Intracellular Transport and Localization of Major Histocompatibility Complex Class II Molecules and Associated Invariant Chain

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Abstract. The intracellular transport and location of major histocompatibility complex (MHC) class II molecules and associated invariant chain (Ii) were investigated in a human melanoma cell line. In contrast to the class II molecules, which remain stable for >4 h after synthesis, the associated Ii is proteolytically processed within 2 h. During or shortly after synthesis the NH_2-terminal cytoplasmic and membrane-spanning segment is in some of the Ii molecules cleaved off; during intracellular transport, class II associated and membrane integrated Ii is processed from its COOH terminus in distinct steps in endocytic compartments.

Immunocytochemical studies at the light and electron microscopic level revealed the presence of class II molecules, but not of Ii on the cell surface. Intracellularly both Ii and class II molecules were localized in three morphologically and kinetically distinct compartments, early endosomes, multivesicular bodies, and prelysosomes. This localization in several distinct endosomal compartments contrasts with the localization of class II molecules in mainly one endocytic compartment in B lymphoblastoid cell lines. As in these lymphoblastoid cell lines Ii is known to be rapidly degraded it is conceivable that the rate of proteolysis of the class II associated Ii and its dissociation from class II molecules modulates the retention of the oligomeric complex in endocytic compartments, and as a consequence the steady-state distribution of these molecules within the endosomal system.

MAJOR histocompatibility complex (MHC)[1] class II molecules consist of two nonidentical glycoproteins, the α-chain and β-chain (for review, see Cresswell et al., 1987). They function in antigen presentation at the surface of a number of cells, including macrophages, B lymphocytes, and some tumor cells (Unanue, 1984). Intracellularly, they are associated with the invariant chain (Ii),[1] and this association occurs directly after insertion into the endoplasmic reticulum (Jones et al., 1978; Kvist et al., 1982). Ii is a transmembrane protein, exposing 30 NH_2-terminal amino acids on the cytoplasmic side and ~160 amino acids on the lumenal side of the membrane (Claesson et al., 1983).

The oligomeric complex of class II molecules and Ii is thought to be transported to an endocytic compartment where Ii dissociates from the complex and class II molecules are then transported further to the plasma membrane (Koch et al., 1989; Long, 1989; Neefjes et al., 1990). Ii remains largely intracellularly and is eventually degraded (Owen et al., 1981). Degradation of Ii can partially be inhibited by the addition of the lysosomotropic agent chloroquine or the protease inhibitor leupeptin (Nowell and Quaranta, 1985; Blum and Cresswell, 1988; Nguyen et al., 1989).

The class II associated Ii has been implicated in the regulation of peptide association to class II molecules (Koch et al., 1989; Long et al., 1989). Two distinct functions of Ii in this process have been proposed. First, Ii could prevent MHC class II molecules from binding peptide prematurely in the endoplasmic reticulum or Golgi complex (Elliott et al., 1987; Roche and Cresswell, 1990). This was suggested from the finding that peptide binding to class II molecules was reduced in the presence of Ii or its lumenal segment (Roche and Cresswell, 1990; Teyton et al., 1990). Second, the association of Ii with class II molecules may regulate the localization of these molecules in endocytic compartments. This was supported by the finding that the cytoplasmic tail of Ii contains a sorting signal for endosomes (Bakke and Dobberstein, 1990; Lotteau et al., 1990).

It has recently been shown, that in the B lymphoblastoid cell line JY class II molecules were localized to a distinct late endosomal, lysosome related compartment (Peters et al., 1991). Ii could only be found in the endoplasmic reticulum, Golgi, and *trans*-Golgi network. The failure to detect Ii in endocytic compartment may reflect the rapid degradation of Ii in B lymphoblastoid cell lines (Blum and Cresswell, 1988; Nguyen et al., 1989).

MHC class II molecules expressed in transfected murine L cells were also shown to be located in a late endocytic com-

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1. *Abbreviations used in this paper*: Ii, invariant chain; MHC, major histocompatibility complex; MPR, mannose-6-phosphate receptor; MVB, multivesicular bodies.

partment (Salamero et al., 1990). As this location was independent on the expression of Ii, it was suggested that MHC class II molecules themselves may contain targeting information for endosomal compartments (Salamero et al., 1990).

We show here that in a human melanoma cell line, in contrast to these previous studies, class II molecules and Ii are localized in three distinct endocytic compartments: early endosomes, multivesicular bodies and prelysosomes. In contrast to B lymphoblastoid cells, in which Ii is degraded rapidly (Blum and Cresswell, 1988; Nguyen et al., 1989), the class II-associated Ii in these melanoma cells is proteolytically processed at a slow rate. This proteolytic cleavage gives rise to several polypeptides which remain associated with class II molecules for >4 h after synthesis and all retain the cytoplasmic tail shown previously to contain endosomal sorting information (Bakke and Dobberstein, 1990; Lotteau et al., 1990). Our results suggest that the localization of MHC class II molecules in the endosomal system can depend on the cell type and possibly on the rate of processing of the class II associated Ii and its dissociation from MHC class II molecules.

Materials and Methods

Materials

Materials were obtained from the following sources: [^{35}S]methionine (sp act 1,300 Ci/mM) and ^{14}C-methylated protein standards were obtained from Amersham International, Amersham, England; protein A-Sepharose was from Pharmacia LKB, Uppsala, Sweden; leupeptin, chymostatin, pepstatin A, aprotinin, and PMSF were from Sigma Chemical Co., St. Louis, MO; endoglycosidase H was from Seikagaku Kogyo Co., LTD, Tokyo, Japan; Neuraminidase (type V) was from Sigma Chemical Co.

Cells and Cell Culture

The human melanoma cell line Mel JuSo was a gift from Dr. Johnson (Institut fur Immunologie Munich, Germany), and described before (Johnson et al., 1981). The cells were cultured in RPMI 1640 medium supplemented with 5% fetal calf serum (RPMI-FCS).

Antibodies

Hybridoma cells L-243 secreting anti-HLA-DR antibodies (Lampson and Levy, 1980) were obtained from the American Type Culture Collection (Rockville, MD). Ascites fluid was obtained by culturing the cells intraperitonally in BALB/c mice. Antiserum 311, a polyclonal antiserum against the α-chain of class II molecules (Sege et al., 1981) was a kind gift of Dr. P. A. Peterson. The polyclonal antisera against fusion proteins of β-galactosidase and parts of Ii expressed in NF1 bacteria were described before (Lipp and Dobberstein, 1986; Wraight et al., 1990). Antisera recognizing an Ii NH_2-terminal portion were raised against a fusion protein containing the NH_2-terminal 73 amino acids (Ii_{1-73}) of Ii and β-galactosidase (anti-IiN); antisera recognizing an Ii COOH-terminal portion were raised against a fusion protein containing amino acids 73-216 of Ii (Ii_{73-216}) and β-galactosidase (anti-IiC); VIC Y1 (Quaranta et al., 1984) is a mouse monoclonal antibody that recognizes an epitope within the NH_2-terminal 30, cytosolic, amino acids of Ii (Wraight et al., 1990), and was a kind gift from Dr. W. Knapp. Clonab LN2 (Biotest AG, Dreieich, Germany) is a mouse monoclonal antibody recognizing an epitope at the outer COOH-terminal portion of Ii (within amino acids 157-216 of Ii; Wraight et al., 1990). AB4, a mouse monoclonal anti-HLA-DR antibody (Kvalheim et al., 1988) was a kind gift from Dr. Funderud. Rabbit polyclonal antiserum against the (cation-independent) mannose-6-phosphate receptor (MPR) (Griffiths et al., 1988) was a gift from Dr. Hoflack. Rabbit polyclonal antiserum against the Golgi enzyme galactosyl transferase (anti-Gal Tf) (Berger et al., 1987) was a gift from Dr. E. Berger.

