

Relationship between Invariant Chain Expression and Major Histocompatibility Complex Class II Transport into Early and Late Endocytic Compartments

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Summary

Invariant chain (Ii), which associates with major histocompatibility complex (MHC) class II molecules in the endoplasmic reticulum, contains a targeting signal for transport to intracellular vesicles in the endocytic pathway. The characteristics of the target vesicles and the relationship between Ii structure and class II localization in distinct endosomal subcompartments have not been well defined. We demonstrate here that in transiently transfected COS cells expressing high levels of the p31 or p41 forms of Ii, uncleaved Ii is transported to and accumulates in transferrin-accessible (early) endosomes. Coexpressed MHC class II is also found in this same compartment. These early endosomes show altered morphology and a slower rate of content movement to later parts of the endocytic pathway. At more moderate levels of Ii expression, or after removal of a highly conserved region in the cytoplasmic tail of Ii, coexpressed class II molecules are found primarily in vesicles with the characteristics of late endosomes/prelysosomes. The Ii chains in these late endocytic vesicles have undergone proteolytic cleavage in the luminal region postulated to control MHC class II peptide binding. These data indicate that the association of class II with Ii results in initial movement to early endosomes. At high levels of Ii expression, egress to later endocytic compartments is delayed and class II-Ii complexes accumulate together with endocytosed material. At lower levels of Ii expression, class II-Ii complexes are found primarily in late endosomes/prelysosomes. These data provide evidence that the route of class II transport to the site of antigen processing and loading involves movement through early endosomes to late endosomes/prelysosomes. Our results also reveal an unexpected ability of intact Ii to modify the structure and function of the early endosomal compartment, which may play a role in regulating this processing pathway.

The function of MHC class II molecules is to present peptides to the α/β receptor of CD4⁺ T lymphocytes. MHC class II molecules primarily present peptides derived from proteins that access the endocytic pathway (1), whether by fluid phase uptake, receptor-mediated uptake, recycling of endogenously synthesized proteins from the plasma membrane, or, in the case of cytosolic proteins, perhaps autophagy (2). Previous functional data supporting this conclusion have recently been bolstered by sequence analysis of peptides eluted from purified MHC class II molecules (3-6). Various methods have been used to study the intracellular maturation and transport of MHC class II molecules and to examine the location and biochemical events involved in processing such proteins into peptides suitable for binding to MHC class II molecules. The bulk of available data indicate that MHC class II-pep-

tide association largely takes place within the endocytic pathway itself (2, 7-10). This latter model is directly supported by the observation that newly synthesized MHC class II molecules undergo a conformational alteration characteristic of peptide binding in an intracellular, post-Golgi compartment (11-13).

Subcellular fractionation has demonstrated that in B cells and B cell tumors, MHC class II molecules are present in light vesicular structures derived from early and/or late endosomes (14). Immunoelectronmicroscopy of mouse peritoneal macrophages and human B cells has revealed some class II-containing vesicular structures with the lucent appearance of early endosomes (7, 14), and others with the multivesicular appearance of carrier vesicles, late endosomes, or prelysosomes (9, 14). In certain nonlymphoid cells, the distribution

appears even broader, extending throughout the endosomal/lysosomal pathway (15).

What controls MHC class II movement into, through, and out of the various vesicular subcompartments of this pathway? A nonpolymorphic type II membrane glycoprotein (16) termed the invariant chain (Ii)¹ (17) appears to be the key to the control of MHC class II availability for peptide binding and intracellular localization. There are several distinct forms of Ii, termed p31, p33, p41, and p43, that arise from a combination of alternative translation initiation (p31 vs. p33, p41 vs. p43) and alternative splicing that results in the presence or absence of a luminal subdomain (p41, p43 vs. p31, p33) (18–20). These various forms of Ii associate with newly synthesized MHC class II α and β chains in the endoplasmic reticulum (ER) (21, 22), possibly as a trimeric core on which three α/β dimers assemble (23). Ii seems to have two major roles in this early stage of class II biosynthesis and transport. First, intact Ii inhibits stable peptide binding to MHC class II molecules (11, 24, 25). Because Ii assembles with the MHC class II α and β chains rapidly after import into the ER, this may constitute a major mechanism precluding effective peptide binding in this early postsynthetic phase of the class II pathway, providing “empty” MHC class II molecules suitable for peptide capture elsewhere in the cell. Second, Ii also acts as a specialized chaperone to facilitate the export of class II molecules from the ER, most likely by preventing retention by resident proteins (26–28).

Recent studies have shown that Ii contains a signal in the NH₂-terminal 30 residue cytoplasmic tail that targets this protein to an endocytic compartment (29, 30), and that Ii coexpression leads to intracellular localization of MHC class II in a vesicular pattern (10, 30). In transfected CV1 cells, the amount of Ii protein increases with time. At relatively low protein levels, Ii was observed to sort to small endosomes, whereas at later times, when Ii expression was greater, a cohort of large vesicular structures was seen (Bakke, O., and B. Dobberstein, unpublished observations). We report here immunofluorescence studies that characterize the majority of these macrovesicular structures as modified early endosomes whose rate of transfer of luminal and membrane contents to later endocytic compartments is markedly slowed by the presence of high levels of Ii. Class II and Ii can also be found in later endocytic locations. The Ii in the early endosomes is largely intact, whereas that accumulating in late endosomes has undergone luminal cleavage. These observations suggest that Ii initially directs class II to early endosomes from which the class II–Ii complexes transit to late endosomes/prelysosomes where the class II binding site becomes available. The documentation of the novel capacity of intact Ii to alter early endosome structure and function, and of these patterns of class II movement and co-ordinate Ii cleavage, provide new insight into the class II antigen presentation pathway.

¹ Abbreviations used in this paper: ER, endoplasmic reticulum; GC, Golgi complex; h, human; Ii, invariant chain; m, mouse.

Materials and Methods

Expression Plasmids and Transfection. All experiments were performed with COS 7.2 cells (31), which were maintained in DMEM/10% FCS. For transient expression, a modified procedure from Lopata et al. (32) was used, as previously described (26). Briefly, COS 7.2 cells plated at 10⁶ per 25 cm² tissue culture flask were washed twice with DMEM/10 mM Hepes. Cells were then incubated in 3 ml DMEM/10 mM Hepes containing 400 μ g/ml DEAE dextran, 100 μ M chloroquine and DNA (1 μ g of each plasmid encoding A α ^b [33] and A β ^b [33], 4 μ g of plasmid encoding mIip31 (34), mIip41 (P. Jones, unpublished data), hIip31 (29), hIip31 Δ 11 (29), hIip31 Δ 20 (29), or human lamp-1 (35). After 4 h at 37°C in 10% CO₂, the cells were treated with 10% DMSO in PBS for 2 min at room temperature, and were then incubated overnight in DMEM/10% FCS. Transfected cells were trypsinized and plated on coverslips pretreated with poly-L-lysine. 24 h later, cells were processed for immunofluorescence.

Indirect Immunofluorescence. Transiently transfected COS 7.2 cells were fixed in 4% paraformaldehyde for 15 min at room temperature. Fixed cells were treated with 0.1 M glycine for 10 min, and were then permeabilized for 15 min with PBS containing 0.2% saponin and 2.5% FCS. Cells were incubated at room temperature for 15 min with primary antibodies (see figure legends). After three washes with PBS, cells were incubated with FITC, rhodamine, or Texas red conjugated secondary antibodies for 15 min at room temperature, washed, and mounted on slides with Fluor SaveTM reagent (Calbiochem Novabiochem Corp., La Jolla, CA). Cells were analyzed using a photomicroscope (Axiophot; Carl Zeiss, Inc., Thornwood, NY) equipped with appropriate filters to gate red and green fluorescence. Images obtained with a \times 100 objective were captured with a video camera (model C2400–08 SIT; Hamamatsu, Inc., Hamamatsu City, Japan), were digitally processed with an image processor (model DVS-3000; Hamamatsu, Inc.) and were then transferred to a computer (Macintosh II; Apple Computer, Inc., Cupertino, CA). Using the Adobe Photoshop program (Adobe Systems, Inc., Mountain View, CA), the eight-bit grey scale images were changed into eight-bit red or green images and printed separately or superimposed using a Mavigraph printer (Sony Corp., Montvale, NJ). For Fig. 1 only, images were obtained using a Confocal Laser Scanning System attachment (Bio-Rad/Analytical Instr. Group, Cambridge, MA) with the Axiophot photomicroscope system. The image planes chosen for the figures in this paper were selected to emphasize intracellular compartments, and deemphasize surface protein staining.

