100 ml of Enlightning with 5% glycerol added (rocking at room temperature for 30 min). Gels were dried in two cycles with continuous vacuum as follows: 2.5 h with a slow increase in temperature to 60°C, and then 2 more h with a rapid rise in temperature to 80°C.

Cell Surface Biotinylation

Radiolabeled cells were pulsed and chased as described above. At the end of the chase period, the cells were washed once in PBS and resuspended at 2×10^6 cells per ml in ice cold bicine-saline, pH 7.5 (BS, 130 mM NaCl, 20 mM bicine, Sigma Chem. Co.). Sulfosuccinimidyl-6-(biotinamido) hexanoate (NHS-LC-Biotin, Pierce Chem. Co.) was added to a final concentration of 0.2 mM and the cells incubated for 30 min on ice. The reaction was stopped by addition of 1 M glycine (Sigma Chem. Co.) to a final concentration of 10 mM and the cells pelleted by centrifugation, 1,000 rpm, 10 min, 4°C. Pellets were stored at -20°C. Cells were then ex-

tracted, precleared, and immunoprecipitated as described above. Pellets were washed four times with TS, pH 7.4, 0.05% TX-100. Protein was eluted from the beads with 100 μ l of elution buffer, pH 11.5 (described above for affinity purification), for 5 min on ice. Supernatants were removed and neutralized by addition of 7 μ l 1 M acetic acid. 1 ml of 0.1% C₁₂E₉ column buffer and BSA to a final concentration of 1% was added to each sample and biotinylated cell surface proteins were specifically precipitated by rocking at 4°C with avidin-agarose beads that had been precoated with FCS to prevent non-specific sticking. Pellets were washed four times with TS, pH 7.4, 0.05% TX-100, and solubilized in 50 ml of tricine sample buffer, before analysis with Tris-tricine acrylamide gels.

Electron Microscopy

For studying the morphology, T1.DR3, T2, and T2.DR3 cells were fixed in Karnovsky and embedded in Epon. Ultrathin sections were stained in uranyl Mg-acetate and Reynolds lead citrate. For immuno-electron microscopy, T1.DR3, T2, and T2.DR3 cells were fixed in a mixture of 2% paraformaldehyde and 0.5% glutaraldehyde (final concentrations) in 0.1 M sodium phosphate, pH 7.4 for 2 h and shipped in 1% paraformaldehyde in the same buffer for further processing. Cell samples were embedded in 10% gelatin which was solidified on ice. Blocks were immersed in 2.3 M sucrose in phosphate buffer for 2 h at 4°C and ultrathin cryosections were single and double immuno-labeled as described (Slot and Geuze, 1985; Slot et al., 1985) with 10- and 15-nm protein-A conjugated colloidal gold probes. The antibodies used were rabbit antibodies against human cathepsin D (Geuze et al., 1988); human CD63 (Metzelaer et al., 1991); human class II (Peters et al., 1991b), DNP (Dakopets, Glostrup, Denmark); CI-MPR (Geuze et al., 1988); I chain (Marks et al., 1990), and a mouse monoclonal antibody (PIN.1) against a synthetic peptide corresponding to the cytosolic tail of I chain (Lamb and Cresswell, 1992).

To study endocytic compartments, T1.DR3 and T2.DR3 were pulse incubated with 5-nm BSA/gold particles (OD, 10) for 10 min, and chased for 0, 30, 60, and 120 min at 37°C. Endocytosis was stopped in ice-cold medium and the cells were washed twice in cold medium to remove most of the surface-adhered gold. Cells were then fixed and processed as above. To detect acidic compartments at the EM level, T1.DR3 and T2.DR3 were incubated with 30 μ M of the weak base 3-(2,4-dinitroanilino)-3-amino-N-methylpropylamine, DAMP (Anderson et al., 1984) at 37°C for 30 min and washed with ice-cold serum-free medium at 0°C for 4 min to remove cytoplasmic DAMP. The DAMP-treated cells were fixed and processed for immunogold labeling as above.

Results

Kinetics of Transport and Processing of HLA-DR3 Molecules

Class II molecules are posttranslationally modified by the addition of N-linked glycans in the ER. As the molecules traverse the Golgi, the oligosaccharides are trimmed and modified (Machamer and Cresswell, 1982). Sialic acid is added in the *trans*-Golgi and serves as a convenient marker for transport to the compartment. Previous studies of the biosynthesis of murine I-A^k molecules in T1 and T2 showed that sialylated class II-invariant chain complexes were generated with similar kinetics in both parent and mutant cell lines (Riberdy and Cresswell, 1992). Similar results have since been obtained in comparisons of DR3 transport in T2 and in wild-type cells (Cresswell, P., and J. M. Riberdy, unpublished results). We also previously showed that I-A^k molecules expressed in T2 cells were transported to a proteolytic compartment, since incubation of T2-I-A^k cells in leupeptin, a cysteine protease inhibitor, causes the accumulation of I chain fragments (leupeptin-induced proteins, or LIP) in association with I-A^k molecules (Riberdy and Cresswell, 1992). LIP is produced by the partial inhibition of I chain proteolysis (Blum and Cresswell, 1988). We have similarly found that LIP is produced in association with HLA-DR3

molecules in leupeptin-treated T2.DR3 cells (Riberdy, J. M., unpublished results). The ultimate I chain proteolysis product in T2.DR3 cells, however, is CLIP.

When isolated from T2.DR3 cells, CLIP is comprised of a nested set of invariant chain-derived peptides 16–24 amino acids in length which are also found associated with a number of class II alleles isolated from wild-type cell lines, suggesting that these peptides are naturally occurring breakdown products from proteolysis of the I chain (Chicz et al., 1992; Rudensky et al., 1991; Hunt et al., 1992). The abnormally high levels of CLIP association found with DR3 isolated from T2.DR3 (Riberdy et al., 1992) prompted us to examine the kinetics of CLIP generation and dissociation in T2.DR3 and wild-type cells. Control Pala and mutant T2.DR3 cells were pulsed with [³⁵S]methionine for 30 min and chased in the presence of 15-fold excess cold methionine for the indicated times. Cells were extracted and immunoprecipitated with the DR-specific monoclonal antibody L243. Immunoprecipitates were analyzed on SDS-PAGE gels using tricine as the trailing ion to better separate small peptides in the 1–5 kD range. The sequence information on CLIP would predict a 2–3 kD peptide species. Fig. 1 shows a distinct band in that range that coprecipitates with class II molecules from both Pala and T2.DR3. In Pala cells the putative CLIP species is transiently associated with class II and then dissociates during the later chase points. In contrast, the DR3 precipitates from T2.DR3 show a striking accumulation of the same low molecular weight species. Interestingly, the putative CLIP species is not detected until 20 h of chase in T2.DR3 cells, suggesting that the rate of proteolysis of I chain in the mutant cells is slower.

To confirm that the small peptide band seen in tricine gels is CLIP, Pala cells were labeled with [³⁵S]methionine for 2 h and chased for 2, 4, or 24 h. DR3 molecules were affinity purified and associated labeled peptides were extracted, mixed with an unlabeled synthetic CLIP 24-mer, and subjected to reversed phase HPLC. The results are shown in Fig. 2. At 2 h, three late-eluting ³⁵S-labeled peaks are seen, eluting at 78 min, 83 min, and 87 min, respectively. Three identical peaks were seen when peptides were similarly isolated from DR3 molecules isolated from [³⁵S]methionine-labeled T2.DR3 cells chased for 18–24 h (data not shown). The 87-min peak eluted coincidentally with the synthetic CLIP 24 mer (not shown), and the two earlier peaks are probably truncated versions of CLIP, previously seen in T2.DR3 cells (Riberdy et al., 1992). It is clear from Fig. 2 that the levels of these peaks are considerably decreased at 24 h, consistent with the tricine gel data shown in Fig. 1.