Metabolic Labeling

Cells were grown on tissue culture dishes, and before labeling the medium was replaced by methionine-free medium. After 1 h this medium was replaced by methionine-free medium containing 0.075 mCi/ml [^{35}S]methionine. After 20 min, the radioactive medium was removed, the cells were washed twice in RPMI-FCS containing 2 mM methionine and incubated at 37°C in the same medium.

At the times indicated, the dishes were placed on ice, washed three times with ice-cold PBS and the cells were lysed in 20 mM Hepes pH 7.5 containing 100 mM NaCl, 5 mM $MgCl_2$, 1% Triton X-100, and 20 μM PMSF. After 10-min incubation on ice, the cell lysates were collected and centrifuged at 13,000 g for 15 min to remove cell debris.

Immunoprecipitation and Electrophoresis

Cell lysates from 1×10^6 cells were incubated with antibody at 4°C for 12 h, followed by the addition of 40 μl protein A-Sepharose (1:1 slurry) and further incubation for 2 h. The beads were washed twice with 1 ml of low salt buffer (10 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.2% NP-40, and 2 mM EDTA), twice with 1 ml of high salt buffer (10 mM Tris-HCl pH 7.5, 500 mM NaCl 0.2% NP-40 and 2 mM EDTA), and twice with 1 ml of 10 mM Tris-HCl pH 7.5. For sequential immunoprecipitation of antigens, antibody L-243 was used for the immunoprecipitation of the class II molecules and associated proteins. This complex was then denatured by boiling the protein A-Sepharose beads in 0.5% SDS and 1 mM β-mercaptoethanol for 7 min. The sample was diluted 10-fold, and the second immunoprecipitation was carried out as described above. The antigens were eluted from the protein A-Sepharose beads by the addition of sample buffer, denatured, and subjected to SDS-PAGE (10–15%) according to Laemmli (1970), fluorography, and autoradiography.

Immunofluorescence Microscopy

Immunofluorescence on live cells was essentially performed as described by Bakke and Dobberstein (1990). Briefly, viable cells grown on coverslips were incubated on ice with the appropriate antibody to label antigens on the plasma membrane. Then, the cells were fixed using 3% paraformaldehyde, washed, and the cell surface molecules were visualized using FITC or Texas Red-conjugated second antibody. To also label intracellular molecules, cells were fixed with methanol for 4 min at −20°C, washed, and labeled using antibodies and fluorescein or Texas Red-conjugated second antibody as described in the figure legends. After labeling, coverslips were mounted in Mowiol, and examined using a Leitz Orthoplan fluorescence photo-microscope equipped with a 63× objective and filters for fluorescein or Texas Red.

Electron Microscopy

For the localization of class II molecules and Ii at the EM level, cells were prepared for cryosectioning, and immunolabeled with antibodies against class II molecules followed by 9-nm gold-conjugated protein A (Geuze et al., 1981).

To identify the various compartments of the endocytic pathway, cells were allowed to internalize different markers, essentially as described by Parton et al. (1989).

For the identification of early endosomes, HRP (10 mg/ml) was allowed to be internalized for 5 or 10 min followed by chase periods up to 30 min to label endosome transport vesicles and the prelysosomes. The HRP was subsequently visualized using anti-HRP antibodies on cryosections, followed by 5 nm protein A-gold complex.

For the identification of lysosomes, a 16-nm BSA-gold complex was internalized for 4 h at 37°C followed by an overnight chase in medium free of BSA-gold. Under this condition the marker distributes between the mannose-6-phosphate receptor (MPR)-enriched prelysosomal compartment (PLC), and the MPR-negative lysosomes, (Griffiths et al., 1988, 1990). These cells were prepared for cryosectioning and immunolabeled with antibodies followed by 5 and 9 nm protein A-gold (Griffiths et al., 1984), as specified in the figure legends.

For quantitation, cells labeled for endocytic compartments were prepared for cryosectioning, and immunolabeled for Ii using anti-IiN followed by 9 nm protein A-gold. Quantitation of the amount of 9 nm gold particles on these cryosections was essentially carried out as described by Griffiths and Hoppeler (1986).

Results

Cleavage of Ii during Intracellular Transport

Ii has previously been shown to dissociate from class II mol-

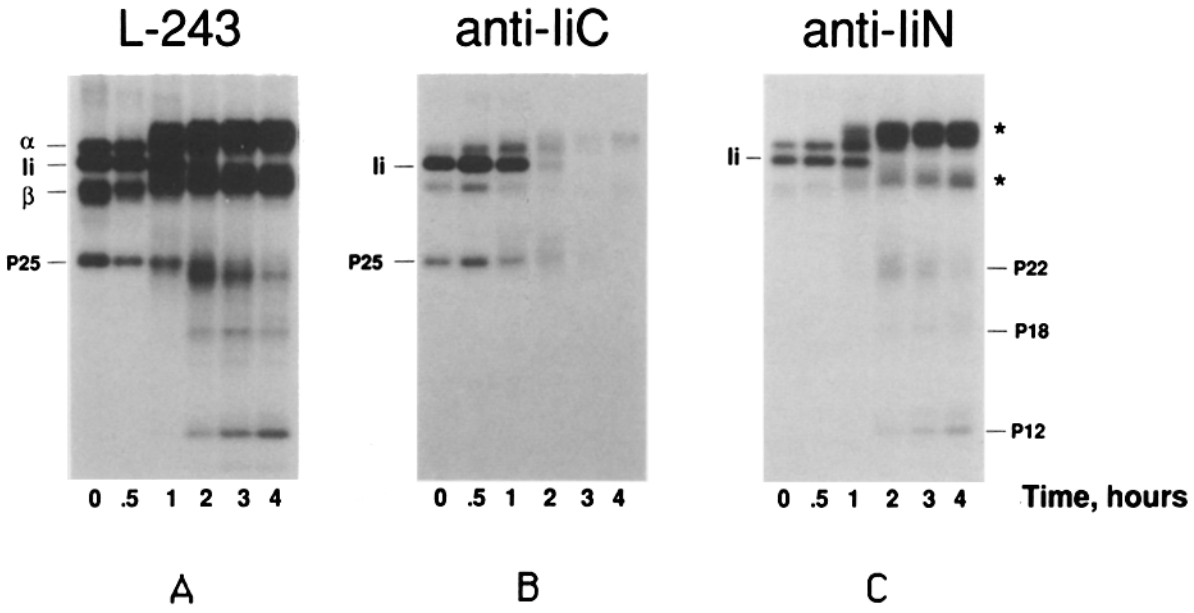


Figure 1. Ii associated with class II molecules is proteolytically processed during intracellular transport. Mel JuSo cells were incubated with [^{35}S]methionine for 20 min, then washed and chased for the times indicated. Proteins were immunoprecipitated with anti-class II antibody (*L-243*; *A*). The immunoadsorbed protein complexes were denatured and reprecipitated with anti-IiC antiserum (*B*), or anti-IiN antiserum (*C*). In *C*, P22, P18, and P12 represent 22-, 18-, and 12-kD Ii-related proteins, respectively. In *C*, an asterisk indicates forms of Ii which probably have acquired complex type carbohydrates. Shown are autoradiographs after SDS-PAGE and fluorography.

ecules during intracellular transport (Kvist et al., 1982; Machamer and Cresswell, 1982). To determine the fate of the class II-associated Ii, pulse-chase studies were performed. Mel JuSo cells were labeled for 20 min and chased for the times indicated in Fig. 1. Proteins were immunoprecipitated with anti-class II antibody (L-243). SDS-PAGE analysis and autoradiography reveal that after 1-h chase, class II α and β chains acquired complex type carbohydrates, as judged by their reduced mobility in SDS-PAGE (Fig. 1 *A*).