Labeling with Endocytic Markers. To label early endosomes, cells were incubated for 45 min at 37°C in PBS containing 20 μ g/ml of iron saturated transferrin (Sigma Immunochemicals, St. Louis, MO). Cells were then washed, fixed, and processed for immunofluorescence. To prelabel lysosomes, cells were incubated for 6–8 h at 37°C in medium containing 1 mg/ml tetramethyl rhodamine isothiocyanate (TRITC)-OVA. Cells were then washed and chased for 24 h in normal medium at 37°C.

The kinetics of accumulation of endocytic marker in lysosomes was studied by monitoring the appearance of FITC-OVA in lysosomes pre-labeled with TRITC-OVA as described above. Cells were incubated in 1 mg/ml FITC-OVA for 1 h at 37°C, washed and chased for 1, 6, and 24 h.

Results

Ii Determines the Intracellular Distribution of MHC Class II Molecules in Transfected COS Cells. Previous studies have

shown that Ii contains (a) sorting signal(s) in its cytoplasmic tail necessary for targeting to and accumulation in incompletely characterized endosomal vesicles (29, 30). The human Ii p31 chain can alter the intracellular distribution of coexpressed human class II α/β dimers in human fibroblasts (10, 30). We examined additional forms and species variants of Ii to determine whether they showed a similar pattern of localization when expressed alone or with mouse class II proteins in monkey cells.

When analyzed with the anti-Ii cytoplasmic tail antibody VIC-Y1, transfected COS cells expressing human (h) Iip31 showed staining of the nuclear envelope, ER, and Golgi complex (GC), as well as very strong staining of central vesicular structures (Fig. 1 A). These data are consistent with morphological and biochemical studies showing that only a portion of the Ii molecules expressed in the absence of MHC class II progress through the GC (23, 29, 30). Using the present fixation conditions, staining with the anti-Ii lumenal-region specific antibody LN2 primarily showed vesicular staining, as previously reported (29) (Fig. 1 B). The human Ii-containing vesicles varied in size and distribution among individual cells in even a single transfection, ranging from large (5–10 μ) and sometimes perinuclear (Fig. 1, A and B) to small ($\sim 1 \mu$) and widely distributed (data not shown). The proportion of cells with large vesicles increased as more DNA was used for transfection or the time after transfection was lengthened, both of which increase the total amount of Ii expression. A similar intracellular staining pattern was observed with either mouse (m) Iip31 (data not shown) or mIip41 (Fig. 1 F), indicating that the localization of Ii to vesicular structures is not a unique property of the human molecule or of the p31 form of the protein.

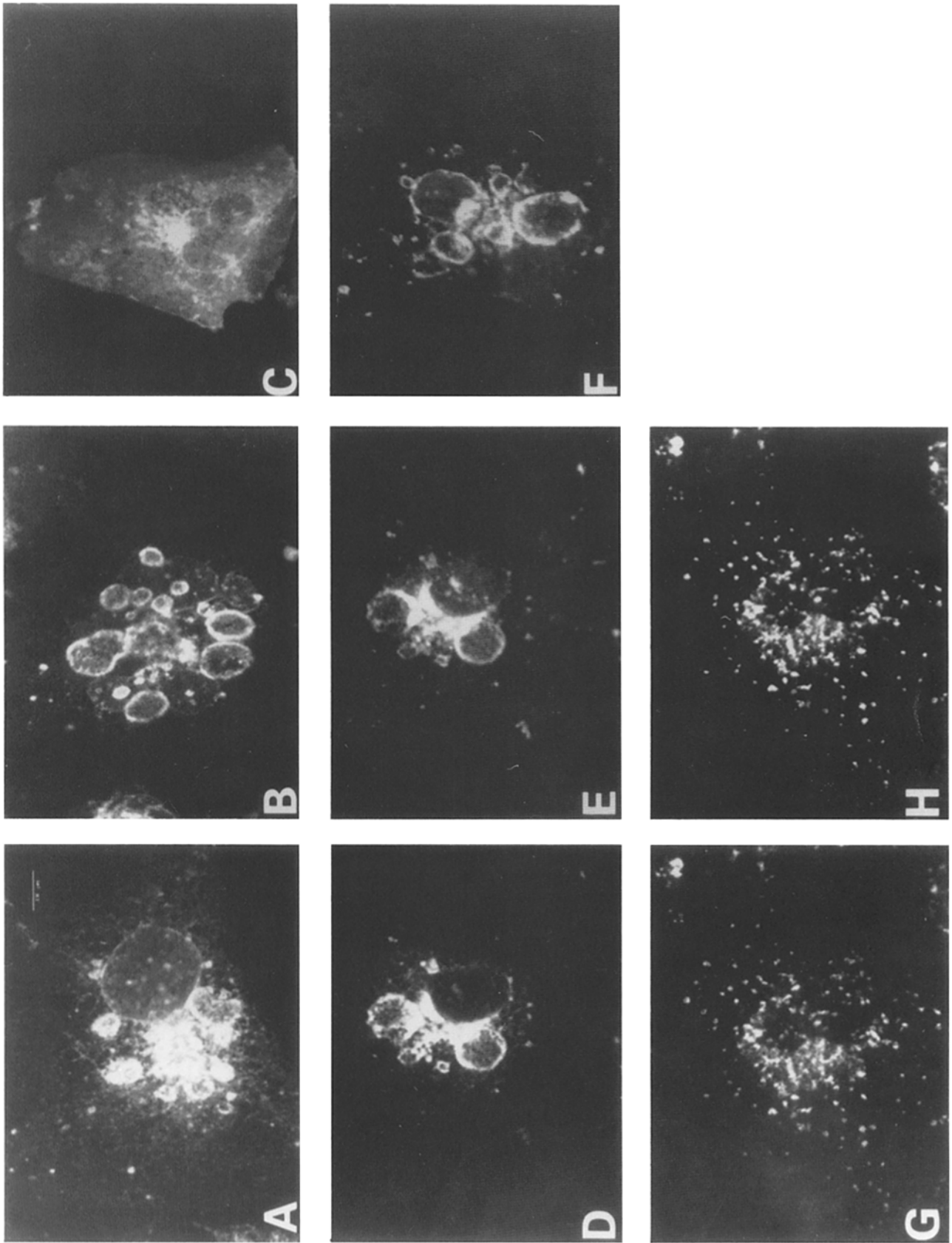
Coexpression of $A\alpha A\beta$ dimers with Ii led to a dramatic change in class II localization. Intracellular staining of cells in which $A\alpha A\beta$ dimers were expressed alone showed strong signals primarily in the ER and GC, with little vesicular localization (Fig. 1 C). Surface class II expression could also be observed as expected (data not shown). Coexpression of Ii altered this pattern. Less staining was seen in the ER and a substantial amount of MHC class II colocalized in large vesicles with human Iip31 (Fig. 1, D and E), as well as appearing on the plasma membrane (data not shown). A vesicular distribution of MHC class II was also seen with mouse Iip41 coexpression, but in this case, small, widely dispersed vesicles were seen (Fig. 1, G and H). The alteration of class II steady state localization from exocytic compartments to vesicular structures upon coexpression with Ii is consistent with the results of Lotteau et al. (30) and Lamb et al. (10) obtained using human Ii, and provide the first demonstration that expression of the p41 form of Ii results in an intracellular distribution of class II similar to that previously reported for the p31 form. The number and size of large vesicles stained with anti-Ii antibody declined in some cases when class II-encoding plasmids were added to Ii transfections. Metabolic labeling experiments (data not shown) suggest that this may not be due solely to plasmid competition and a reduction in absolute Ii protein levels, implying that a high level

of free (non- α/β complexed) Ii may be necessary for the appearance of the large vesicles. This apparent dependence on free Ii for macrosome formation may explain the differences observed in cells coexpressing class II with human Iip31 vs. mouse Iip41, as the former achieves higher levels of expression in the COS system than the latter (data not shown).