As a further confirmation that the 2–3-kD band seen associated with HLA-DR3 molecules by tricine electrophoresis was CLIP, the three late-eluting [³⁵S]methionine-labeled peaks, derived from T2.DR3 cells, were collected and analyzed by Tris-tricine gel electrophoresis. The mobility of the purified radiolabeled peptides on a tricine gel was identical to CLIP coprecipitated with HLA-DR3 from T2.DR3 by L243 (Fig. 3). The tricine gel system presumably is incapable of resolving the minor *M_r* differences between the individual peptides. Finally, in an attempt to definitively show that the Pala-derived peaks were CLIP, the final peak (87 min elution) was subjected to Edman sequencing and the individual cycles collected and counted for ³⁵S-content. The CLIP 24-mer has methionine residues at the 11th and 13th

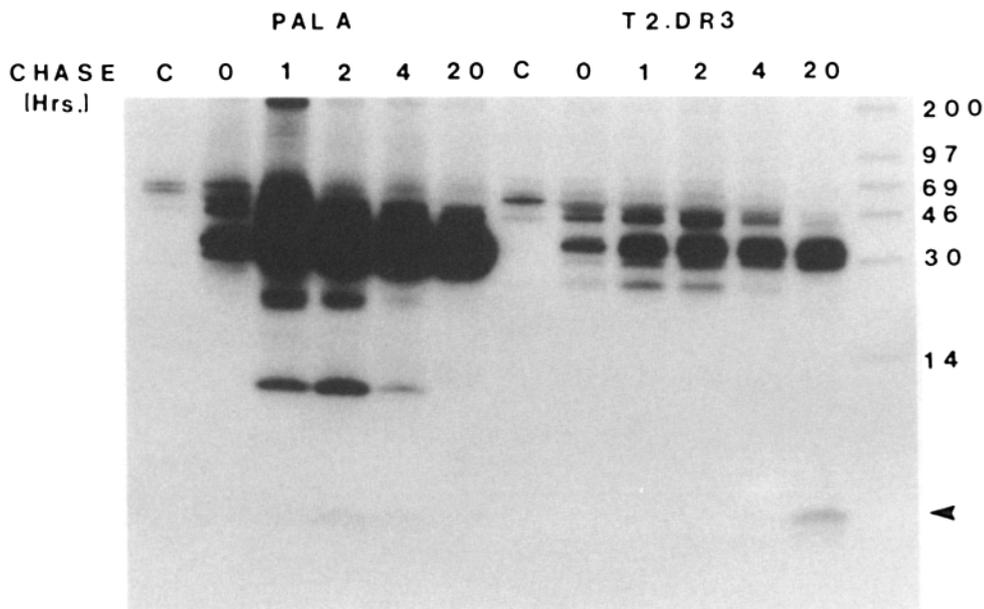


Figure 1. Pulse-chase analysis of CLIP association. Pala and T2.DR3 cells were labeled with [³⁵S]methionine for 15 min and chased in the presence of a 15-fold excess of cold methionine for the indicated times. Immunoprecipitates of cell extracts were performed with a negative control antibody GAP.A3 or an anti-HLA-DR antibody L243 and subjected to Tris-tricine gel electrophoresis as described in Materials and Methods. The arrowhead indicates a low molecular weight peptide species that coprecipitates with HLA-DR3 and was later identified as CLIP (see below).

positions (Riberdy et al., 1992). ³⁵S was clearly obtained at the 11th cycle, consistent with the peptide being CLIP (data not shown). ³⁵S was also present in the 13th cycle, although a clear "trough" with zero counts was not obtained on the 12th cycle. This may be a consequence of some contamination with a peptide with an additional residue on the NH₂-terminus, or incomplete cleavage on the 11th cycle. Nevertheless, overall these data validate the pulse-chase experiment seen in Fig. 1, suggesting that CLIP is generated

during intracellular transport and is transiently associated with class II complexes in wild-type DR3-positive cells, whereas αβCLIP complexes accumulate later during biosynthesis in T2.DR3 cells.

Intracellular Distribution of Class II and I Chain

The above biochemical analyses clearly demonstrated an alteration in the proteolysis and/or dissociation of I chain during biosynthesis in the mutant cell line T2.DR3. To determine if this affected the intracellular distribution of class II molecules and associated I chain, the cells were examined

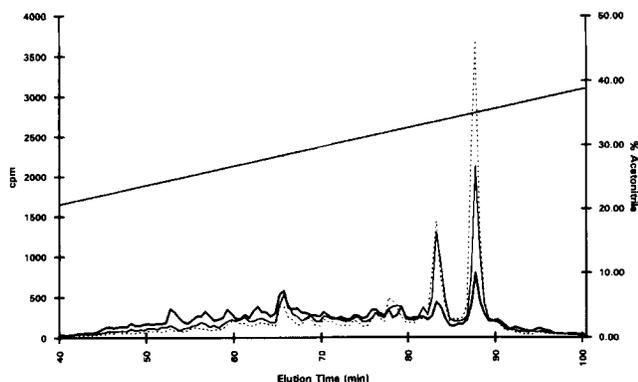


Figure 2. Identification of CLIP during pulse-chase analysis. Pala cells were labeled for 2 h with [³⁵S]methionine and chased in the presence of a 15-fold excess of cold methionine for 2, 4, and 24 h, respectively. Cell lysates were subjected to affinity chromatography with an L243 anti-HLA-DR affinity column. The peak radioactive fractions were pooled. Acetic acid was added to a final concentration of 10% and peptides smaller than 10 kD were separated by filtration through a Centricon 10 filter unit. Synthetic CLIP 81-104 (30 μg) was added to the radioactive peptides and the preparations were separated by reversed phase HPLC. 30-s fractions were collected and assayed for [³⁵S]methionine cpm in a liquid scintillation counter. Absorbance of the synthetic CLIP peptide was monitored at 210 nm and eluted with a retention time equivalent to the radioactivity collected in the last radioactive peak. The profile from the 2-h chase is represented by the dotted line, that from the 4-h chase by the solid line, and that from the 24-h chase by the thick solid line.