Several polypeptides are associated with the class II molecules throughout the chase. To determine whether these proteins are immunologically related to Ii, we denatured the complex by boiling in SDS and β-mercaptoethanol. This procedure dissociates the complex into separate polypeptide

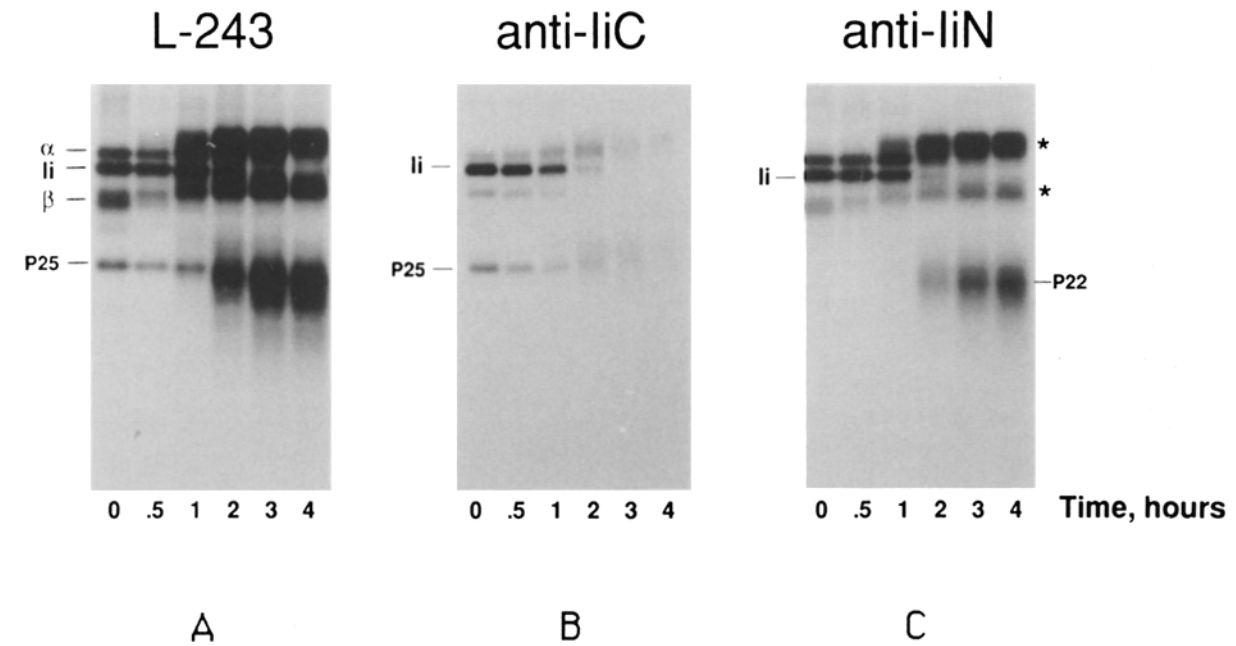


Figure 2. Proteolytic processing of class II-associated Ii to P18 and P12, but not to P22 is inhibited by leupeptin, Mel JuSo cells were incubated with 0.3 mM leupeptin 4 h before labeling. Cells were pulse-labeled and chased for the times indicated in the presence of 0.3 mM leupeptin. Proteins were immunoprecipitated using anti-class II antibody L-243 (*A*). The immunoadsorbed protein complexes were denatured and reprecipitated with anti-IiC antiserum (*B*), or anti-IiN antiserum (*C*). In *C*, asterisks indicate forms of Ii that have acquired complex type carbohydrates. Shown are autoradiographs after SDS-PAGE and fluorography.

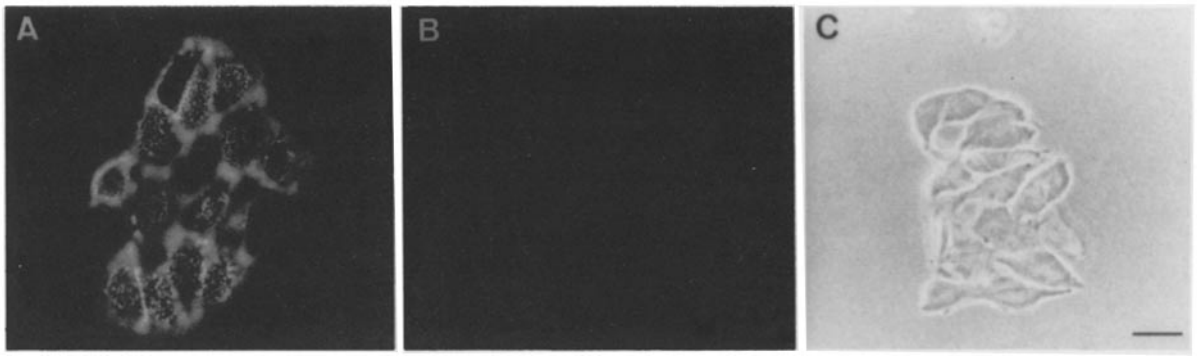


Figure 3. Plasma membrane immunolocalization of class II molecules and Ii in Mel JuSo cells. Cells grown on coverslips were labeled on ice for class II molecules using mAb AB4 (*A*) or for Ii using mAb LN2 (*B*), and a fluorescein-conjugated second antibody. Thereafter, the cells were fixed with paraformaldehyde. *C* shows a phase-contrast photograph of the cells labeled for Ii shown in *B*. Bar, 20 μm.

chains. A subsequent immunoprecipitation, using antisera recognizing either the lumenal Ii COOH-terminal portion (anti-IiC) or the cytoplasmic Ii NH_2-terminal part (anti-IiN), allows thus to identify Ii-related polypeptides associated with class II molecules.

P25 results from an early cleavage in the endoplasmic reticulum. It is not recognized by anti-IiN but by anti-IiC antiserum (Fig. 1). Therefore we conclude that P25 lacks the NH_2-terminal region of Ii, most likely including the membrane-spanning region. The amount of cell-associated P25 decreased during the chase and a portion of P25 can be detected in the culture medium after 2-h chase (Fig. 1 *B*; Pieters, J., and B. Dobberstein, unpublished data).