Active Generation of a Unique Set of Macrocytic Vesicles after Expression of Intact Ii. Macroosomes were not seen after transfection of MHC class II plasmids alone (Fig. 1 C), nor after transfection of a variety of plasmids encoding various type I integral membrane proteins (e.g., CD4, CD8, MHC class I; data not shown). Because these latter proteins are not selectively targeted to the endosomal/lysosomal pathway, we also expressed in COS cells human lamp-1, a lysosomal membrane protein that when highly expressed, traffics through the early and late endocytic compartments (35). Only small- to medium-size ($\sim 1 \mu$), widely dispersed vesicles were seen in cells transfected with a plasmid encoding human lamp-1 (Fig. 2 A), the same staining pattern seen for endogenous simian lamp-1 (Fig. 2 B). Thus, mere overexpression of endosomally targeted proteins in the COS transfection system does not nonspecifically result in macrosome formation. Furthermore, transfection of truncated cDNAs that encoded Ii lacking the endosomal targeting signal in the cytoplasmic tail (hIip31 Δ 20), although producing as much Ii protein as the plasmid encoding intact Ii, did not result in the visualization of macroosomal structures (data not shown). Thus, Ii macroosomes are specifically detected in cells expressing intact Ii.

The macroosomal staining pattern could be due either to the accumulation of Ii in a preexisting set of large vesicular structures, now made detectable by staining for Ii, or could reflect the creation of a new, previously absent cohort of vesicles. We therefore examined COS cells transfected with plasmids encoding intact Ii or control plasmids for the presence of macrovesicular structures using methods that did not rely on detection of Ii itself. Cells exposed to high concentrations of fluid phase endocytic tracers such as FITC-OVA or TRITC-OVA showed distinct patterns of tracer accumulation, depending on the presence or absence of Ii. Cells lacking Ii show numerous small dispersed vesicles (Fig. 3 A), whereas cells expressing Ii had the tracer in macroosomes (Fig. 3 B). Phase contrast observation revealed that cells without Ii did not have a significant number of large vesicular/vacuolar structures, whereas cells expressing Ii contained numerous large lucent vesicles in the perinuclear region (data not shown). Acridine orange staining showed that cells lacking Ii had a relatively tight cluster of small vesicles staining red with this dye, indicating their acidic nature, whereas those expressing Ii had large acidic vesicles corresponding in size and location to the macroosomes revealed by staining for Ii (data not shown). These observations indicate that macroosomes do not preexist in COS cells, but rather they are actively generated by the presence of high levels of Ii and are part of the endocytic pathway.

Most Ii Macroosomes Are Altered Early Endosomes. There is general consensus that the endocytic pathway can be subdivided into at least three major compartments: early endo-



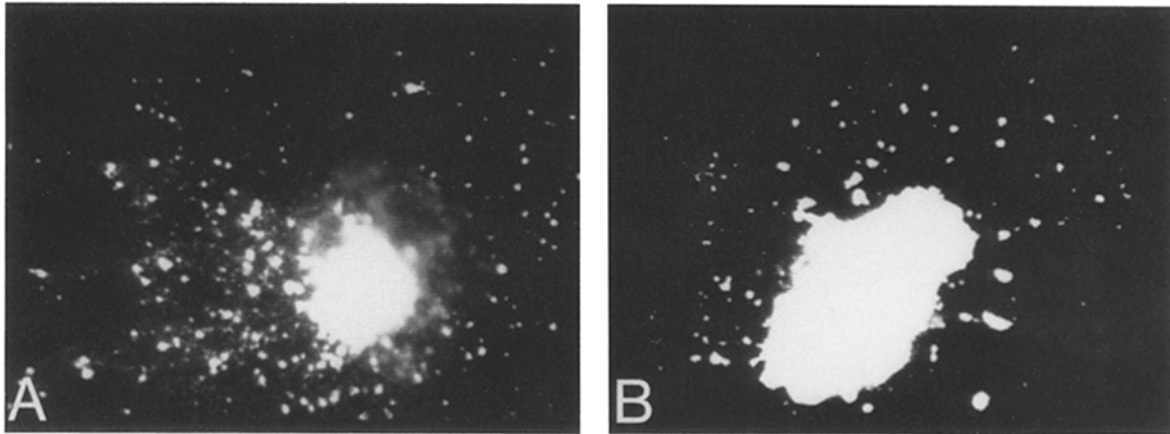


Figure 2. Immunofluorescence staining of human lamp-1 (A) in transfected COS 7.2 cells. The human protein was specifically detected with the mAb H5G11 (41). The staining pattern was compared with endogenous lamp-1 (B) detected with the mAb BB6 (35) in mock-transfected COS cells.

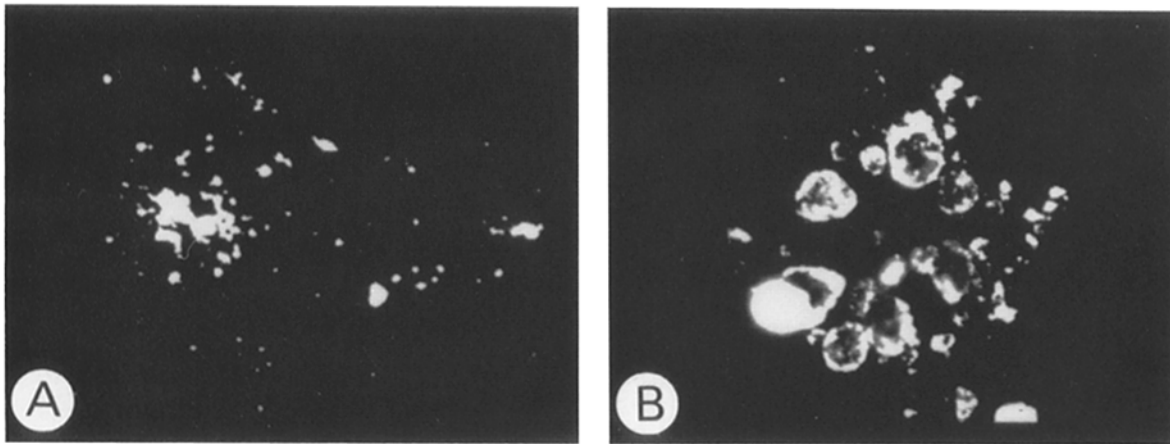


Figure 3. Invariant chain-independent visualization of Ii-macrosomes. 48 h after transfection COS 7.2 cells expressing A α^b A β^b (A) or hIip31 (B) were examined after FITC-OVA uptake. Cells were pulsed for 1 h with FITC-OVA (1 mg/ml), washed, and the internalized marker was chased for 1 h at 37°C. Cells were then fixed and analyzed.

somes, late endosomes/prelysosomes, and lysosomes (42, 43). Ii-transfected COS cells stained with antibody to α adaptin present in clathrin-coated vesicles trafficking to early endosomes, showed a pattern distinct from and nonoverlapping with that of Ii, which did not correspond to the macrosome pattern (data not shown). However, when transfected COS cells expressing hIi were incubated for 30 min with iron-saturated human transferrin, extensive colocalization of transferrin and Ii in macrosomes was observed (Fig. 4, A and B). The macrosome-localized transferrin could be chased out completely within 20 min (Fig. 4, C and D). In addition, a mAb to an epitope generated by the pH-dependent change of

influenza hemagglutinin conformation in the acidic environment of (early) endosomes (45) demonstrated the presence of recently internalized virus in the macrosomes (Fig. 5). In some cells, a minority of macrosomes was observed that failed to show transferrin accumulation (data not shown), suggesting that the effects on Ii on endosomal structure were not confined solely to the early sorting endosome. Nevertheless, these data strongly suggest that most macrosomes correspond to a modification of the early endocytic compartment.