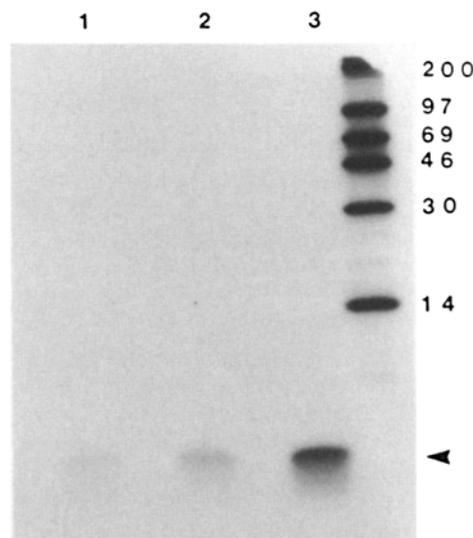
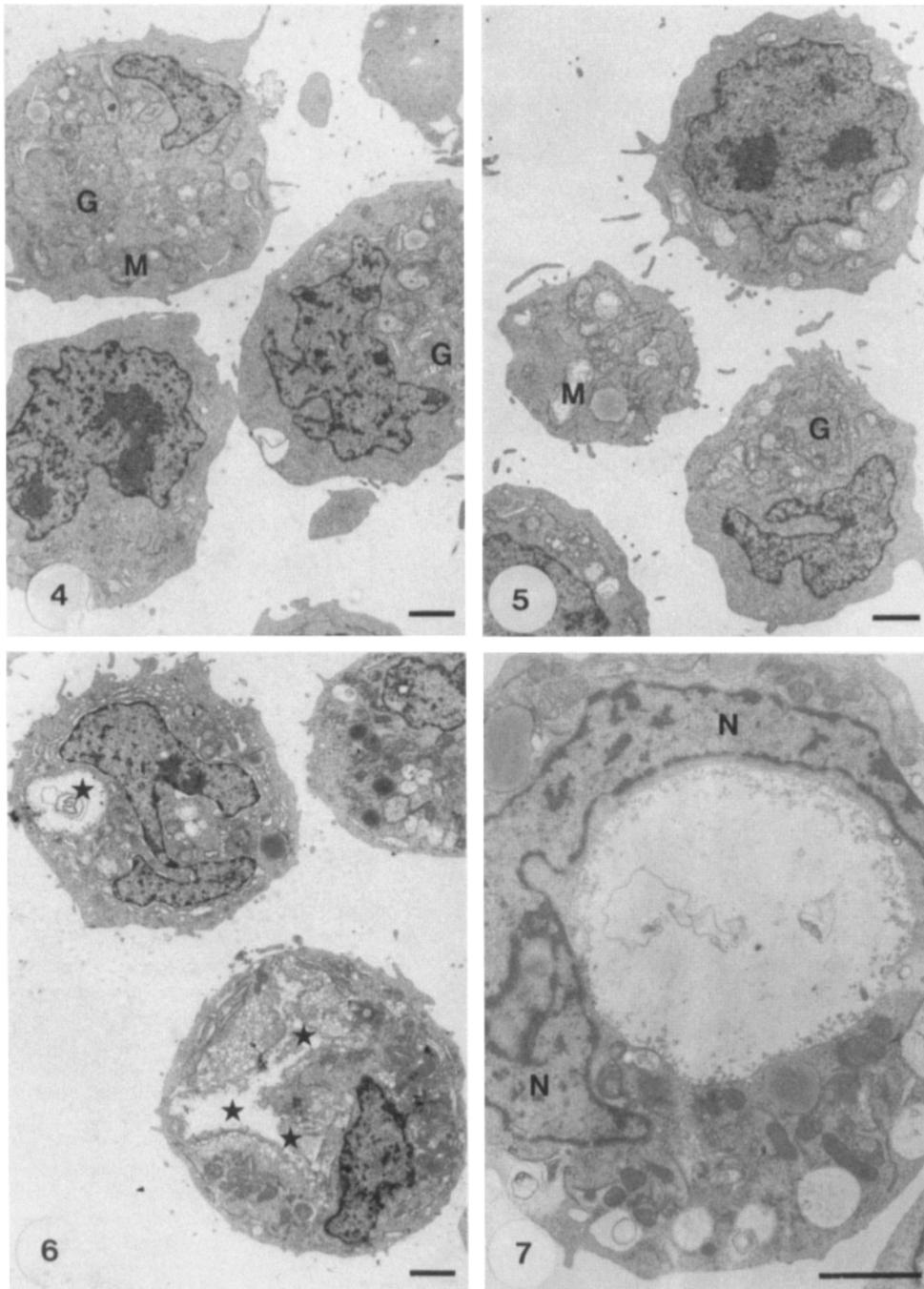


Figure 3. Purification of radiolabeled CLIP and Tris-tricine gel electrophoresis. DR3 was purified from [³⁵S]methionine labeled T2.DR3, denatured by addition of acetic acid and peptides separated by filtration through a Centricon 10 filter unit and reversed phase HPLC. The fractions corresponding to the three individual radioactive peaks in Fig. 4 were collected, lyophilized, and subjected to Tris-tricine gel electrophoresis.

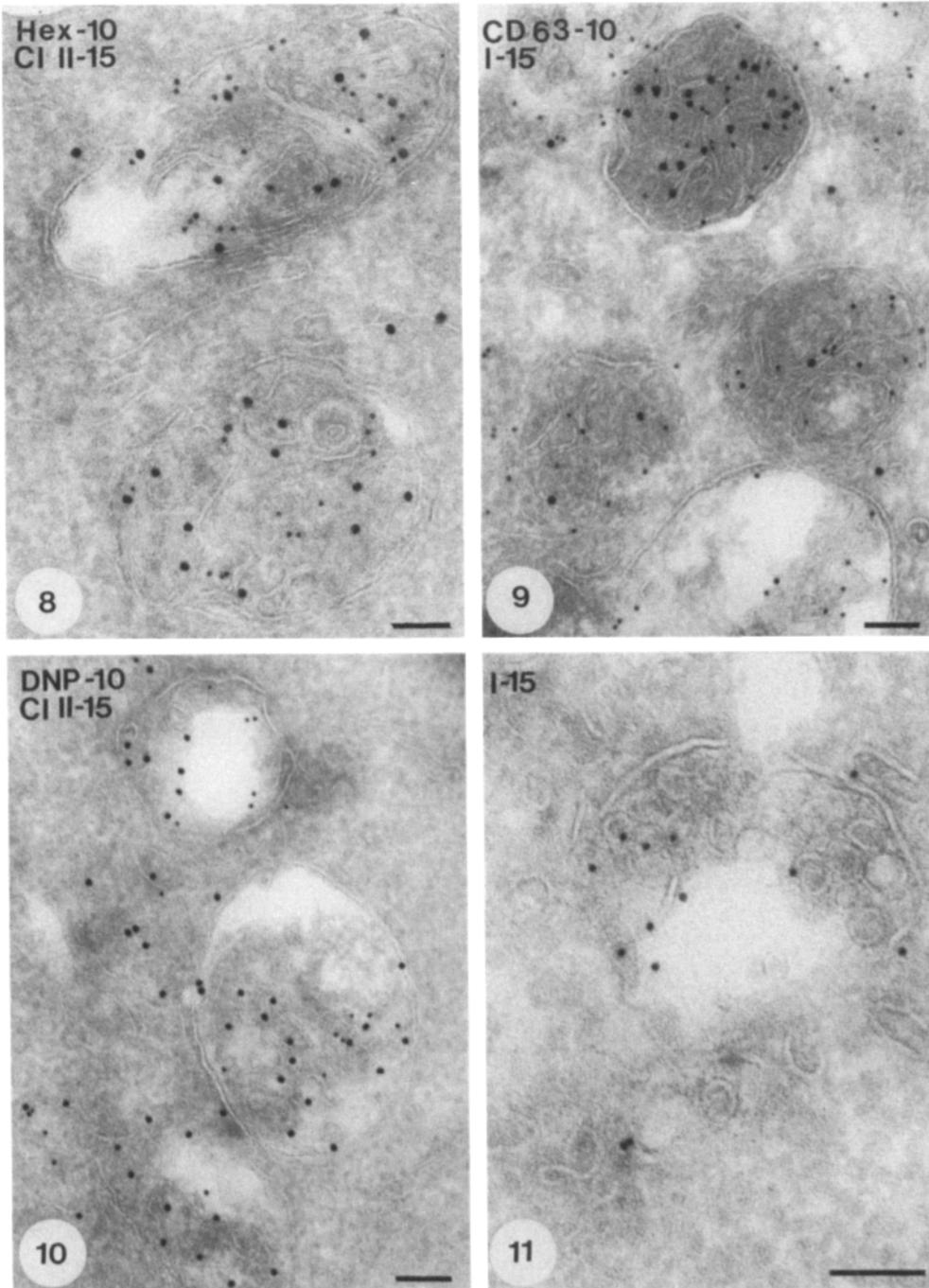


Figures 4-7. Electron micrographs of T1.DR3 (Fig. 4), T2 (Fig. 5), and T2.DR3 (Figs. 6 and 7). Cells were fixed in Karnovsky and embedded in Epon. Ultrathin sections were stained in uranyl Mg-acetate and Reynolds lead citrate. The T2.DR3 show huge vacuoles with internal membrane sheets and vesicles (*asterisks*) and surrounded by numerous larger vesicles, which were absent from T1.DR3 and T2. *G*, Golgi complex; *M*, mitochondrion; *N*, nucleus; Bars, 2 μm .

by electron microscopy. For a wild-type control cell in these experiments T1.DR3, a DR3 β -transfectant of T1, the immediate parent of T2, was used. This cell expresses both DR3 and DR7, but like T2 cells, is a TxB hybrid and therefore a superior control to the Pala B-cell line for morphological studies. Figs. 4 and 5 show that T1.DR3 and T2 cells, respectively, have the same general morphology as that previously described in human B-cell lines (Peters et al., 1991b). Comparison of the control cell lines with mutant T2.DR3 cells at low magnification reveals large, often irregularly shaped vacuoles with a diameter of up to 7 μm only in the mutant cells (see Figs. 4-7). Numerous internal vesicles and membrane sheets are seen within these distended vacuoles (Fig.