During the chase period, several small molecular mass proteins appeared after 2 h in the MHC class II immunocomplexes (Fig. 1 *A*). As Ii associated with class II molecules is degraded during intracellular transport (Blum and Cresswell, 1988; Nguyen and Humphreys, 1989), we suspected that these small molecular weight proteins might be derived from Ii.

Fig. 1, *B* and *C* shows that after denaturation of the class II immunocomplexes and reprecipitation with Ii specific antisera, the small molecular mass proteins are immunoprecipitated by the antiserum against the NH_2-terminal part of Ii (anti-IiN), but not by the antiserum against the COOH-terminal part (anti-IiC) (Fig. 1 *C*). These small molecular weight proteins have molecular masses of 22 (P22), 18 (P18) and 12 kD (P12). As all these molecules react with anti-IiN antiserum, which recognizes the extreme NH_2-terminal portion of Ii, these molecules must lack increasing portions from the COOH-terminal side. The amount of P22 decreases between 2 and 4 h of chase, whereas the amount of P12 increases during the same time. This suggests that P22 is processed sequentially to P18 and to P12.

Effect of Leupeptin on Ii Processing

The accumulation of P22, P18 and P12 after 2 h of chase and the presence of complex type carbohydrates suggest that cleavage of Ii occurs in a *trans*-Golgi or post-Golgi compartment. It is known that at least some endocytic compartments possess proteolytic activity (Kornfeld and Mellman, 1989; Diment et al., 1989). Furthermore, it is known that in B lymphoblastoid cells degradation of Ii can be inhibited by the addition of the protease inhibitor leupeptin (Blum and Cresswell, 1988; Nguyen et al., 1989). To test whether proteolytic processing of Ii in Mel JuSo cells occurs in an endocytic compartment, we added leupeptin to the culture medium. Cells were incubated for 4 h in the presence of 0.3 mM leupeptin, labeled for 20 min and chased for the times indicated in Fig. 2. Proteins were immunoprecipitated using anti-class II antibody (L-243), followed by denaturation and immunoprecipitation using anti-IiC and anti-IiN antisera. Fig. 2 shows that the incubation of the cells with leupeptin resulted in accumulation of complexes of class II molecules and P22 during the chase period. No processing to P18 and P12 is seen (Fig. 2, *A* and *C*). Leupeptin had, as expected, no effect on the appearance of P25 (Fig. 2 *B*).

Incubation of the cells in the presence of a cocktail of the protease inhibitors leupeptin, chymostatin, and pepstatin A did not prevent cleavage of Ii to P22 (data not shown).

Localization of Class II Molecules and Ii by Light Microscopy

The biochemical analysis of Ii chains assembled with class II molecules strongly suggested their transport to an endocytic, proteolytic compartment. To localize class II molecules and Ii in Mel JuSo cells, we used immunofluorescence microscopy.

Class II molecules are found at the plasma membrane in Mel JuSo cells, and this is shown in Fig. 3 *A*. In contrast, no Ii could be detected at the plasma membrane (Fig. *B* and *C*), using the monoclonal antibody LN2, recognizing an epitope located at the COOH-terminal portion of Ii (Wraight et al., 1990). After permeabilization of the cells this antibody strongly labeled intracellularly located Ii molecules (results not shown).

As our biochemical data indicate that during intracellular transport of the oligomeric complex of class II molecules and Ii, the lumenal, COOH-terminal part of Ii is degraded, we used antibodies against the NH_2-terminal part of Ii in the following studies. To analyze the localization of Ii in relation to different markers of intracellular compartments, cells were permeabilized and Ii was visualized using VIC Y1, an antibody recognizing an NH_2-terminal, cytoplasmic determinant (Wraight et al., 1990). Ii could be localized in the perinuclear region and in a punctated pattern throughout the cytoplasm (Fig. 4 *A*). As reference, we used a marker for the Golgi region. Cells were double labeled for Ii and galactosyl transferase, a marker of the *trans*-Golgi (Berger et al., 1987) (Fig. 4, *A* and *B*). As can be seen in Fig. 4, *A* and *B*,

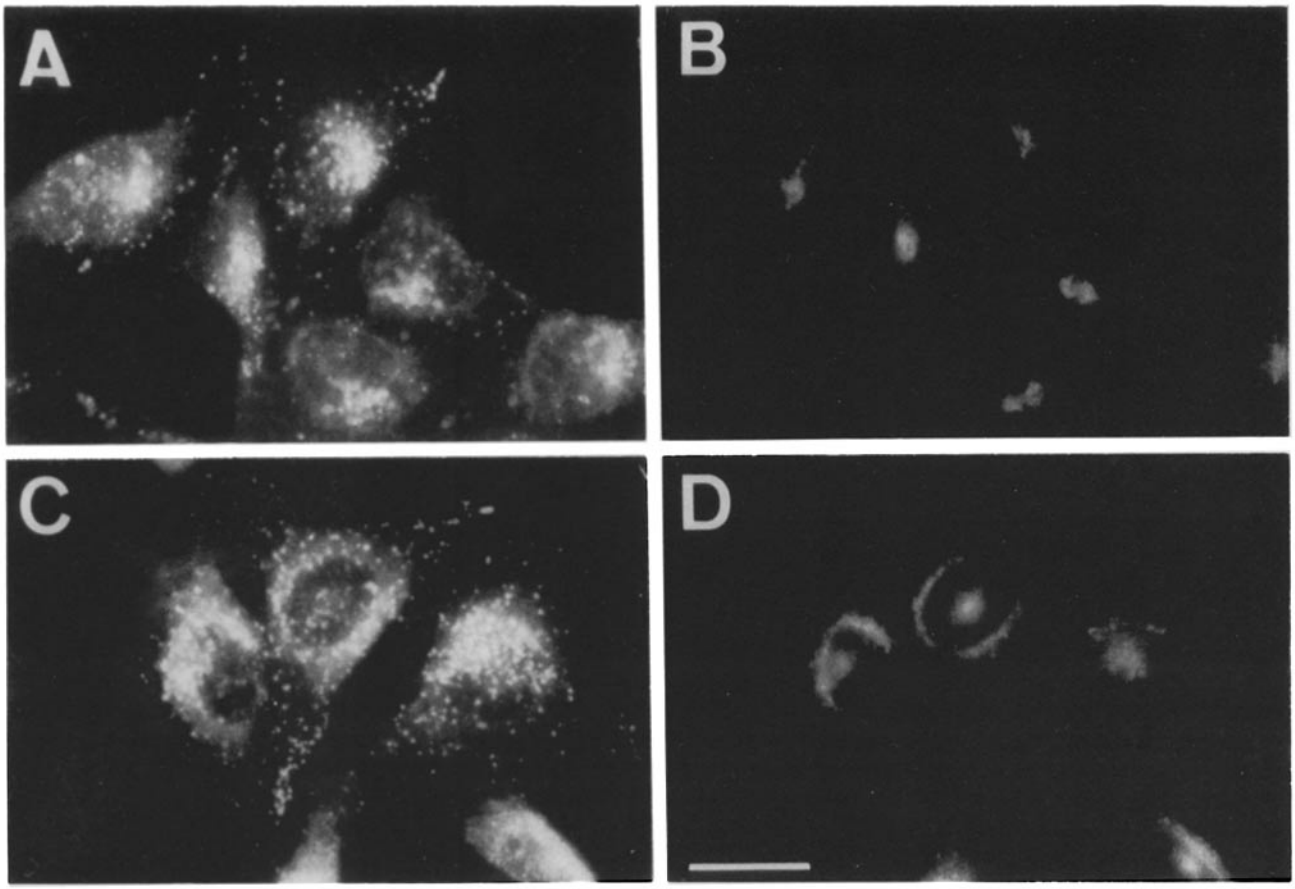


Figure 4. Intracellular localization of Ii in Mel JuSo cells. Cells grown on coverslips were fixed with methanol, and double labeled for Ii (*A*) and galactosyl transferase (*Gal Tf*; *B*), or for Ii (*C*), and the cation-independent mannose-6-phosphate receptor (*MPR*; *D*). Ii was labeled with mAb VIC Y1 and a FITC-conjugated second antibody. Gal Tf and MPR were labeled with polyclonal antisera and visualized using Texas Red–conjugated second antibody. Bar, 20 μm.