We also stained the Ii transfected cells with anti- γ adaptin antibodies to identify the *trans*-Golgi network (TGN) and with anti-CI-M6PR antibodies to identify the TGN and

Figure 1. Localization of Ii, and MHC class II α/β dimers in COS 7.2 cells transfected with plasmids encoding hIip31 (A and B); A α^b A β^b (C); hIip31 and A α^b A β^b (D and E); mIip41 (F); mIip41 and A α^b A β^b (G and H). Class II molecules were stained with the mAb M5/114 (36) (C and E) or with the mAb Y3P (37) (H); hIip31 was stained with the mAb VIC-Y1 (38) (A and D) or LN2 (39) (B). mIip41 (F and G) was detected with the mAb In-1 (40). FITC-labeled second Abs against mouse or rat IgG were used. For double-staining (D, E, and G, H) TRITC-labeled Abs against mouse IgG were used in D and G, and FITC-labeled antibodies against rat IgG in E and H. Bar in A, 10 μ .

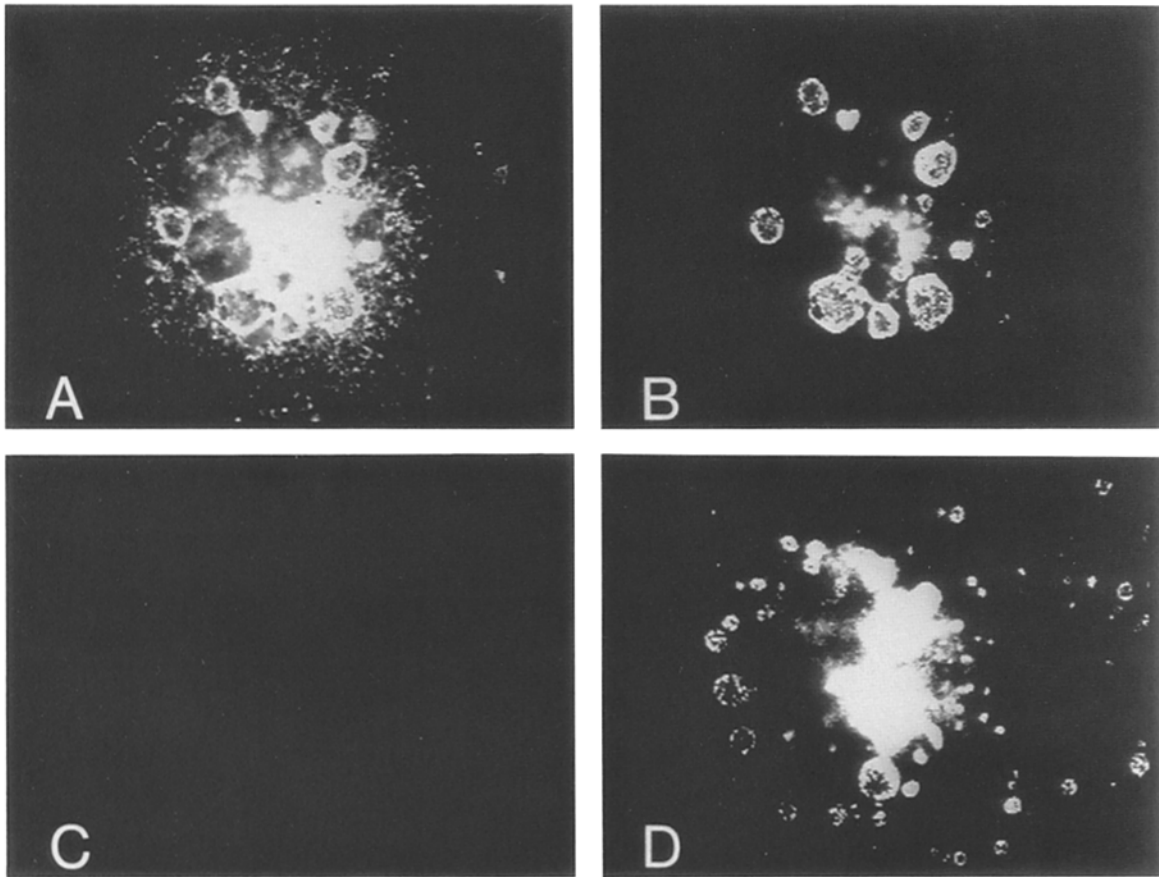


Figure 4. Double-staining of transferrin and Ii in COS 7.2 cells expressing hIip31. Cells were pulsed for 45 min at 37°C with iron-saturated human transferrin (20 µg/ml), then either fixed (A and B) or washed, and normal serum-containing medium added to chase the human transferrin with bovine transferrin (C and D). After fixation, cells were permeabilized and stained with FITC-labeled sheep antibodies to human transferrin (44) (A and C) and the mAb LN2 (B and D). TRITC-labeled anti-mouse IgG antibodies were used to detect the anti-Ii antibody (B and D).

sorting compartment between TGN and late endosomes/prelysosomes. Neither reagent showed significant colocalization with Ii in macrosomes in these cells. Additional staining revealed that Ii macrosomes contain a very small but detect-

able amount of proteolytic enzymes such as cathepsin D, which has been recently described as trafficking through early endosomes (46), but lack significant amounts of MHC class I molecules (data not shown), which segregate from MHC

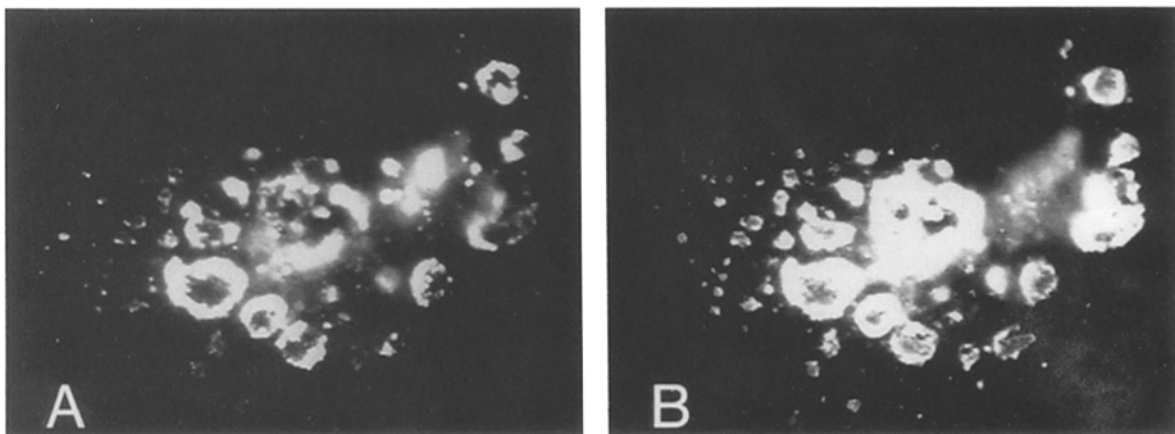


Figure 5. Localization of internalized influenza virus in Ii macrosomes. 48 h after transfection, COS 7.2 cells were incubated with heat-inactivated influenza virus in the presence of protein synthesis inhibitors as described. After fixation and permeabilization, hemagglutinin was detected with the mAb Y8-10C2 (45) (A) and mIip41 with the mAb In-1 (B). TRITC-labeled anti-mouse IgG and FITC-labeled anti-rat IgG were used in the second step.