7). Interestingly these structures are only found in T2 cells that have been transfected with DR3 genes and not in untransfected T2 cells (see Fig. 5). While we have not examined other class II transfectants of T2 by electron microscopy, we have found swollen vesicles which contain I-A^k in T2.I-A^k cells by immunofluorescence (Robbins, N., unpublished data). Thus, the presence of such vesicles is not a peculiarity of DR3 molecules themselves or the particular transfectant used.

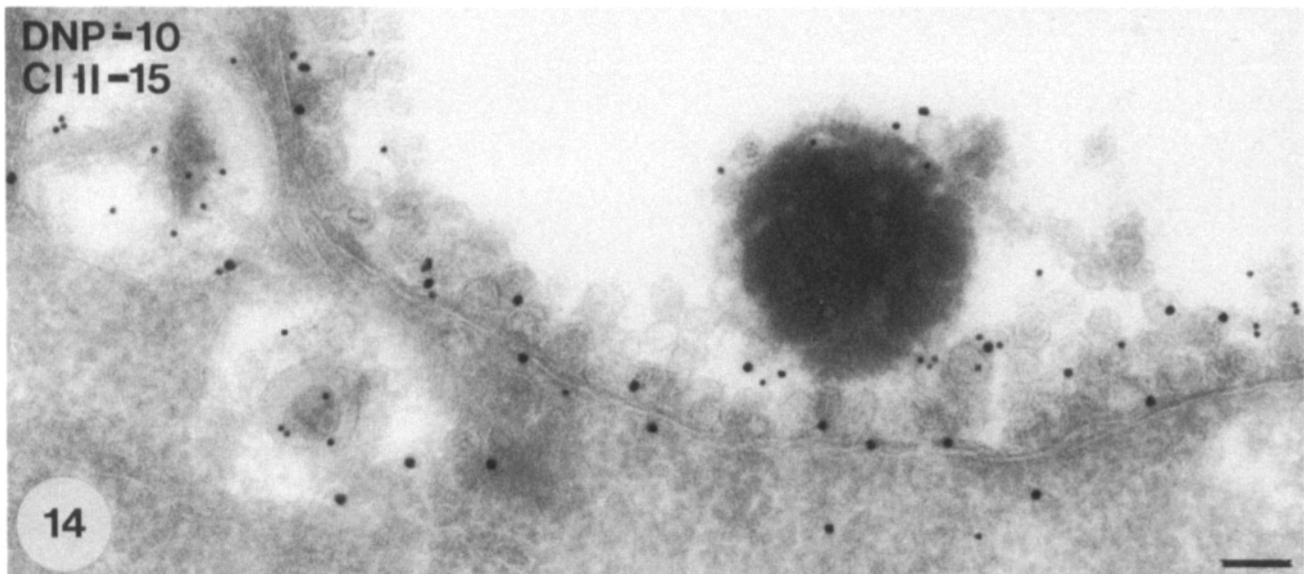
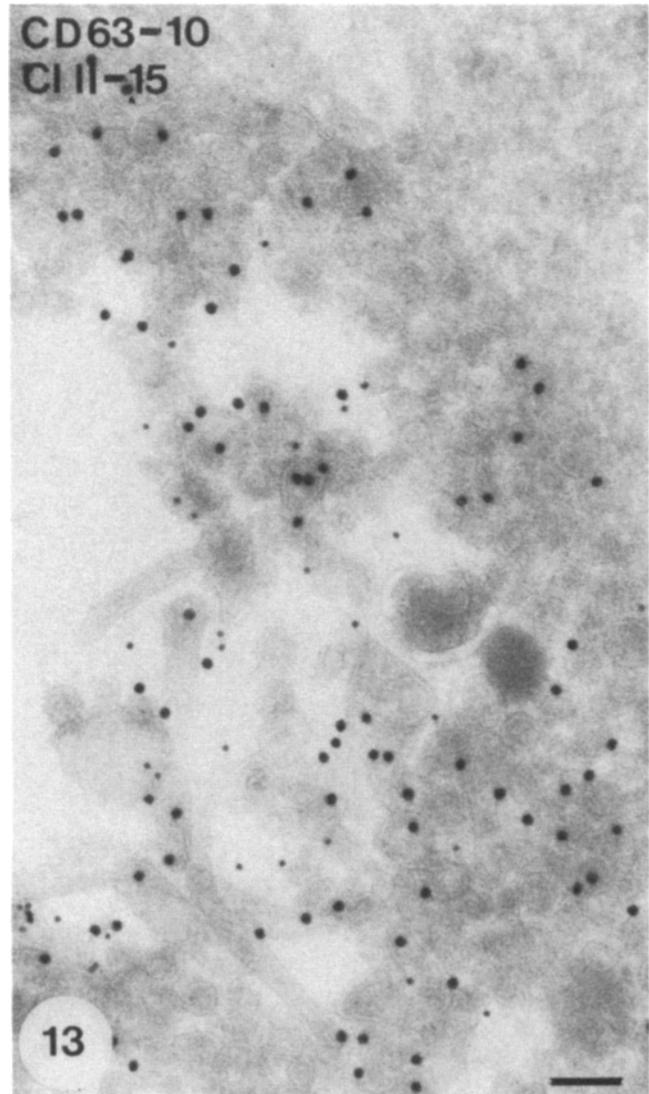
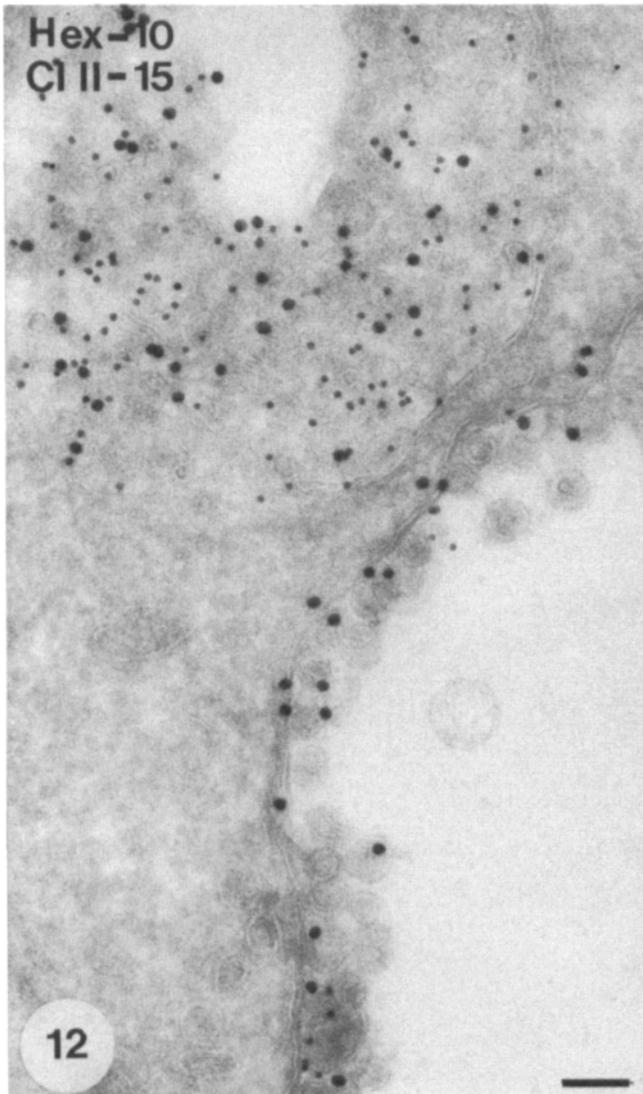
In normal B-cell lines, most of the intracellular class II is present in MHCs which have typical lysosomal characteristics, i.e., contain lysosomal enzymes, lysosomal membrane proteins, lack mannose-6-phosphate receptors (MPR), are