Ii is localized in the Golgi area, but also in other distinct structures in the cell periphery.

Inhibition of Ii processing by leupeptin suggested transport of the complex of class II molecules and Ii to an endocytic compartment with proteolytic activity. As the (cation-independent) mannose-6-phosphate receptor (MPR) is known to reside largely in a late endosomal compartment containing proteolytic enzymes (i.e., prelysosomes/late endosomes; Kornfeld and Mellman, 1989; Griffiths et al., 1988; Griffiths et al., 1990), we compared its location with that of Ii. Mel JuSo cells were permeabilized and labeled for both Ii, using VIC Y1, and MPR, using rabbit anti-MPR. As is depicted in Fig. 4, *C* and *D*, some Ii seems to colocalize with the MPR, but the bulk of the vesicular structures in which Ii is localized, is MPR-negative.

Localization of Class II Molecules and Ii at the Electron Microscopy Level

To further characterize the compartments in which class II molecules and Ii are present, we used electron microscopy.

Endocytic compartments were identified by the internalization of HRP for different times (Parton et al., 1989). After 5 min of internalization, HRP is localized to the early endosomes. After 10–15 min of internalization significant amounts of HRP is also found in typical membrane rich, spherical structures (Gruenberg et al., 1989). These structures are especially prominent in Mel JuSo cells, and show a multivesicular body like morphology, therefore, we name them multivesicular bodies (MVB). When HRP is pulsed for 10 min followed by a 30 min chase, HRP is then found distributed between the endosome transport vesicles and the

*Table I. Amount of Ii Present in Double-labeled Cryosections of Mel JuSo Cells as Determined by Immunogold Labeling Using Anti-IiN Antiserum Followed by 9 nm Protein A-Gold**

Compartment	Gold per area organelle		
	Anti-IiN	Anti-IiC	
Early endosome‡	3.4 ± 0.9	10.6 ± 1.4	gold/μm^2
MVBs§	5.5 ± 1.2	4.0 ± 0.5	gold/μm^2
Prelysosome‖	3.3 ± 0.7	13.3 ± 1.8	gold/μm^2
Lysosome¶	<0.5	<0.5	gold/μm^2
Golgi complex**	4.7 ± 1.7	15.5 ± 2.7	gold/μm^2

The labeling of the plasma membrane (0.08 ± 0.01 gold per linear micrometer for anti-IiN and 0.06 ± 0.01 for anti-IiC) while low was over five times higher than background (labeling with an irrelevant antibody gave 0.016 ± 0.004 gold/μm plasma membrane.

* Thirty random profiles of each structure were counted, except for lysosomes (n = 12).

‡ Defined as HRP or 5 nm gold positive after 5 min internalization.

§ Membrane rich spherical structures: defined by characteristic morphology.

‖ MPR-enriched, containing 16 nm gold-BSA after 4-h label followed by overnight chase.

¶ MPR-negative, containing 16 nm gold-BSA after 4-h label followed by overnight chase.

** Defined by characteristic morphology.

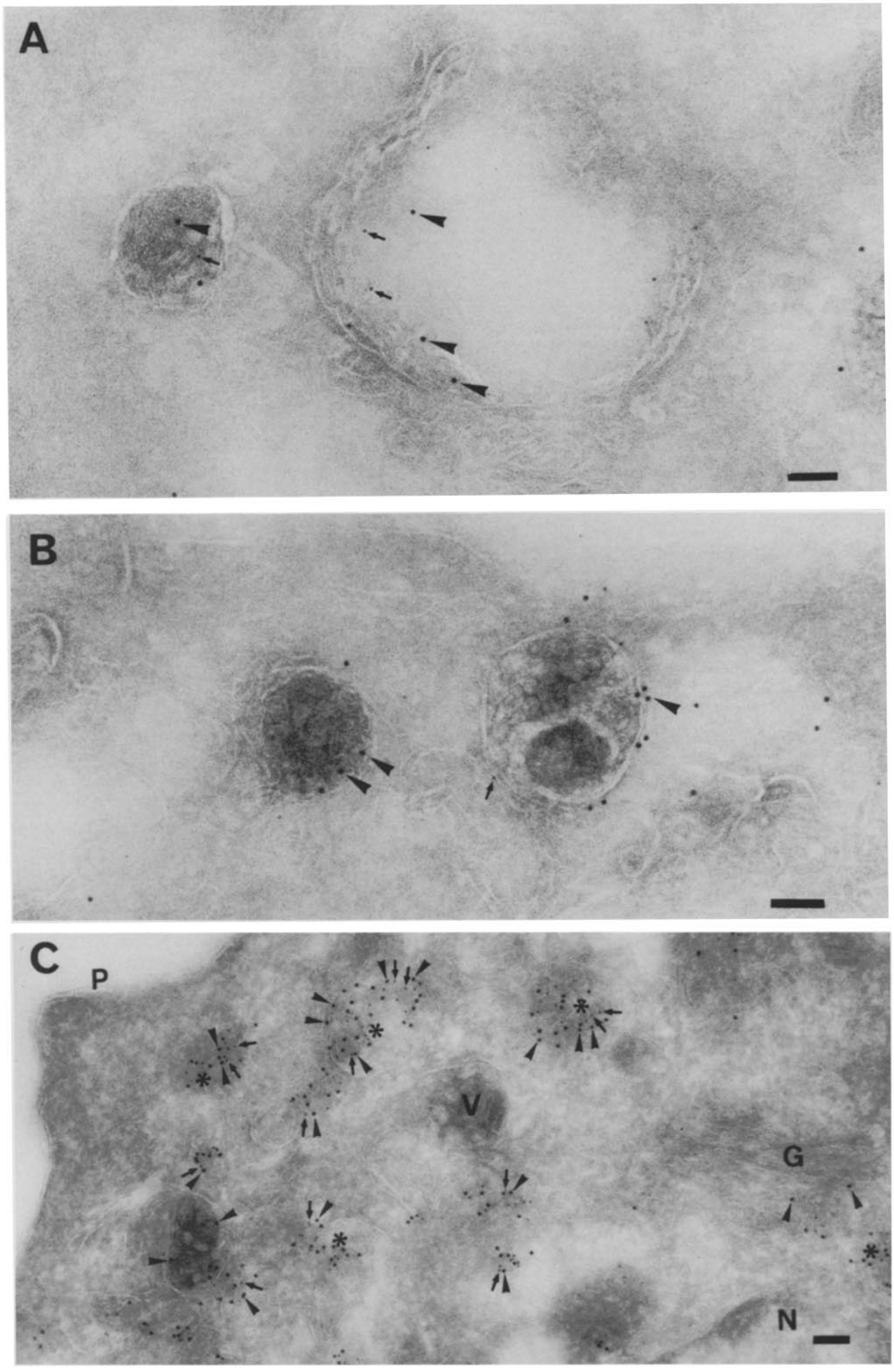

A
B
C
P
V
G
N

*Table II. Amount of MHC Class II Molecules Present in Double-labeled Cryosections of Mel JuSo Cells as Determined by Immunogold Labeling Using Antiserum against the α-Chain followed by 9 nm Protein A-Gold**