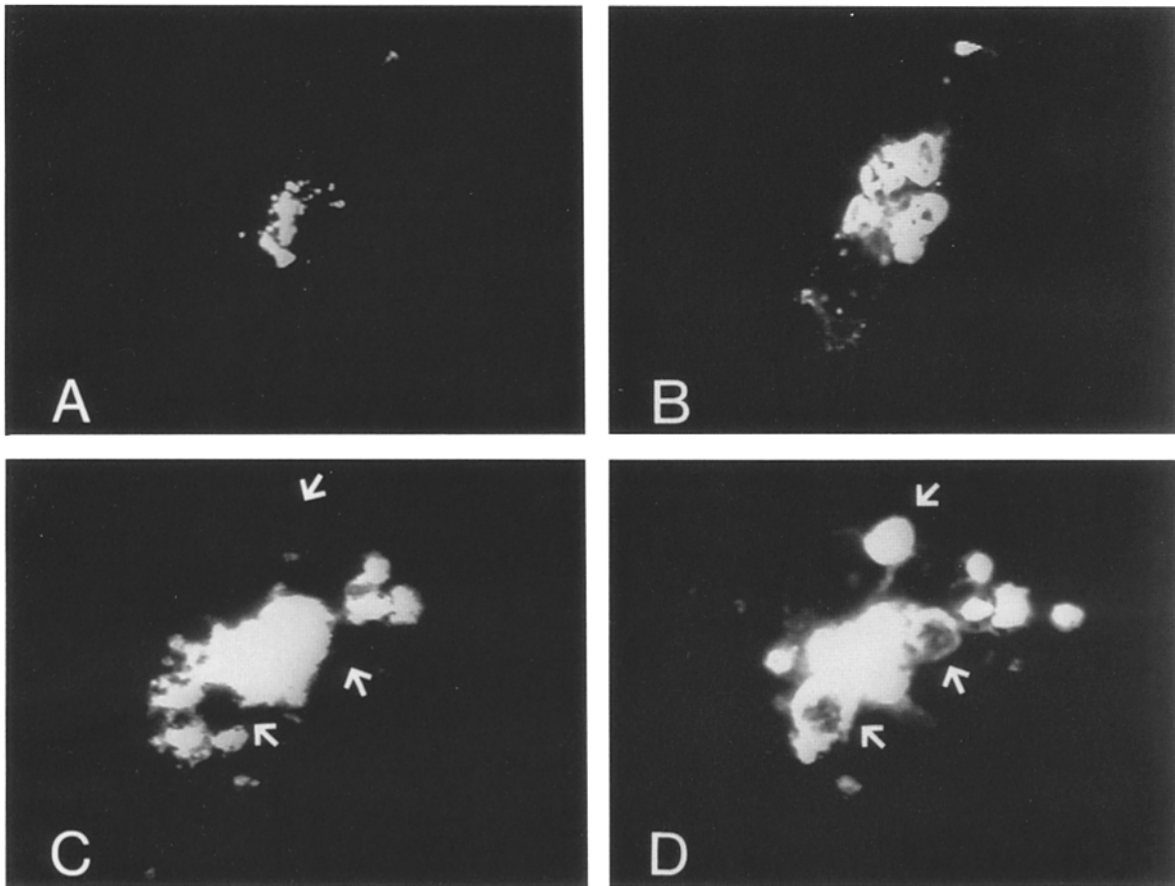


Figure 6. Double-staining of Ii and prelysosomal/lysosomal markers in COS 7.2 cells expressing hIip31. Transfected cells preloaded with TRIC-OVA (pulse 3 h, chase 20 h) (A) were fixed, permeabilized, and stained with anti-Ii mAb VIC-Y1. FITC-labeled anti-mouse IgG was used in the second step to reveal Ii staining (B). Endogenous lamp-1, a marker of prelysosomes and lysosomes, was detected with the mAb BB6 and TRITC-labeled anti-mouse IgG for the second step (C). After blocking with mouse IgG, Ii was detected with directly fluoresceinated VIC-Y1 (D). Ii macroosomes are BB6-negative. (Arrows) Positions of Ii-positive, lamp-1-negative macroosomes (C and D).

class II in the TGN (8). The accumulation in macroosomes of endocytic tracers such as FITC-OVA occurred readily at 20°C (data not shown), again consistent with this compartment representing altered early endosomes (47).

To identify mature lysosomes, cells were incubated in labeled dextran or OVA for several hours, then washed, and the internalized label chased for 20–24 h to permit complete transport to and accumulation in terminal lysosomes. Fig. 6, A and B show that Ii does not colocalize with the lysosomes marked by this method. Similar results were obtained for cells coexpressing MHC class II and Ii (data not shown). High lamp-1 content is characteristic of both late endosomes/prelysosomes, and lysosomes (35, 48). Consistent with this, COS cells labeled with dextran as just described, then stained for endogenous lamp-1 show a significant overlap in location of the two markers, although a cohort of lamp-1-positive, tracer-negative vesicles is always observed (data not shown). Lamp-1 was not found in Ii macroosomes (Fig. 6, C and D, arrows), consistent with their characterization as modified early endosomes. There was clear colocalization in

smaller vesicles of lamp-1 with Ii detected using a cytoplasmic tail specific reagent (VIC-Y1), which will be discussed below.

Ii-Macroosomes Show a Markedly Decreased Rate of Transport of Luminal and Membrane-associated Material to Later Endocytic Compartments. The morphologic modification of early endosomes by Ii prompted us to examine whether the transit of molecules through this compartment was also altered by the presence of Ii. COS cells either possessing or lacking Ii macroosomes were examined for the transfer of OVA through this early compartment to later stages in the endocytic pathway. Transfected COS cells were first labeled with TRITC-OVA by incubation for 5 h, followed by a chase for 24 h, resulting in accumulation of the label in lamp-1⁺ lysosomes. When COS cells expressing MHC class II only were incubated in FITC-OVA for 1 h, washed, and reincubated for 1, 6, or 24 h at 37°C, the FITC-OVA began to show colocalization with the TRITC-OVA-marked lysosomes as early as 1 h of chase, with further increases over time (Fig. 7, A–C). In contrast, COS cells also expressing hIip31, although demonstrating a large accumulation of FITC-OVA in macroosomes, did not

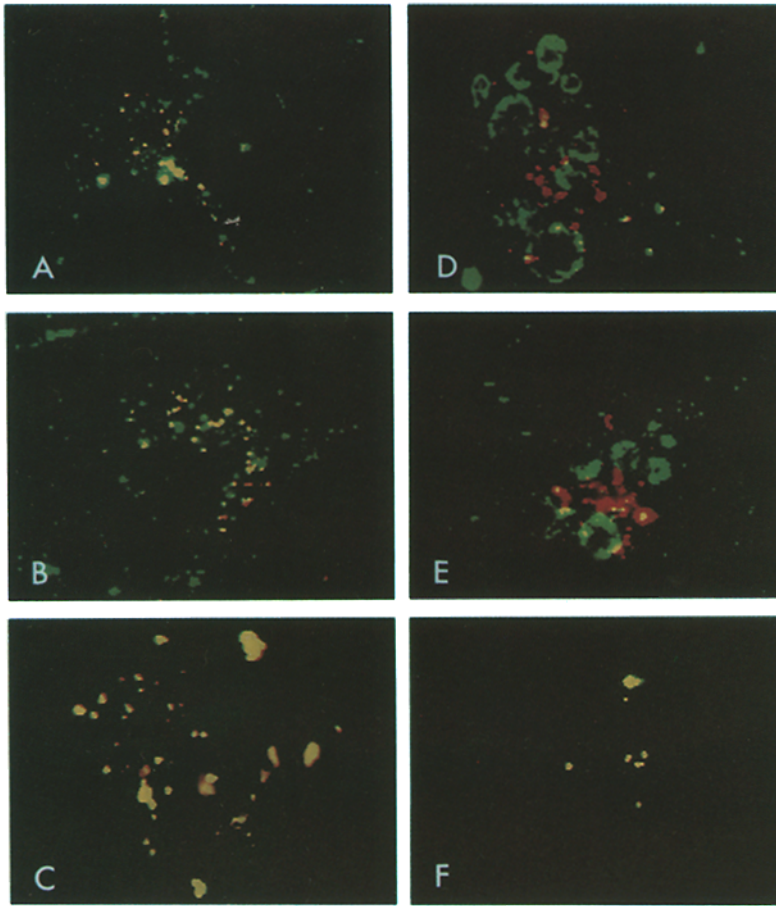


Figure 7. Rate of transport of OVA to lysosomes in COS 7.2 cells expressing either $A\alpha^bA\beta^b$ (A–C) or hIip31 (D–F). COS 7.2 cells were preloaded with TRITC-OVA in conditions allowing lysosome labeling. Cells were then exposed to FITC-OVA at 1 mg/ml for 1 h and reincubated in normal medium at 37°C for 1 h (A and D), 6 h (B and E), and 24 h (C and F). Only transfected cells showing macroosomes by phase contrast were included in this analysis.

show significant colocalization of this marker with TRITC-OVA at the 1- or 6-h time points. Transfer to lysosomes was observed after an overnight chase (Fig. 7, D–F). At this late time, FITC-OVA was also depleted from the macroosomes. This is in contrast to transferrin's rapid entry into and exit from macroosomes, indicating that the Ii-induced altera-

tion in flow selectively involves antegrade, not retrograde, movement.