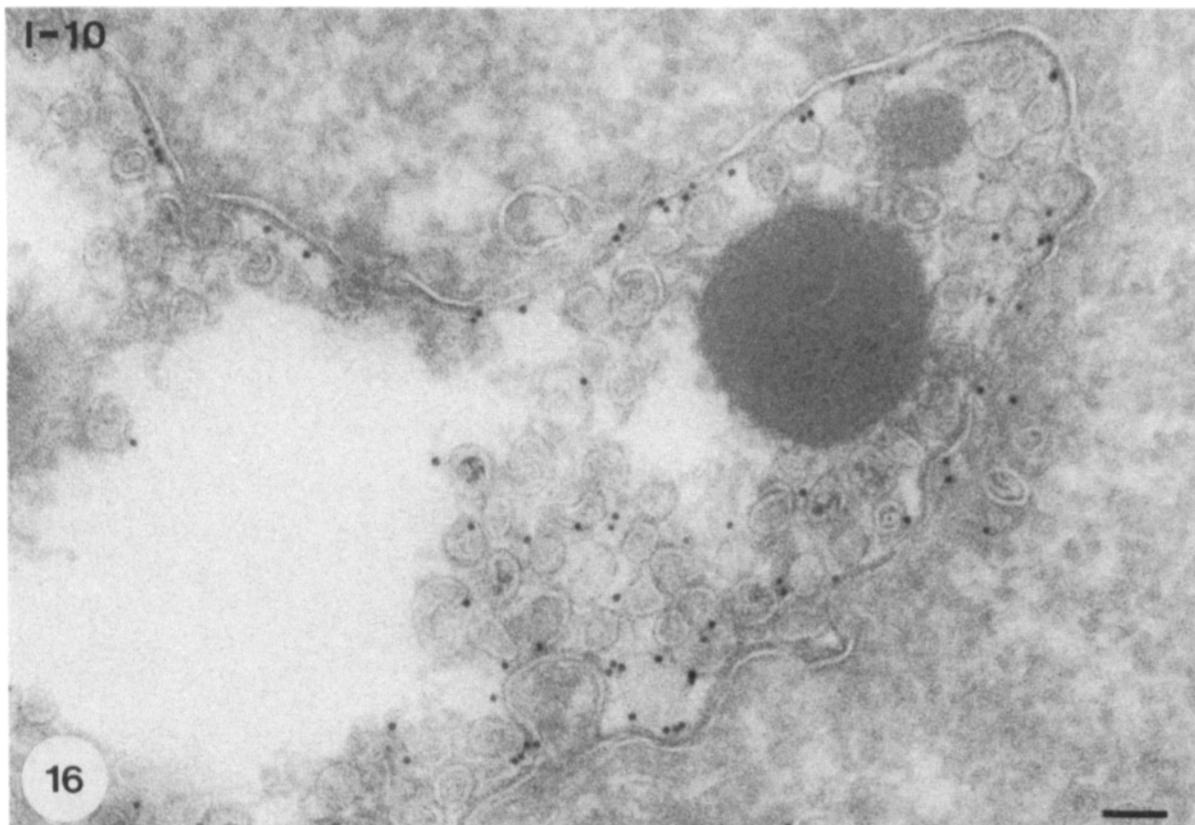
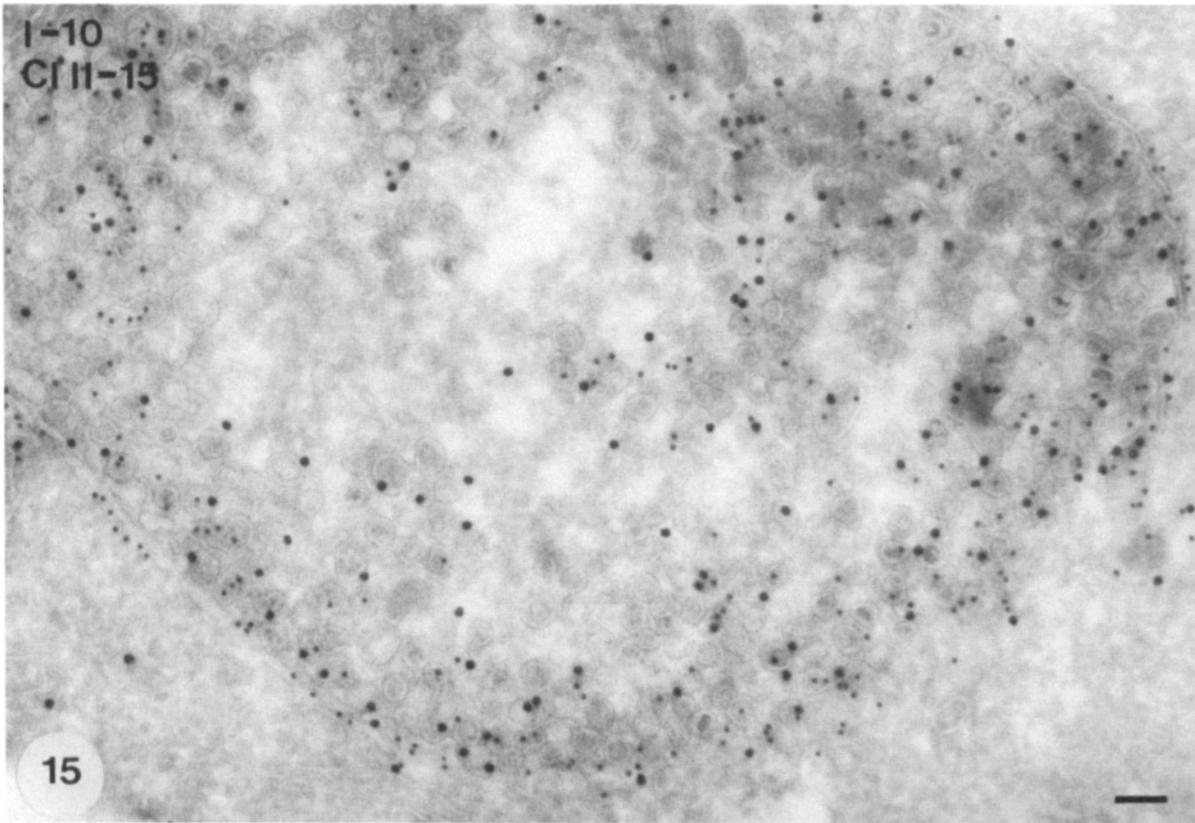
Figures 8-11. Ultrathin cryosections of T1.DR3 (Figs. 8-10) and of T2 (Fig. 11). Cells were fixed in 2% paraformaldehyde, 0.5% glutaraldehyde in 0.1 M sodium phosphate, pH 7.4 for 2 h and stored in 1% paraformaldehyde, 0.1 M sodium phosphate, pH 7.4. Cells were embedded in 10% gelatin, solidified on ice and blocks were immersed in 2.3 M sucrose in phosphate buffer for 2 h at 4°C. Ultrathin cryosections of T1.DR3 or T2 cells were labeled with gold particles (either 10 nm or 15 nm) directly conjugated to antibodies against β -hexosaminidase, class II molecules, CD63, and I chain, respectively. T1.DR3 cells were incubated with 30 mM DAMP at 37°C for 30 min and washed with cold serum-free medium at 0°C for 4 min before immunogold labeling with anti-DNP antibodies which will recognize internalized DAMP in acidic compartments, as described above. Bars, 0.1 μ m.