Compartment	Anti-α-chain	
Plasma membrane	9.8 ± 1.3	gold/μm
Early endosome	5.9 ± 1.4	gold/μm
MVBs‡	5.6 ± 2.1	gold/μm
Prelysosome‡	4.8 ± 0.9	gold/μm
Lysosome	1.7 ± 0.4	gold/μm
Golgi complex	0.2 ± 0.05	gold/μm

* For experimental details see legend to Table I.
‡ Gold particles present on the outer membranes only were quantitated.

MPR enriched PLC in BHK cells (Gruenberg et al., 1989). This could be confirmed for Mel JuSo cells by double labeling with anti-MPR antibodies (not shown). Lysosomes were identified by the presence of 16 nM BSA-gold conjugate that was given to the cells for 4 h followed by an overnight chase (Griffiths et al., 1990).

Mel JuSo cells in which various compartments of the endocytic pathway had been labeled as described above were prepared for cryosectioning and antibody labeling. The amount of Ii label on cryosections present in the various subcellular compartments was quantified using anti-IiN and anti-IiC antiserum, and the results are shown in Table I. Ii was found to be present in early endosomes, late endosomes or prelysosomes and MVBs. Very little Ii was found on the plasma membrane.

The quantitative analysis of the labeling of Ii supports the qualitative data showing similar amounts in early endosomes, MVBs and prelysosomes, with negligible amounts in lysosomes (cf. below). To directly compare the amounts of labeling between the different compartments it is necessary to assume that the labeling efficiencies are the same, that is that each antibody has equal access to antigen in the different structures. This may not always be the case (Griffiths and Hoppeler, 1986) and the relatively lower labeling with anti-IiC in the MVBs, for example, may reflect less access of this antibody to the antigen. Alternatively, part of the MVB population may already contain proteolytic activity which leads to degradation of Ii COOH-terminal fragments (see Figs. 1 and 2). Nevertheless, taken together the data for both antibodies suggest that the amount of Ii in the early endosome, MVB and prelysosome is similar.

Examples of intracellular compartments which are Ii positive are shown in Fig. 5. Fig. 5 *A* shows a typical early endosome (Griffiths et al., 1990; Gruenberg et al., 1989) in which the lumen is clearly labeled with Ii and HRP, taken up by the cells for 10 min. Also present in this figure is a multivesicular body, in which both the HRP and Ii can be visualized. Fig. 5 *B* shows two of these MVBs, labeled with both Ii and internalized HRP.

Immunofluorescence microscopy indicated some colocalization of Ii and MPR. This finding was also supported by electron microscopy. Fig. 5 *C* shows colocalization of Ii with MPR in structures which most probably represent a prelysosome. Also MVBs are shown containing both Ii and MPR. In addition, a MVB is indicated devoid of both Ii and MPR. The plasma membrane, as expected, is unlabeled.

As class II molecules and Ii remain associated in Mel Juso cells for >4 h after synthesis and Ii does not appear on the plasma membrane, we were also interested to localize class II molecules in intracellular compartments. Intracellular compartments were identified in a similar manner as described above for Ii.

The amount of class II labeling on cryosections present in the various subcellular compartments was quantitated using the anti-α-chain antiserum. The results are presented in Table II. To be able to compare directly the amount of label on the plasma membrane with that over internal structures we expressed the class II labeling on a per linear micrometer scale. The plasma membrane, as expected, abundantly contained class II molecules; furthermore, the class II molecules were found to be present in equal amounts in the early endosomes, MVBs, and prelysosomes. Lysosomes contained a low but significant amount of class II molecules, whereas the Golgi complex was poorly labeled. The significance of this low Golgi labeling is not known.

Micrographs showing examples of class II molecules localized in the various compartments are shown in Figs. 6 and 7. Fig. 6 *A* shows colocalization of class II molecules with HRP endocytosed for 5 min in an early endosome. Fig. 7, *A* and *B* show localization of class II molecules on the plasma membrane (*P*) and in MPR-rich compartments presumed to be prelysosomes. Some of these vesicular structures were also labeled with a 16 nm gold-BSA complex given to the cells for 4 h and then chased overnight. Not all MVBs which were found positive for class II molecules were also found positive for MPR. This is particular evident in Fig. 7 *B*, which shows two MVBs devoid of MPR-specific labeling and one which contains relatively large amounts of MPR. Note also that MPR is mostly found at the internal membranes of the MVBs whereas class II molecules are nearly exclusively localized on the peripheral membrane.

The relative distribution of the class II molecules in the endocytic compartments and MVBs is very similar to the distribution of Ii as determined using anti-IiN and anti-IiC antisera.

As apparently there are distinct populations of MVBs, as judged by the absence or presence of MPR, we were interested to see whether both Ii and class II molecules colocalized to the same MVBs. Fig. 6 *B* shows that indeed Ii and class II molecules can be identified within the same MVB.

Discussion

In this study we analyze the intracellular transport and loca-

Figure 5. Localization of Ii in endocytic compartments (*A* and *B*). Mel JuSo cells were allowed to internalize HRP for 10 min to label endocytic compartments. Cryosections were prepared and double labeled with anti-IiN and 9 nm gold (*arrowhead*) and anti-HRP and 5 nm gold (*arrow*). (*A*) Labeling of early endosomes as well as MVBs with Ii and HRP. (*B*) Labeling of MVBs with Ii and HRP. (*C*) Cryosections of Mel JuSo were double labeled with anti-IiC and 9-nm gold (*arrowhead*) and anti-MPR and 5-nm gold (*arrows*). Shown are MVBs and prelysosomes with Ii and MPR. (*) Indicate MVBs; (*P*) plasma membrane; (*G*) Golgi complex; (*V*) unlabeled MVB; (*N*) nucleus. Bars, 100 nm.