A modification of transport through early endosomes was also observed if cells were cotransfected with Ii and lamp-1. In cells expressing high levels of lamp-1 molecules, a fraction reaches the plasma membrane and then undergoes rapid en-

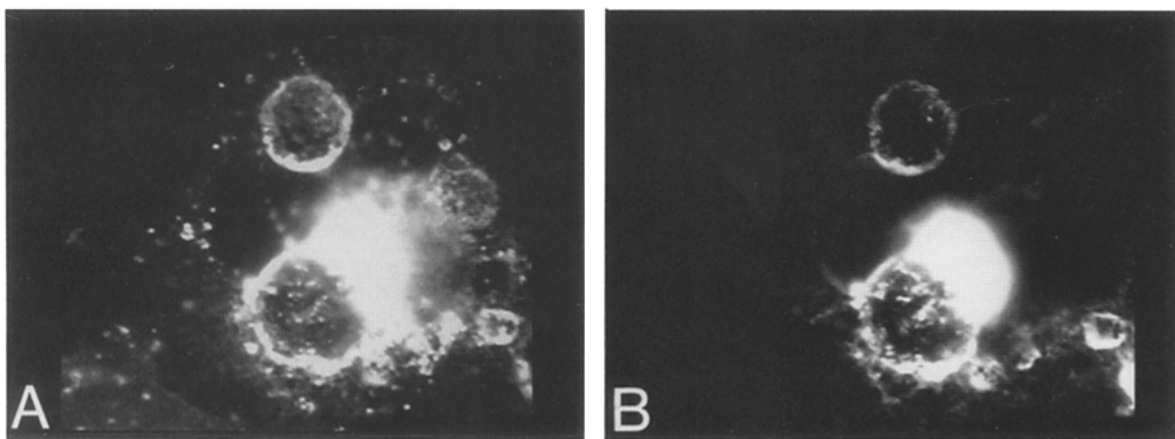


Figure 8. Colocalization of human lamp-1 and hIip31 in Ii-macroosomes. COS 7.2 cells expressing human lamp-1 and hIip31 were fixed and permeabilized. Human lamp-1 was specifically detected with the mAb H5G11. TRITC-labeled anti-mouse IgG were used in the second step (A). After blocking free binding sites with mouse IgG, Ii was detected with directly fluoresceinated VIC-Y1 (B).

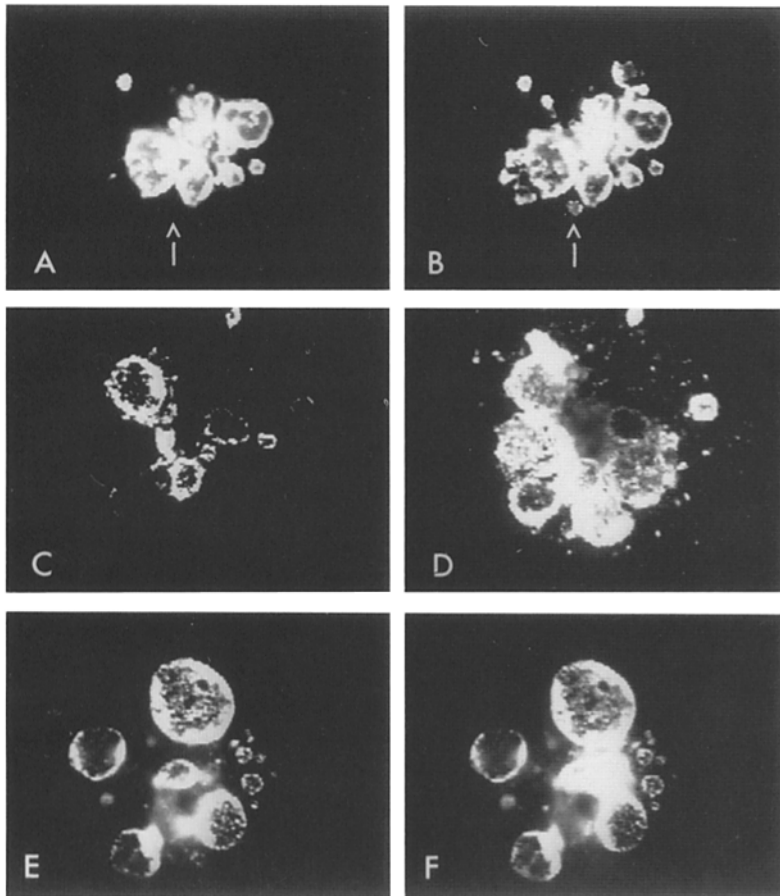


Figure 9. Different forms of Ii are present in distinct vesicular structures. mIip41-expressing cells were double-stained with the mAbs P4H5 (A) and In-1 (B). (Arrow) In-1⁺/P4H5⁻ vesicle. In cells coexpressing A α^b A β^b and hIip31 and grown with (E and F) or without (C and D) leupeptin, Ii was detected with the mAb LN2 (C and E) and A α^b A β^b with the mAb M5/114 (D and F).

docytosis and movement through the endocytic pathway to lysosomes (35, 48). In contrast to the results described above (Fig. 2 A) for cells transfected with human lamp-1 only, coexpression of hIip31 with human lamp-1 (Fig. 8 A) resulted in lamp-1 accumulation in the Ii-containing macromosomal structures (Fig. 8 B). This indicates that egress of membrane-associated proteins from this site to late endosomes/lysosomes is also retarded.

Distinct Populations of Endocytic Vesicles Contain Epitopically Intact and COOH Terminally Degraded Ii. The activity of MHC class II molecules in antigen presentation requires the binding of processed antigenic peptides, an event that is dependent on the removal of (intact) Ii from the MHC class II molecule (24). Ii is known from metabolic labeling studies to undergo progressive proteolytic cleavage after transport from the late Golgi (49, 50), and intracellular peptide binding to MHC class II is only seen after such cleavage has occurred (11). Recent data suggest that processing of antigen in the class II pathway may occur most efficiently in late endosomes/lysosomes (51), and the bulk of MHC class II in EBV-transformed cells is found in vesicles with the characteristics of late endosomes/prelysosomes (9). We therefore carefully examined the transfected COS cells for the presence of MHC class II and Ii in vesicular structures other than modified early endosomes. In some cells coexpressing Ii and MHC class II,

we noted not only macromosomes staining for both proteins, but also vesicles positive for MHC class II but failing to stain with LN2, a mAb directed against the luminal part of Ii. Because the detection of MHC class II molecules in vesicular structures in COS cells is dependent on Ii coexpression, and it is known that Ii is proteolytically processed from the COOH terminus (luminal segment) when complexed with MHC class II molecules (50), it thus seemed likely that the Ii in these class II-positive vesicles had been posttranslationally altered or degraded.

