Journal of Cell Science 103, 811-822 (1992)
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A block in degradation of MHC class II-associated invariant chain correlates with a reduction in transport from endosome carrier vesicles to the prelysosome compartment

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Summary

Invariant chain (Ii) associated with MHC class II molecule is processed proteolytically via several distinct intermediates during its intracellular transport through endosomal compartments. Leupeptin added to the culture medium blocks processing of Ii, prevents its dissociation from the class II molecules and leads to an intracellular accumulation of a 22 kDa intermediate form of Ii. We show here that leupeptin has a very general effect on protein transport in the endocytic pathway. When added to Mel Juso cells leupeptin reduces the transport of endocytosed material from multivesicular body-like, endosome carrier vesicles (ECV) to the prelysosomal compartment (late endosome) and leads to a concomitant increase in the number of ECV. Our results argue that one effect of leupeptin, related to antigen processing and presentation, is to block transport of antigen and/or MHC class II molecules to prelysosomal compartments.

Key words: leupeptin, invariant chain (Ii), endosome carrier vesicle (ECV), endocytosis.

Introduction

Class II major histocompatibility complex (MHC) molecules expose specific peptides at the cell surface of antigen-presenting cells to CD4-expressing T cells (Unanue, 1984). These peptides are generated by proteolysis of larger protein antigens taken up from the extracellular medium via the endocytic pathway. Subsequently the peptides bind class II molecules and the complex is routed to the cell surface. While it is clear that both the proteolytic processing events and the binding of specific peptide fragments to class II molecules occur somewhere in the endocytic pathway, the precise site(s) where these events occur remains to be established (Brodsky, 1992).

MHC class II molecules consist of polymorphic α and β chains, which associate with invariant chain (Ii) directly after insertion into the ER membrane (Kvist et al., 1982; Jones et al., 1978). Ii is a type II transmembrane protein exposing 30 N-terminal residues on the cytoplasmic side and a large C-terminal domain of ~ 160 amino acids on the exoplasmic side of the membrane (Claesson et al., 1983). Recent findings suggest that Ii may play an important role in the regulation of peptide binding to MHC class II molecules (Koch et al., 1989; Long et al., 1989). Since MHC class II molecules with assembled Ii were found to be unable to bind peptides, it was concluded that Ii prevents peptide binding in the early compartments of the biosynthetic pathway by occupying the peptide binding site on MHC class II molecules. The second proposed function of Ii is to sort MHC class II molecules into endocytic compartments. This is supported by the finding that the N-terminal tail of Ii contains an endosomal sorting signal (Bakke and Dobberstein, 1990; Lotteau et al., 1990).

In a recent immunocytochemical study of Mel Juso cells, both MHC class II molecules and Ii could be detected in three distinct endocytic compartments. These were the early endosomes, the multivesicular body-like endosome carrier vesicle (ECV) and the cation-independent mannose 6-phosphate (MPR)-enriched prelysosomal compartment (PLC) (Pieters et al., 1991). In B-lymphoblastoid cells, class II molecules are found mainly in structures classified as lysosomes (Peters et al., 1991).

During intracellular transport Ii is proteolytically processed and dissociates from the complex with class II molecules at an intracellular site. Processing occurs in distinct steps from the C terminus of the molecule (Pieters et al., 1991). In B-lymphoblastoid cells processing intermediates of Ii can only be seen after inhibition of proteolysis with leupeptin (Kvist et al., 1982; Blum and Cresswell, 1988). In the human melanoma cell line, Mel Juso, this proteolysis results in the sequential formation of the processing intermediates P22, P18 and P12, each lacking increasingly larger portions from the C-terminal side (Pieters et al., 1991).

Processing of Ii can be inhibited by protease inhibitors, such as leupeptin, added to the culture medium, or by agents which increase the pH of endosomes, such as chloroquine (Blum and Cresswell, 1988). Leupeptin mainly inhibits thiol proteases such as cathepsin B, plasmin and papain, but has little or no activity in inhibiting the thiol protease cathepsin A and the acid protease cathepsin D (Aoyagi and Umezawa, 1975). In Mel Juso cells, leupeptin treatment over 4 h leads to an accumulation of P22, the first degradation product of Ii. In this paper we describe the effect of leupeptin on the localization of the MHC class II-associated Ii in Mel Juso cells. Our data show that leupeptin significantly retards the transport of a fluid-phase marker horseradish peroxidase (HRP) from the ECV to the PLC. A consequence of this block is that the total number of ECV per cell increases about two-fold.