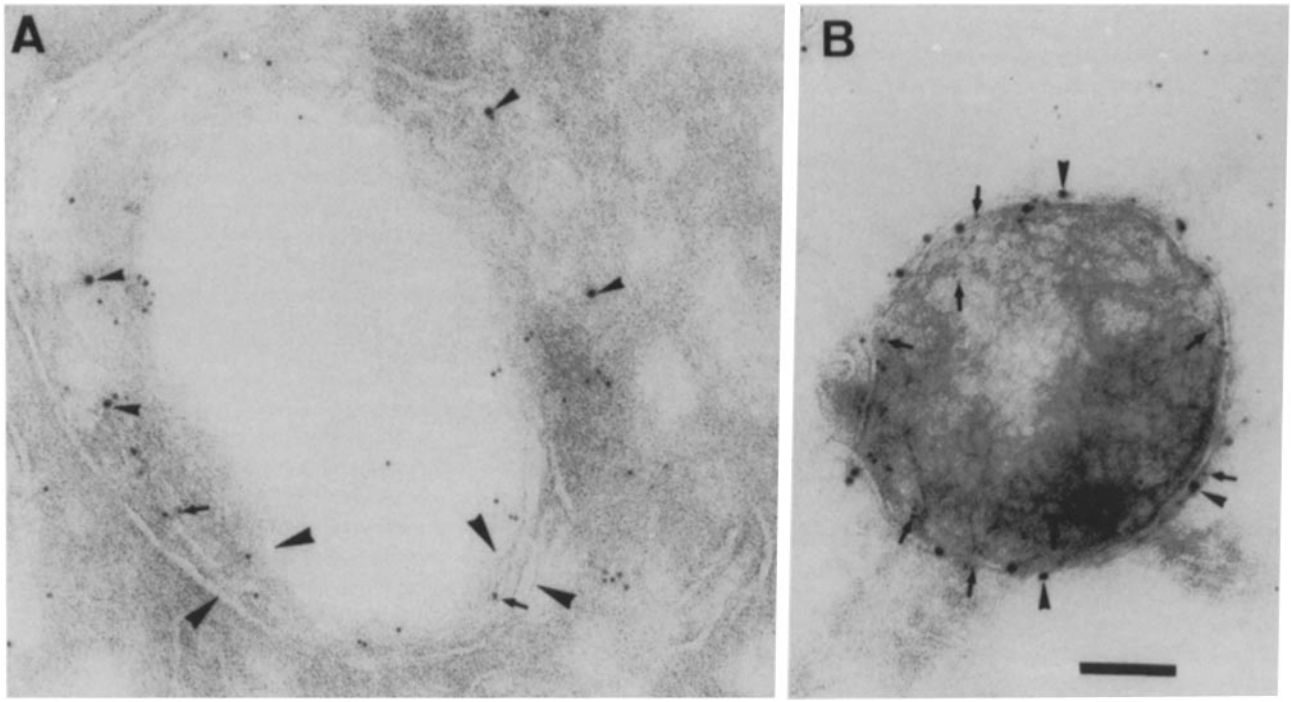


Figure 6. (*A*) Localization of class II molecules in early endosomes. Mel JuSo cells were incubated with HRP for 5 min. Cryosections were prepared and labeled with antibody against the α-chain of class II (Ab 311) and 9-nm gold (*small arrowhead*), and an antibody against HRP and 5 nm gold (*small arrows*). Large arrowheads indicate the limiting membranes. (*B*) Colocalization of class II molecules and Ii in a typical multivesicular body. Cryosections of Mel JuSo cells were labeled with an antibody against the α chain of class II molecules (Ab. 311) and 5-nm gold (*arrows*), and anti-IiN antiserum and 9 nm gold (*arrowheads*). Bar, 100 nm.

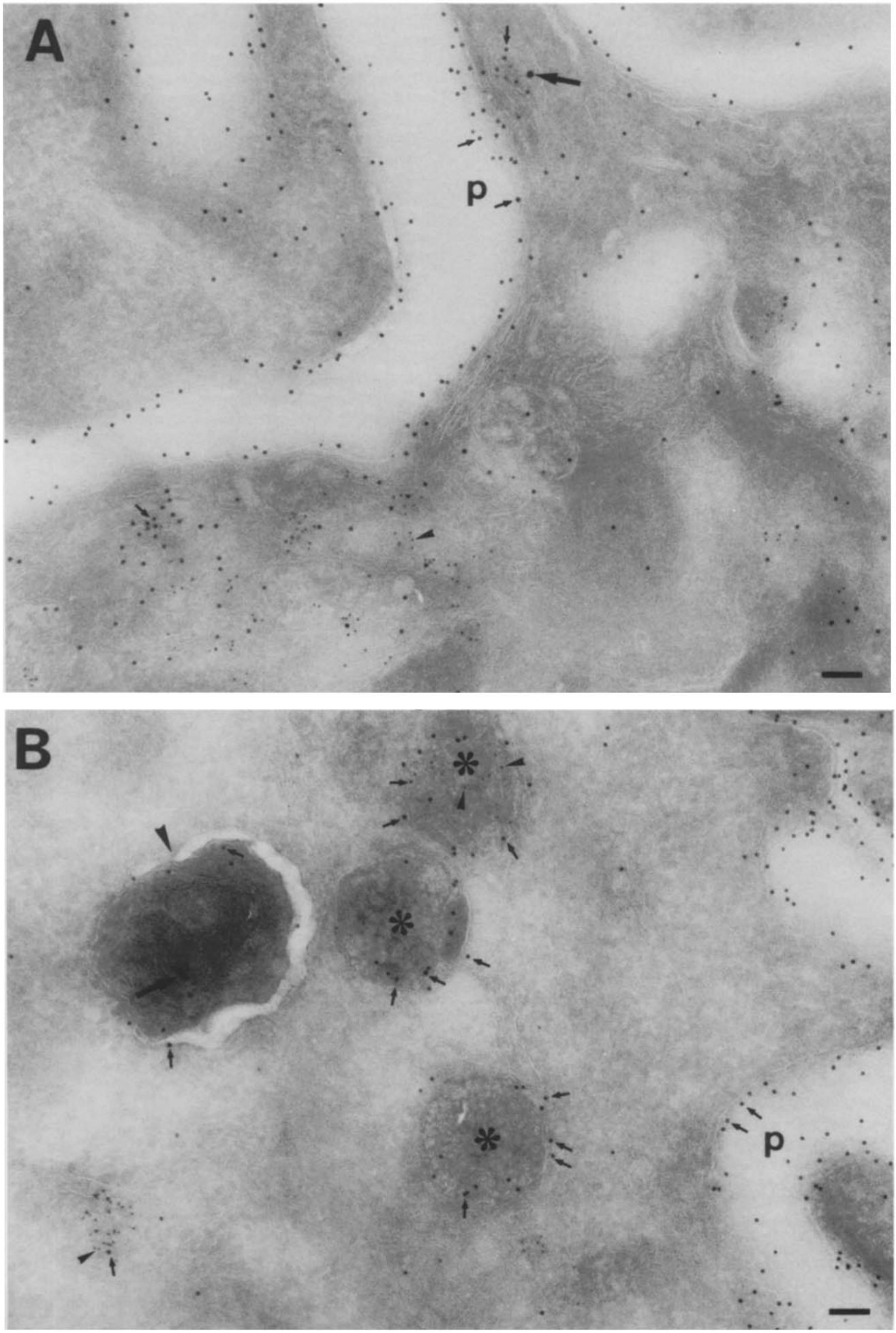
tion of MHC class II molecules and associated invariant chain in a human melanoma cell line. We show that after 2 h, the class II-associated Ii is processed to distinct fragments. This processing occurs from the COOH terminus and results in the formation of P22, P18, and P12, each representing a different segment of the NH_2-terminal part of Ii (Fig. 8). As complex type carbohydrates can be detected on the class II molecules and intact Ii already after 1 h of synthesis, this suggests that cleavage from the Ii COOH terminus occurs in a post-Golgi compartment. In addition, incubation of the cells in the presence of leupeptin prevented cleavage of Ii to P18 and P12, but not to P22. This indicates that P18 and P12 are generated in an endosomal compartment (Fig. 8). As processing of Ii to P22 could not be prevented by the addition of several other protease inhibitors, this cleavage either occurs in a compartment distinct from the endocytic pathway, or is insensitive to the protease inhibitors used.