To examine this issue, we took advantage of two sets of monoclonal anti-Ii Abs: (a) P4H5, which reacts with the luminal region of mIi (52), and In-1, which reacts with the cytoplasmic region of mIi (21); and (b) LN2, which reacts with the COOH-terminal, luminal region of hIi (39), and VIC-Y1, which reacts with the NH₂-terminal, cytoplasmic region of hIi (38). After transfection of either mIip31 (data not shown) or mIip41, vesicles failing to stain with the lumen-specific antibody (Fig. 9 A) but reactive with the anti-cytoplasmic tail reagent (Fig. 9 B) were observed, though not the converse. Likewise, in cells expressing hIip31, NH₂-terminal epitope-positive but COOH-terminal epitope-negative vesicles were found (data not shown). In transfectants coexpressing hIip31 and A α^b A β^b , class II and Ii colocalize in all vesicles when examined using the antibody to the

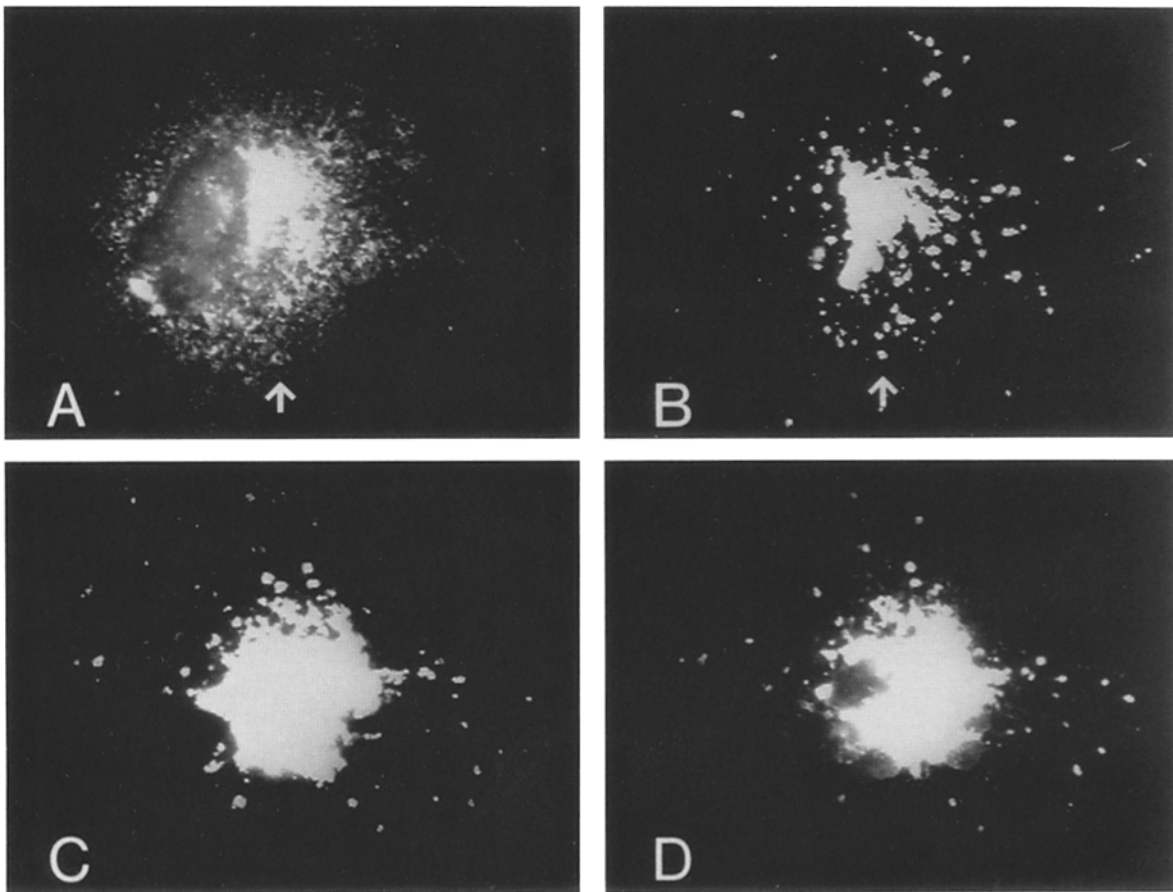


Figure 10. Characterization of hIip31 Δ 11⁺ vesicles. (A and B) COS 7.2 cells expressing hIip31 Δ 11 were pulsed for 30 min at 37°C with iron-saturated human transferrin (20 μ g/ml). Cells were then fixed, permeabilized, and stained with FITC-labeled sheep antibodies to human transferrin (A) and the mAb VIC-Y1 (B). TRITC-labeled anti-mouse IgG Abs were used in the second step. (Arrow) Single transferrin and Ii-containing vesicle. (C and D) hIip31 Δ 11-expressing cells were double-stained with the mAb BB6 (C) and the mAb VIC-Y1 (D).

NH₂-terminal cytoplasmic tail (Fig. 9, C and D). However, a cohort of vesicles lacking staining with the anti-Ii lumen-specific antibody (Fig. 9 C), but containing MHC class II (Fig. 9 D) was found. As seen in cells coexpressing A α^b A β^b and hIip31, cells with A α^b A β^b and either mIip31 or mIip41 showed that all class II-containing vesicles had Ii as assessed with an antibody to the cytoplasmic tail, but a subset of vesicles with class II lacked reactivity with the anti-Ii luminal epitope antibody (data not shown).

Cells transfected with hIip31 plus A α^b A β^b and grown in the presence of leupeptin, an inhibitor of one of the enzymes (cathepsin B) believed responsible for the intracellular proteolysis of Ii (50), showed a complete overlap of staining with anti-class II and the anti-Ii antibody to the luminal epitope (Fig. 9, E and F). This confirms that the difference between the two sets of vesicles described above is the extent of proteolytic cleavage of the Ii in its COOH-terminal (luminal) region.

Characterization of Vesicles Containing Ii with N- or C-Region Truncations. The vesicles containing LN2 (luminal epitope)-negative Ii were frequently smaller than those containing Ii that stained with LN2. This, plus the discrete nature of the

difference in staining corresponding to individual vesicles, implied that the structures containing cleaved Ii were a part of the endocytic pathway distinct from the modified early endosomes. Antibody to the hIi cytoplasmic tail stained a subpopulation of smaller vesicles that also could be stained by anti-lamp-1 (Fig. 6, C and D). Furthermore, this reagent identified a cohort of vesicles that was not readily accessible to transferrin, and that was not accessible to OVA at 20°C (data not shown). Because no localization of Ii or class II was seen in terminal lysosomes (see above), these data identify the vesicles containing cleaved Ii as late endosomes/prelysosomes, the same compartment shown to be the site of the highest steady state level of MHC class II in transformed B cells (9). When leupeptin was added to prevent destruction of the COOH-terminal part of Ii, some of the vesicles containing Ii reactive with antibody to the luminal segment were not readily accessible to transferrin (data not shown). This suggests that the COOH-terminal cleavage accompanies, but does not control, movement from early to late endocytic compartments.

Another form of Ii was also observed in this late compartment. hIip31 with a deletion of the residues 2–11 of the cy-

toplasmic tail (hIip31 Δ 11) failed to generate Ii macrosomes and was found in smaller vesicles. These vesicles were largely inaccessible to transferrin, costained with lamp-1 (Fig. 10), and were not accessible to fluid phase markers at 20°C (data not shown). Thus, they have the same properties as the vesicles with COOH terminally cleaved Ii found after expression of the full-length Ii (Iip31). The bulk of coexpressed MHC class II colocalizes in these late endosomes/prelysosomes (data not shown), replicating the distribution seen in transformed B cells that are efficient at antigen processing and presentation via class II molecules.