acidic and have a late position in the endocytic pathway (Peters et al., 1991b). To investigate the nature of MIICs in T2.DR3 cells, immunogold labeling with antibodies against class II α - and β -chains, I chain and a number of lysosomal/MIIC markers was performed on ultrathin cryosections of T2.DR3 cells, as well as the control cell lines T1.DR3 and T2. T1.DR3 cells contain typical MIICs with abundant internal vesicles and membrane sheets (Figs. 8-10). The MIICs in T1.DR3 cells contain the lysosomal enzymes β -hexosaminidase (Fig. 8) and cathepsin-D (not shown), the lysosomal membrane protein CD63 (Fig. 9), lack CI-MPR (not shown), are acidic as indicated by the accumulation of the

weak base 3-(2,4-dinitroanilino)-3-amino-N-methylpropylamine, DAMP (labeled with an anti-DNP antibody, Fig. 10), and contain abundant class II molecules (Figs. 8, 10, and 11 inset). Interestingly, MIICs containing I chain were also evident in T2 cells, but lack the same distended morphology as the irregular vacuoles found in T2.DR3 cells (Fig. 11). The MIICs of T1.DR3 and T2 show variable labeling for the I chain cytoplasmic domain. Some MIICs are strongly labeled for I chain while others show hardly if any labeling (Fig. 9). Most of the I chain labeling is associated with the content or with membranes in it, whereas the limiting membranes of the MIICs in T1.DR3 (Fig. 9) and T2 (Fig. 11) are



Figures 12–14. Ultrathin cryosections of T2.DR3. T2.DR3 cells were fixed and ultrathin cryosections were prepared for immunogold labeling as described for Figs. 8–11. Sections were immunogold labeled with antibodies against β -hexosaminidase, CD63, DNP, and class II molecules, as indicated. Only small segments of the large MIICs are depicted illustrating the size differences between MIICs in T1.DR3 and T2. Bars, 0.1 μ m.



Figures 15-16. Ultrathin cryosections of T2.DR3. T2.DR3 cells were fixed and ultrathin cryosections were prepared for immunogold labeling as described for Figs. 8-11. Sections were immunogold labeled with antibodies against class II molecules and I chain, as indicated. The MIIC profile in Fig. 16 contains two dense granules. Bars, 0.1 μm .

almost devoid of I chain label. The clear observation of I chain in MIICs in these experiments is probably because an antibody to the cytoplasmic tail was used, which can detect I chain after proteolysis of the luminal region. Antibodies against the luminal part of I chain only moderately label MIICs.

In any aspect studied except morphology, the huge vacuoles in T2.DR3 were characteristic of MIICs. Similar to the MIICs in T1.DR3, MIICs in T2.DR3 were positive for β -hexosaminidase (Fig. 12), CD 63 (Fig. 13), lack CI-MPR (not shown), and are acidic (Fig. 14), illustrating their lysosomal nature. Additionally, the MIICs in T2.DR3 contained abundant class II molecules and I chain (Figs. 12–15, 17). I chain labeling in T2.DR3 was different from that in T1.DR3 in two aspects. First, MIICs in T2.DR3 contain a much larger fraction of the intracellular I chain. Secondly, most of the I chain in T2.DR3 was associated with the limiting membrane of the MIICs rather than with the content as in T1.DR3 (Fig. 9) and T2 (Fig. 11). I chain labeling using antibodies to either cytoplasmic or luminal domains was absent from the cell surface. The MIICs in T1.DR3 and T2 were often connected to and surrounded by tubules that label for I chain (Fig. 11). The large vacuoles in T2.DR3 lack the tubules but are instead accompanied by numerous larger vesicles (Fig. 6).

In favorably contrasted sections the luminal face of the MIIC membranes, in particular of the limiting membrane, showed an electron dense layer (Fig. 16) which was less apparent in T1.DR3 and T2. Furthermore, MIICs in T2.DR3 contained electron dense globules (Figs. 14 and 16), which are reminiscent of those in the secretory lysosomes of cytotoxic T-cells (Peters et al., 1990, 1991a).

The position of MIICs in the endocytic pathway was studied by pulse-chase incubation of both T2.DR3 and T1.DR3 cells with 5-nm BSA/gold particles for 10+0, 10+30, 10+60, and 10+120 min. Only a few endocytosed particles could be detected in the MIICs in T2.DR3 after 10+30 min (Fig. 17), whereas in T1.DR3 the MIICs already contain many particles after this period of endocytosis (see inset of Fig. 17). This is somewhat earlier than found previously in the B-cell line JY where BSA/gold reaches MIICs only after 60 min (Peters et al., 1991b). Thus, the microscopy studies show that T2.DR3 cells contain extraordinarily large, acidic MIICs with normal marker characteristics in which class II molecules and I chain seem to accumulate, and which are positioned late in the endocytic pathway.

CLIP Peptides at the Cell Surface

Fig. 1 demonstrates that CLIP accumulates with class II molecules in T2.DR3. It was unclear if $\alpha\beta$ CLIP primarily accumulates in the endosomal compartment defined by electron microscopy or whether $\alpha\beta$ CLIP was also found at the cell surface. Pala and T2.DR3 cells were labeled with [³⁵S]methionine for 6 h and chased in the presence of 15-fold excess of cold methionine for 3 and 20 h, respectively. At the end of each chase period, the cell surface proteins were biotinylated with NHS-LC-biotin, a membrane impermeable reagent reactive with primary amino groups. Duplicate samples were extracted and immunoprecipitated with a negative control mAb (GAP.A3), an anti-I chain antiserum that exclusively recognizes intracellular I chain (R.p35N), or an anti-DR monoclonal antibody (L243). One

set of samples was analyzed by Tris-tricine electrophoresis as a control for the total amount of protein precipitated by the individual antibodies. Both the R.p35N and L243 samples showed significant amounts of protein precipitated with each antibody (data not shown). The remaining set of samples were eluted from the protein A beads by alkaline elution (pH 11.5) and the supernatants were neutralized by addition of acetic acid before dilution with 10-fold excess lysis buffer. The cell surface proteins were then specifically precipitated with avidin-agarose beads and the samples were analyzed on Tris-tricine gels. Fig. 18 clearly shows that cell surface DR3 complexes isolated from T2.DR3 molecules are associated with CLIP, while DR3 molecules from the Pala sample do not contain CLIP. The lack of avidin-precipitable protein bands isolated from the anti-p35N precipitates shows that the technique is not recognizing intracellular proteins. Thus, CLIP is found at the cell surface in T2.DR3 cells, while wild-type Pala cells do not express $\alpha\beta$ CLIP at the cell surface in amounts detectable by this procedure.

Discussion

The data described in this paper argue that the early stages of class II-invariant chain complex assembly and transport are similar in T2.DR3 and wild-type cells. After assembly the complexes are transported through the Golgi. In wild-type B-cell lines and in the parental hybrid T1.DR3, the class II-invariant chain complexes are then delayed in transit, probably in MIIC, the prelysosomal or lysosomal compartment previously defined by electron microscopy. In T2.DR3 cells the dominant pool of intracellular class II molecules is found in the large multivesicular compartments described in this paper. These vacuoles contain the same complement of markers as the previously defined MIIC, and are like wild-type MIIC except for their morphology. We consider the most likely explanation for their origin to be that they represent MIIC which are swollen and distended. Presumably this gross morphological change is a consequence of the mutation in the T2.DR3 cell line responsible for defective antigen processing.