Materials and methods

Cell culture

The Mel Juso cell line is derived from a human malignant melanoma (Johnson et al., 1981) and was obtained from Dr. G. Riethmüller, University of Munich. The cells were cultured in RPMI 1640 medium supplemented with 10% foetal calf serum (RPMI-FCS).

Antibodies

The hybridoma cell line L-243 secreting anti-HLA-DR antibodies (Lampson and Levy, 1980) was obtained from the American Type Culture Collection. Ascites fluid was obtained by culturing the cells intraperitonally in Balb/c mice. VIC Y1 (Quaranta et al., 1984) is a mouse monoclonal antibody that recognizes a cytosolic epitope within the 30 N-terminal amino acids of Ii. LN2 (Biotest AG, FRG) is a mouse monoclonal antibody specific for an epitope in the outer C-terminal portion of Ii (within amino acids 157-216 of Ii; Wraight et al., 1990). The polyclonal rabbit antibody, anti-IiN, recognizes the N-terminal cytoplasmic segment of Ii. The antiserum was raised against a fusion protein of β-galactosidase and the N-terminal 73 amino acids (Ii_{1-73}) of Ii as described previously (Lipp and Dobberstein, 1986).

Rabbit polyclonal antisera specific for the (cation-independent) mannose 6-phosphate receptor (MPR) and for horseradish peroxidase (HRP) were gifts from Drs. B. Hoflack and J. Gruenberg, respectively.

Immunofluorescence studies

Mel Juso cells were grown on coverslips (1 cm × 1 cm) and for leupeptin experiments 150 μg/ml leupeptin (Sigma) was added to the medium. The immunofluorescence labelling was carried out as previously described by Bakke and Dobberstein (1990). Briefly, the cells were fixed in 3% paraformaldehyde (PFA) and permeabilized with 0.1% Triton X-100. They were labelled with the appropriate antibody (VIC Y1 or LN2) on ice and Ii was visualized by using a FITC-conjugated second antibody. After labelling, the cells were mounted in Moviol and examined using a Leitz Orthoplan fluorescence photo-microscope equipped with a ×63 objective and filters for FITC.

Metabolic labelling and pulse-chase protocol

Mel Juso cells, cultured in 35 mm dishes, were incubated in prewarmed, methionine-free medium. After 30 min this medium was replaced by methionine-free medium containing 0.08 mCi [^{35}S]methionine. After 30 min pulse labelling the cells were washed twice and further incubated in prewarmed chase medium (RPMI-FCS with 4 mM methionine). After the chase times indicated in the figure legend, the cells were placed on ice and washed 2× with ice-cold PBS. The cells were lysed for 10 min on ice with ice-cold lysis buffer (1% Triton X-100, 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1 mM PMSF). The cell lysates were centrifuged at 10,000 ***g*** for 2 min at 4°C to remove cell debris and antigens immunoprecipitated from the supernatants.

Leupeptin treatment

Cells were metabolically labelled as described above using medium containing 150 μg/ml leupeptin for 4 h. The medium was subsequently replaced by methionine-free medium with the same concentration of leupeptin. All of the following steps were conducted as in the usual protocol, except that all media additionally contained 150 μg/ml leupeptin.

Immunoprecipitation and electrophoresis

A 40 μl sample of Protein A-Sepharose (1:1 slurry) equilibrated in buffer A (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 2 mM EDTA, 0.2% NP-40) was added to the cell lysates and adsorbed for 30 min at 4°C. The beads were removed by centrifugation and 1 μl of the anti-MHC class II antibody L-243 was added to the supernatant and incubated for 2 h at 4°C. A further 20 μl Protein A-Sepharose was added, followed by a 1 h incubation. The beads were washed twice in buffer A, twice in buffer A containing 500 mM NaCl, and twice in 10 mM Tris-HCl, pH 7.5. The antigens were eluted from the beads by the addition of sample buffer, denatured and analyzed by electrophoresis on a 10% to 15% gradient polyacrylamide gel and subjected to fluorography and autoradiography.