Discussion

The observations that Ii possesses a cytoplasmic signal responsible for transport to endocytic structures and that MHC class II molecules localize in endocytic compartments when coexpressed with Ii containing this cytoplasmic signal has led to a model in which the movement of MHC class II to the endocytic pathway for peptide capture is mediated by association with Ii (10, 29, 30). The present report confirms and extends these initial observations, demonstrating that such Ii-dependent deviation of MHC class II from the default exocytic pathway is observed in other cell types, with other MHC class II isotypes, and for various forms of Ii. Endocytic localization is observed for Ii alone or in association with complete α/β dimers, indicating that MHC class II is not essential to the formation of the targeting signal, although differences in the localization pattern seen in the presence and absence of MHC class II that suggest its presence can modify the localization properties of Ii (10, 30). These experiments also provide the first direct demonstration that the p41 form of Ii can independently enter the endocytic pathway and that it can direct class II to this site. Whether subtle differences exist in the rate of movement, site of accumulation, or rate of dissociation of Iip31-class II vs. Iip41-class II complexes remains to be determined. Such differences might explain the distinct effects of these two forms of Ii in presentation of certain antigens (53).

Previous studies on the intracellular localization of Ii have not examined the state of this molecule in the various compartments in which it can be detected. This is a critical issue, as it is clear that proteolytic modification of Ii is essential to the proper function of MHC class II molecules in antigen presentation. We demonstrate here that Ii present in early endocytic vesicles is largely intact. Most Ii molecules in late endosomes/prelysosomes have undergone luminal cleavage by the time they have accumulated in this compartment. Studies with the protease inhibitor leupeptin suggest that this cleavage may be a consequence of proteolytic activity in this compartment, rather than a necessary prelude to movement to this site. The MHC class II molecules in late endosomes/prelysosomes are in any case clearly associated with a form of Ii that has undergone initial cleavage in the region most likely to play a major role in regulating peptide binding, thus providing them with the opportunity to initiate peptide acquisition.

Under conditions of high Ii expression, class II and intact

Ii colocalized in a transferrin-accessible compartment with additional characteristics of early endosomes, implying that this was the site of entry into the endosomal pathway, as suggested previously by Cresswell (54). It is unlikely that accumulation of class II and Ii in early endosomes was the result of "spillover" into this compartment after saturation of a transport system that normally results in direct movement from the *trans*-Golgi to late endosomes/prelysosomes (9, 30). This latter model would predict that all transfectants would show class II localization in lamp-1-positive, transferrin-inaccessible vesicles as a consequence of "correct" transport before overloading of this putative transport pathway. Yet in cells with large numbers of macrosomes, such late endocytic localization was rarely seen, arguing against an "overflow" explanation of our observations.

The accumulation of human lamp-1 in early endosomes when coexpressed with Ii, and the failure to visualize this molecule in such vesicles in the absence of Ii, is best explained by a rapid flux through and low steady state concentration of human lamp-1 in early endosomes when expressed alone. The detection of class II and Ii in late but not early endosomes in the presence of moderate levels of Ii is thus also likely to result from the rapid passage of MHC class II-Ii complexes through the early endocytic compartment and the slower egress of these molecules from late endosomes/prelysosomes under these conditions. These findings emphasize the distinction between the compartments a molecule may transiently access during intracellular movement, and the sites of high steady state accumulation readily seen by immunofluorescence or electron microscopy.

A striking and previously unrecognized property of Ii documented here is its ability to actively alter both the structure and transport properties of the early endocytic compartment. Macrocytic vesicles were formed in COS cells expressing Ii with an intact cytoplasmic tail in the absence or, in certain cases, the presence of MHC class II. Transferrin entry into and exit from macrosomes occurred rapidly, indicating that vesicular movement from the cell membrane to early endosomes and from these endosomes back to the membrane was largely unaffected by the introduction of Ii. However, antegrade movement to later endocytic compartments was markedly slowed as assessed fluid phase marker studies, and this diminished rate of transport also seemed to affect membrane proteins moving through this compartment, for example, lamp-1 overproduced after gene transfer.

Is this effect of Ii on endocytic structure and function an artifact of the high protein expression level of the COS cell model, or does it reflect an important biological property of Ii? High level expression in COS cells of various proteins destined for default exocytic movement fails to cause such alterations. Furthermore, overexpression of other molecules normally accumulating in late endocytic compartments but trafficking through early endosomes (e.g., lamp-1) also fails to induce these modifications, providing evidence that non-specific protein overloading of endosomes is not the cause of this effect. An Ii mutant lacking NH₂-terminal residues 2-11 also does not induce macrosomes, despite being trans-

ported to the endocytic pathway. Because this cytoplasmically truncated form of Ii is synthesized at the same rate as intact Ii, these data indicate that saturation of a proteolytic process required for Ii cleavage that protects the early endocytic compartment from disruption cannot explain the present observations. Furthermore, the observation that removal of the highly conserved residues 2–11 from the NH₂ terminus of Ii prevents macroosome formation and early endosomal retention is consistent with the capacity to alter endosomal function being an important property of Ii. This region is not essential to endosomal targeting, as the Δ 2–11 form of Ii still deviates from the default pathway to accumulate in late endosomes. These residues of the NH₂ terminus of Ii are well conserved in evolution (29). Such conservation in the absence of its requirement for endosomal targeting suggests that this sequence has an additional role, which may be to regulate transport of MHC class II and other proteins from early to late endosomes.

The extent of Ii expression does dictate the size and number of the altered early endosomes and coexpression of MHC class II reduces the extent of enlargement and/or the number of such structures. Typical MHC class II-positive hematopoietic cells produce Ii in molar excess over MHC class II (23, 55) and free Ii is present in such cells. However, in both human cells expressing Ii but lacking class II (23, 30) or class II-negative mouse cells transfected with an Ii expression construct (56), only a small fraction of Ii (perhaps 5%) leaves the ER. In CV-1 cells transfected with the hIip31 construct used in the present experiment, a larger fraction of the expressed Ii (15%) develops N-linked glycans resistant to endoglycosidase H digestion (29), indicating passage through the medial Golgi. These results suggest that dramatic alterations in early endosome structure and function would be seen only in cells with very high Ii synthetic rates in excess of class II production, consistent with the failure to observe macroosome-like structures in most class II-expressing cells. Freshly isolated Langerhans cells have an extraordinarily high rate of Ii and MHC

class II biosynthesis (57) and significant surface Ii expression (58). Associated with this high rate synthesis is the presence of large lucent acidic vesicles with the characteristics of early endosomes (59). Although no direct evidence establishes that the extremely high rate of Ii synthesis is responsible for these structures in Langerhans cells, the COS data suggest this possibility, which is consistent with the loss of these structures upon culture as Ii levels decline. It is possible that the levels of Ii seen in other class II-expressing cells such as B lymphocytes may result in more subtle changes in endosomal function. In either case, these changes might enhance class II presentation by preventing excessive degradation of some antigens or by increasing the opportunity for endosomal vesicle fusion, assuring more access of newly synthesized class II to endocytosed antigen.

The capacity of Ii to alter early endosomal structure and activity raises the question as to the molecular mechanism by which this occurs. Two distinct possibilities can be considered. Ii is known to undergo multimerization and when free of MHC class II, higher order aggregation (23). Thus, a physical change in membrane structure due to the creation of large sheets of Ii could interfere with membrane trafficking, leading to the gross enlargement that characterizes macroosomes. Alternatively, Ii might contain specific structural features that interact with the machinery responsible for intercompartmental vesicular traffic, changing the formation or movement of vesicles, or the fusion competence of vesicular structures. The recent reports that both levels of expression of and ligand interaction with ERD-2 (60) or ELP-1 (61) control of the rate of retrograde traffic between the intermediate compartment (*cis*-Golgi network) and ER provides an intriguing model for active regulation of intercompartmental transit. Thus, further study of Ii might provide new insights into the biochemical regulation of intracellular transport in general, and the ways in which differentiated cells modify the basic transport machinery for specialized functions.

This paper is dedicated to the memory of Corine Layet, who passed away during the course of this study. The authors wish to especially thank Jack Bennink for his help with the photomicrography and digital image processing; M. Fukuda, E. Long, S. Kornfeld, W. Knapp, S. Cullen, N. Koch, J. August, E. Ungewickell, and F. Brodsky for generously providing antibodies and/or plasmids necessary for completion of this study; and Eric Long, Jennifer Lippincott-Schwartz, and Peter Peters for critical reading of the manuscript.

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Received for publication 21 August 1992 and in revised form 30 October 1992.

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