The normal proteolysis of invariant chain is severely affected in T2.DR3 cells. The pulse-chase studies shown in Fig. 1 demonstrate this clearly. In the wild-type cells a number of lower molecular weight class II-associated bands appear during the chase which are characteristic of a protein undergoing progressive proteolytic degradation, and these bands are presumably fragments of the invariant chain. It has previously been shown that $\alpha\beta$ dimers associated with LIP, a leupeptin-induced partial proteolytic fragment of the invariant chain retaining the cytoplasmic domain, are reactive with L243, the mAb used for these experiments (Blum and Cresswell, 1988). One of the bands has an M_r similar to that of a previously described 11-kD I chain fragment (Nguyen et al., 1989). Between 2–4 h the lowest M_r fragment transiently appears. We have characterized this as CLIP, a nested set of I chain-derived peptides from residues 81–104 (Figs. 2 and 3). Since CLIP acts as a competitive inhibitor of antigenic peptide binding (Riberdy et al., 1992), the removal of this peptide from class II molecules is presumably essential for antigen presentation to occur. Its ultimate disappearance from DR3 molecules in the wild-type cell

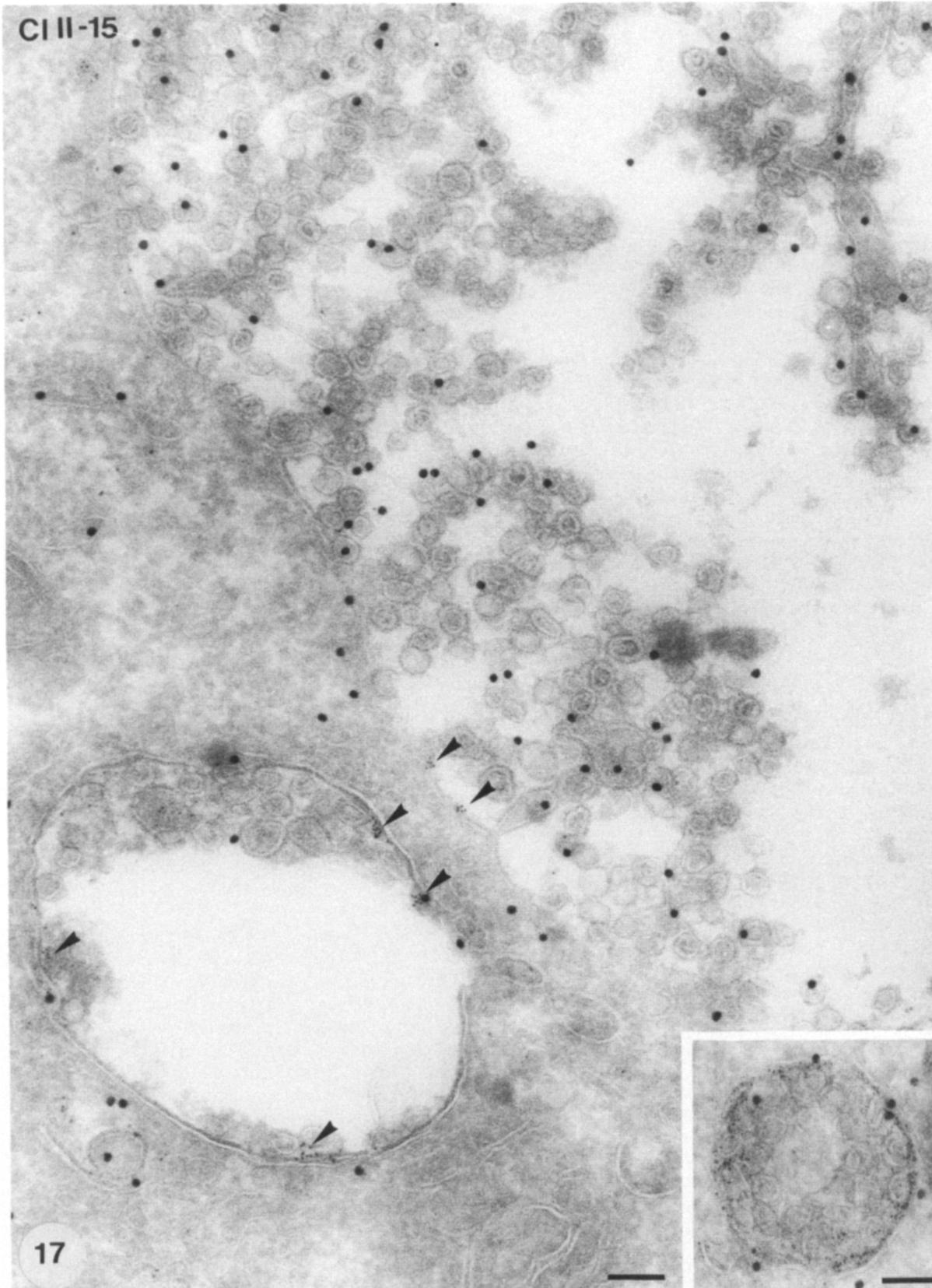


Figure 17. Ultrathin cryosections of T2.DR3 and T1.DR3 (*inset*). T1.DR3 and T2.DR3 cells were pulse incubated with BSA/gold particles for 10 min and chased for 30 min at 37°C. Endocytosis was arrested by cooling the cells with 4°C medium and excess gold that may have adhered to the cell surface was extensively washed away. Note that only a few particles (*arrowheads*) had reached the MIICs in T2.DR3 in contrast to T1.DR3, which shows numerous endocytosed particles in the MIIC at this time. Bars, 0.1 μ m.

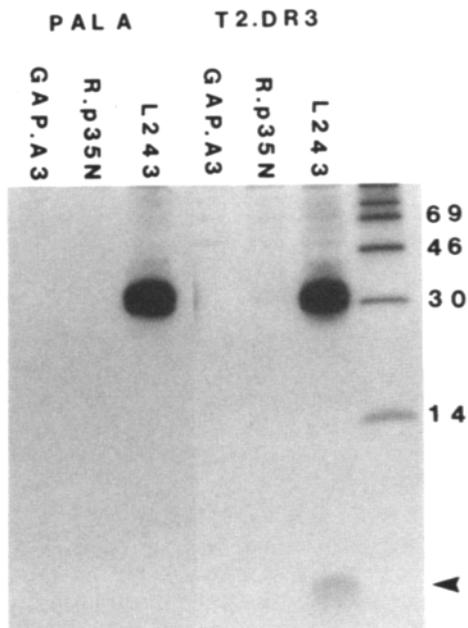


Figure 18. Identification of cell surface CLIP. Pala and T2.DR3 cells were labeled with [³⁵S]methionine for 6 h and chased in the presence of a 15-fold excess of cold methionine for 3 and 20 h, respectively. At the end of each chase point, the cell surface proteins were biotinylated with the amino-reactive biotin derivative, NHS-LC-biotin. Duplicate cell extracts were immunoprecipitated with a negative control antibody, GAP.A3, an antibody recognizing only intracellular p35 I chain, R.Ip35N, or an HLA-DR specific antibody L243. One set of immunoprecipitates was subjected to Tris-tricine gel electrophoresis (data not shown). The other set of immunoprecipitates were eluted off the protein A-Sepharose beads by alkaline elution, pH 11.5. Eluates were neutralized by addition of 7 ml 1 M acetic acid and diluted to 1 ml in 0.1% C₁₂E₉ column buffer. Cell surface proteins were specifically precipitated with avidin-agarose beads and subjected to Tris-tricine gel electrophoresis.

probably reflects the loading of antigenic peptides and the acquisition of an SDS-stable phenotype for a large proportion of them. In the T2.DR3 cells, the fragmentation pattern of invariant chain is radically different, and CLIP is generated at a much slower rate. The slow generation of CLIP is consistent with the observed accumulation of I chain in T2.DR3 vesicles relative to T1.DR3 MIIC (Figs. 15 and 16). Despite the differences in the degradation pattern of I chain in T2.DR3 cells, CLIP is ultimately generated. However, CLIP does not efficiently dissociate during biosynthesis (Fig. 1). The inability to efficiently eliminate CLIP may be associated with the inability to properly load antigenic peptide in the enlarged MIIC compartment.