Studies from several laboratories have shown, that in contrast to Mel JuSo cells, no COOH-terminal proteolytically processed forms were observed in B lymphoblastoid cells in the absence of protease inhibitors (Kvist et al., 1982; Blum and Cresswell, 1988; Nguyen et al., 1989). Only in their presence could P22 (P21, LIP) and P12 (P10) be detected (Nguyen et al., 1989; Blum and Cresswell, 1988). This suggests that in Mel JuSo cells either intracellular transport of class II molecules and Ii is relatively slow, or that these cells lack or contain very low amounts of certain proteases. Experiments in which distinct endosomal proteases are added to the cells may allow to discriminate between these possibilities.

At the light microscopy level, Ii appeared to be present in vesicular structures throughout the cell and in particular in the Golgi area. Some colocalization of MPR and Ii was found. Electron microscopy analysis showed that both Ii and class II molecules are present in three distinct compartments of the endocytic pathway: the early endosomes, membrane-rich spherical compartments (MVBs), and prelysosomes.

Both Ii and class II molecules were found largely at the pe-

Figure 7. Localization of class II molecules on the plasma membrane, in MVBs and lysosomes; colocalization of class II molecules with MPR in some compartments. Cryosections were prepared of Mel JuSo cells that had internalized 16-nm gold-BSA for 4 h followed by an overnight chase in normal medium. This marker together with MPR defines prelysosomes and lysosomes. The sections were double labeled with an antibody against the α-chain of class II molecules (Ab. 311) and 9-nm gold (*arrows*) and anti-MPR antibody and 5-nm gold (*small arrowheads*). In both *A* and *B* the bulk of the class II labeling is seen on the plasma membrane (*P*). In *A* there is extensive colocalization of class II molecules and MPR in many vesicular profiles, one of which contains the internalized 16-nm gold (*large arrow*). In *B* three MVBs are seen (*). In all class II molecules can be found, almost exclusively on the peripheral membrane. One of these multivesicular bodies (*upper one*) has significant MPR labeling and may therefore have already fused with the prelysosomal compartment. A profile of the latter is evident in the bottom left part of the figure, in which also class II molecules can be localized. The large arrowhead indicates a lysosome containing class II labeling. Bars, 100 nm.


A
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B
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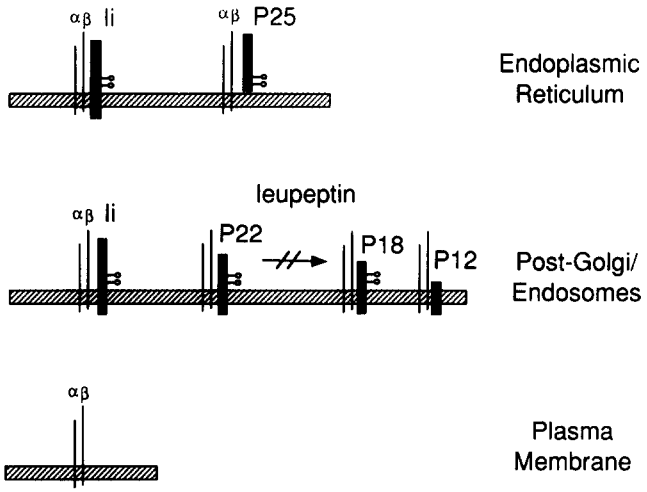

Figure 8. Schematic representation of the processing steps of class II associated Ii at various stages of intracellular transport in Mel JuSo cells. α and β represent the α-chain and the β-chain of MHC class II molecules, respectively; –○, represents carbohydrate side chains; *P25*, *P22*, *P18*, and *P12* represent processed forms of Ii. In the ER class II molecules ($\alpha\beta$) are found associated with membrane integrated Ii and with an NH_2-terminal deletion of Ii, P25. During intracellular transport Ii is proteolytically processed to P22, P18, and P12. Processing of P22 to P18 and P12 can be inhibited by culturing cells in medium containing the protease inhibitor leupeptin. On the plasma membrane class II molecules can be found but very little Ii.

riphery of the MVBs in contrast to MPR which was found more in the centre of these organelles. The functional significance of this differential distribution in the MVB remains to be elucidated but may be related to their different fates (Felder et al., 1990).

The presence of class II molecules in a late endocytic, lysosome-related compartment has recently been demonstrated in the B lymphoblastoid cell line JY (Peters et al., 1991). Ii was not detected in endocytic compartments, probably due to the rapid degradation of Ii in lymphoblastoid cells (Blum and Cresswell, 1988; Nguyen et al., 1989). Using a different lymphoblastoid cell line, Guagliardi et al. (1990) localized class II molecules and Ii in an early endocytic compartment.

Clearly in Mel JuSo cells we find a different distribution of class II molecules and Ii. Both of these molecules are found in early as well as late endosomes, and, in addition in MVBs. In contrast to the rapid degradation of Ii in B lymphoblastoid cell lines, Ii NH_2-terminal fragments remain associated with class II molecules >4 h after synthesis in Mel JuSo cells. As the NH_2-terminal cytoplasmic fragment of Ii has been shown to contain a sorting signal for endosomal compartments (Bakke and Dobberstein, 1990; Lotteau et al., 1990), the steady-state location of class II molecules in a given cell line might depend on the rate of processing of Ii and on the dissociation of Ii from class II molecules.

In transfected mouse L cells, Salamero et al. (1990) showed that class II molecules can be found in late endosomes independent of the expression of Ii. This would suggest that class II molecules may contain themselves targeting information for late endosomes. It remains to be established which pathway is followed by class II molecules to endocytic compartments. Depending on the cell type, class II molecules may enter the endosomal system directly from the *trans*-Golgi network *en route* to the plasma membrane (Neefjes et al., 1990), or by internalization after being transported to the plasma membrane (Reid and Watts, 1990). At present, the relative contributions of the signals in Ii and class II molecules to the followed pathway to endosomes are not known.

The association of Ii or its NH_2-terminal fragments with class II molecules might also modulate the distribution of these molecules in the endosomal system. This would explain the finding that class II molecules expressed in cells in which Ii is rapidly degraded (e.g., lymphoblastoid cell lines) are localized in late endosomes/prelysosomes only (Peters et al., 1991). Our results, using a melanoma cell line, suggest that prolonged association of Ii or its NH_2-terminal fragments may be responsible for the localization of class II molecules in several distinct endosomal compartments. Differences in Ii proteolysis in different cell lines may therefore explain the apparent discrepancy in class II localization described before (Guagliardi et al., 1990; Peters et al., 1991).

We conclude that knowledge of the type of Ii molecules at various stages of transport in a given cell line may be essential for understanding transport and localization of MHC class II molecules.

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