Electron microscopy

Epon sections

In order to identify all the compartments of the endocytic pathway, cells were incubated for 10 h at 37°C in prewarmed RPMI (5% FCS; ± 150 μg/ml leupeptin) containing 5 mg/ml HRP (Sigma, type VI). After 10 h endocytosis was stopped by removing the medium, the dishes were then placed on ice and washed twice in ice-cold PBS. Preparation of cells for plastic embedding using Epon 812 was performed as described previously (Griffiths et al., 1984a), except that after osmium tetroxide treatment the cells were treated with 1.5% uranyl acetate in 50 mM Tris-maleate buffer, pH 5, for 30 min. The visualization of HRP with diamino-benzidine (DAB; Sigma Chemical Co.) was done as described by Griffiths et al. (1989).

Cryomicrotomy and immunocytochemistry

Dishes with a nearly confluent monolayer were placed on ice and cells were removed by treatment with proteinase K (50 μg/ml in PBS) and fixed in 8% formaldehyde in 250 mM Hepes-buffer (pH 7.4). The preparation of cryo-sections as well as single and double labelling with antibodies and Protein A-gold were performed as described (Griffiths et al., 1984b, 1988).

Markers for endocytosis

For identification of the various compartments belonging to the endocytic pathway, cells were incubated for various times in medium containing BSA-gold complexes of different sizes. To identify early and late endosomes, cells were allowed to internalize a BSA-5 nm gold complex for 30 min. To identify late endocytic structures, a BSA-16 nm gold complex was internalized for

4 h, followed by an overnight chase in medium without BSA-gold. Under these conditions this marker is distributed between the MPR-enriched prelysosomal compartments (PLCs), and the MPR-negative lysosomes (Griffiths et al., 1988, 1990).

Morphometric analysis

Cells

Control cells were cultured in normal medium (RPMI-10% FCS) and for leupeptin treatment the cells were incubated for 10 h in medium with 150 μg/ml leupeptin before processing.

Mean cell volume

The methods for estimating the mean cell volume have been described in detail previously (Griffiths et al., 1984a, 1989). The 'volume weighted' nuclear volume (V_{nuc}) of Mel Juso cells was estimated by using the 'linear intercept method', developed by Gundersen and Jensen (1985). For this method to be accurate the mean cell volume of all the cells in the population must be approximately the same. In this paper we have assumed this to be the case: any deviation from this assumption will be reflected in a corresponding overestimation of the mean cell volume. The volume fraction of nuclei (volume density, $Vv_{nuc, ce}$) was then estimated by point counting. For this, random sections of Epon-embedded cells (± leupeptin) were photographed at a magnification of ×4,000. All measurements were made on negatives enlarged ×4.1 on an EMBL-designed enlarger. A lattice grid (D64) was placed over the projected negatives and the $Vv_{nuc, ce}$ was estimated by relating the points over the nucleus to those over the cell. Knowing the values for the nuclear volume and the fraction of the cell occupied by the nucleus enabled us to estimate the mean cell volume.

Diameter and volume of ECVs

Diameters and volumes of ECVs were determined as described by Weibel (1979). Cryosection of Mel Juso cells (± leupeptin) with ECVs were photographed at a magnification of ×16,000. Diameters of the ECVs were determined on ×4.1 enlarged negatives. Smaller ECV profiles were not readily distinguishable from other vesicle types, since their typical membrane-rich morphology is not so obvious. Therefore, the measured diameters were grouped into classes to make a histogram showing the distribution of the ECV diameters. On the basis of the histogram, the 'missing' values for smaller ECVs were extrapolated (for details see Weibel, 1979). The calculation for the diameter was performed as discussed by DeHoff (1968), which enabled us to determine the mean volume of a spherical ECV.