Degradation of the invariant chain resulting in removal of the cytoplasmic domain is probably required for the delivery of mature $\alpha\beta$ dimers to the cell surface (Blum and Cresswell, 1988; Neefjes and Ploegh, 1992). It has been argued that the invariant chain contains an endosomal retention signal in the cytoplasmic domain as well as a targeting signal (Bakke and Dobberstein, 1990). Ploegh and co-workers have also shown that DR molecules associated with LIP are not transported from the endosomal system to the plasma membrane (Neefjes and Ploegh, 1992). Thus, a reduced rate of invariant

chain degradation would be expected to result in prolonged retention of class II molecules in the endosomal system. We have attempted by various techniques to precisely determine the transit time from ER synthesis to the plasma membrane for DR3 molecules in T2.DR3 but have been unsuccessful. In wild-type Pala cells, using a cell surface biotinylation approach, we were able to detect some DR3 molecules on the cell surface within 30 min, with complete transport in 2–4 h (data not shown). This is somewhat faster than has been previously seen in other B-cell lines (Cresswell and Blum, 1988). In similar experiments we could not detect any cell surface expression in T2.DR3 within 8 h. Cell surface expression is clearly detectable at 24 h (Fig. 18), but we have not quantitated the percentage of the class II molecules which successfully complete the journey from the ER. Overall, the data argue that class II expression on the cell surface is delayed in T2.DR3 relative to wild-type cells, that the delay probably results from a reduced rate of invariant chain proteolysis, and that the class II accumulates in the vacuoles defined by electron microscopy which have characteristics of MIIC but are morphologically dramatically different.

The precise reason for the generation of the large vesicles in T2.DR3 is unknown. It is conceivable that they enlarge simply because class II-invariant chain complexes accumulate in them, as has been proposed to occur in transfected cell lines expressing invariant chain (Romagnoli et al., 1993). The presence of class II is clearly necessary since untransfected T2 cells which have I chain, but lack class II genes, do not contain distended MIIC. This would argue that the enlarged MIIC are not merely a general defect, but are specifically associated with the accumulation of class II molecules within that compartment. The swollen MIIC cannot be due to a simple defect in acidification as immunogold labeling with DNP clearly shows that the compartment is acidic (Fig. 10). Presumably, enlarged vesicles containing exclusively I chain do not occur in untransfected T2 cells because I chain, without class II molecules, predominantly remains in the ER (Marks et al., 1990; Lamb and Cresswell, 1992). The small number of normal MIICs in T2 (Fig. 11) may result from the very small fraction of invariant chain (p33 homotrimers) which successfully escapes the ER (Lamb and Cresswell, 1992). The fact that MIICs form in T2 argues that their formation is independent of class II expression, and suggests that the invariant chain itself may be responsible for generating the structure.

At least two plausible explanations exist to explain the *in vivo* association of CLIP with class II molecules derived from wild-type cells. One is that CLIP is a free peptide generated during the proteolysis of I chain which has a significant binding affinity for a range of class II alleles. Class II binding peptides generated from the normal complement of exogenous proteins would then compete with CLIP for the binding cleft of the newly liberated $\alpha\beta$ heterodimer. Incomplete competition would result in some $\alpha\beta$ -CLIP complexes being formed. Alternatively, CLIP could remain as a residual-associated fragment of I chain after its proteolysis. This could be a region of the I chain in contact with $\alpha\beta$ dimers in $\alpha\beta$ I complexes, bound in the peptide-binding groove in the same manner as a conventional peptide. Induction of CLIP dissociation by unknown mechanisms would then be required to generate $\alpha\beta$ dimers competent to bind peptide. If the dissociation was incomplete, $\alpha\beta$ -CLIP com-

plexes would remain. The pulse-chase studies (Fig. 1) indicate that CLIP is generated during the biosynthesis of class II molecules, is transiently associated with the class II complex, and dissociates at later time points. This result is probably more consistent with CLIP remaining as a residual class II-associated fragment after invariant chain proteolysis. Whatever mechanism is responsible for inducing CLIP dissociation and/or binding endocytically generated peptides is presumably defective in T2.DR3 and may be associated with the distention of the MIIC in that cell line.

To draw any conclusions about the kinetics of CLIP generation and association in wild-type cells, it was crucial to show that the smallest molecular weight band seen in Fig. 1 was CLIP. The purification of metabolically labeled class II-associated peptides from T2.DR3 and Pala cells that had been pulsed and chased showed that at least one of the peptide peaks from the reversed phase HPLC profiles comigrated precisely with non-labeled synthetic CLIP, and this peak had a methionine residue in the expected 11th position. The synthetic 24-mer used was the longest of the CLIP peptides that were originally sequenced (Riberdy et al., 1992) and the last to be eluted from the C18 column. Thus, comigration with the last radioactive peak is expected. Also, the original steady state profiles of endogenous peptides associated with DR3 from T2.DR3 showed four distinct peptide peaks, all containing a CLIP derivative. Thus, the characteristic profile of multiple radiolabeled peptide peaks associated with class II molecules purified from Pala and T2.DR3 is consistent with all peaks containing CLIP. The fact that the radioactive samples generate chromatograms containing three peaks instead of the four peaks previously observed is most likely due to a loss in resolution because the radioactive peaks are actually collected fractions, not continuous absorbance readings. Together, these data confirm that the labeled peptides seen on Tris-tricine gels that are associated with the class II molecules in T2.DR3 and Pala are CLIP and thereby validate the pulse-chase experiment examining the kinetics of CLIP generation.

CLIP is clearly associated with surface DR3 in T2.DR3 (Fig. 18). The apparent lack of CLIP associated with the class II complexes at the cell surface in Pala suggests that in wild-type cells CLIP may dissociate before arrival at the cell surface. The obvious extension of these observations is that in wild-type cells, class II molecules bind endocytic peptides and/or simultaneously dissociate from CLIP before their arrival at the cell surface. We have previously shown that an acidic pH facilitates the binding of an antigenic peptide to DR3 molecules purified from T2.DR3, consistent with this occurring in an endosomal compartment. However, CLIP dissociation fails to occur in T2.DR3 *in vivo*, suggesting that acidification is not the only requirement. Also, the level of sensitivity of the biotinylation assay used to determine the lack of CLIP at the cell surface is uncertain and a small percentage of $\alpha\beta$ dimers at the cell surface in wild-type cells could contain CLIP. The results shown in Fig. 18 do show, however, that CLIP dissociation is not an absolute requirement for transport of HLA-DR3 molecules to the cell surface.

Recent reports by Romagnoli et al. (1993) suggest that there may well be more than one post-Golgi compartment that class II molecules traverse during biosynthesis in which antigen processing is facilitated. These data suggest that cer-

tain events such as complete and efficient invariant chain proteolysis or antigenic peptide loading may need to occur in order to have efficient transport to the next biosynthetic compartment. Since T2.DR3 cells do not process and present antigenic proteins well, nor do they bind a normal repertoire of peptides intracellularly, it is reasonable to propose that the defect in these cells is in accessibility to peptides. MIICs have been shown to be part of both the biosynthetic pathway of class II molecules and the endocytic pathway, and are probably positioned at the intersection of these routes (Neefjes et al., 1992). They contain hydrolytic enzymes and are acidic and are therefore candidates for a compartment in which antigen is processed and peptide is loaded on to the class II molecules. Indeed, recent studies on macrophages have shown that antigenic peptides are efficiently generated and may be loaded on to class II molecules in a late endocytic compartment with characteristics of MIIC (Harding et al., 1991; Harding and Geuze, 1993). How MIICs are formed from post-Golgi intermediates has not yet been elucidated. It is not known in what type of vesicles $\alpha\beta$ complexes exit the Golgi en route to the next station in transport, presumably the MIIC. The absence of small tubules and vesicles normally present around MIICs and the accumulation of larger vesicles (see Fig. 6) in T2.DR3 may reflect a defect in the formation of MIICs resulting in an improper sorting of class II complexes to the compartment where endocytically generated peptides reside. Conversely, there may be defects in transporting peptides to the compartment where the liberated class II $\alpha\beta$ dimers reside. Any of these scenarios could result in the accumulation of class II molecules in MIIC and ultimately a defect in class II antigen presentation. Further investigation of the processes involved in dissociating CLIP and binding peptide in the MIIC are needed to determine the mechanisms which generate the distended MIIC in these cells.

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