Number of ECVs per cell

The volume density of ECVs in the cytoplasm ($Vv_{ecv, cy}$) was determined in Mel Juso cells (± leupeptin) using point counting method (Weibel, 1979). A total of 29 micrographs were taken at random from cryo-sections. Using a double-lattice grid (D64) the $Vv_{ecv, cy}$ was calculated by relating the number of points over ECVs to the total number of points over the cytoplasm. Together with the known values of cytoplasmic and ECV volumes, the number of ECVs per cell could then be determined.

Quantification of immunolabelling

Cells were prepared for cryo-section and labelled with the appropriate antibodies followed by Protein A-gold. The number of gold particles per linear μm of ECV peripheral membrane profile was determined by intersection counting, and the number of gold particles per μm^2 surface area of ECVs and PLCs was estimated by point counting. The PLC was defined as profiles on sections with at least three gold particles labelling the MPR. The quantification of the density of gold particles was carried out as described by Griffiths and Hoppler (1986). For background labelling the number of gold particles over the nucleus was counted and substracted from the total number.

Results

After their transport from the Golgi complex to endocytic compartments the newly synthesized Ii molecules are sequentially degraded in Mel Juso cells. As shown previously, three distinct electrophoretic forms of Ii, still associated with class II molecules, could be identified: the 33 kDa mature Ii is sequentially cleaved into P22, P18 and finally P12 (Pieters et al., 1991). In the latter study it was shown that MHC class II molecules with associated P22 accumulated when cells were pretreated with leupeptin, pulse labelled and chased for 4 h. In order to discover whether the MHC class II complex with the processing intermediate P22 could be maintained for longer times we chased the cells for up to 17 h in the presence of leupeptin. Antigens from the cell lysates were immunoprecipitated with an anti-MHC class II antibody (L-243), which recognizes only oligomeric MHC class II molecules. As expected

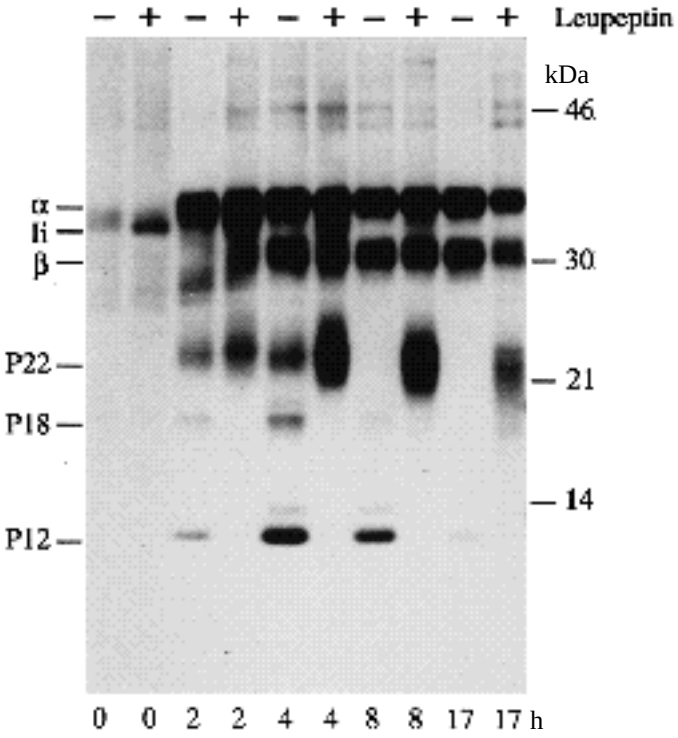


Fig. 1. Effect of leupeptin on the proteolytic processing of the MHC class II-associated invariant chain (Ii). Cells were incubated for 4 h in medium (±) leupeptin (150 μg/ml) and then pulse labelled for 30 min with [^{35}S]methionine and chased (± leupeptin) for the indicated times. Proteins were immunoprecipitated with anti-class II antibody L-243. Shown are autoradiographs after SDS-PAGE and fluorography. The migration of molecular mass markers (in kDa) is indicated on the right side of the lanes. The α and β chain of MHC class II and Ii and its proteolytically processed forms P22, P18 and P12 are indicated on the left side.

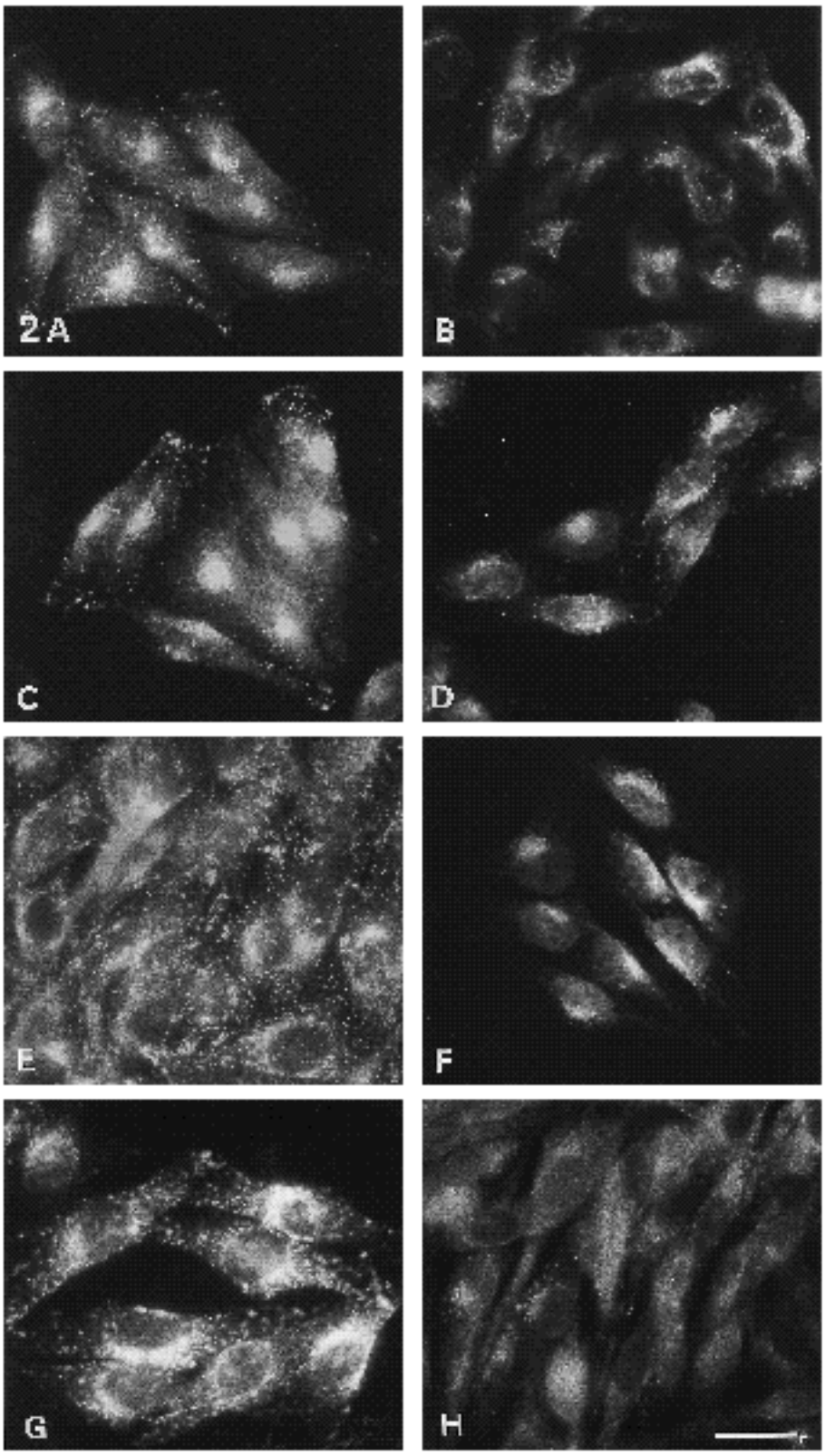


Fig. 2. Intracellular localization of Ii in Mel Juso cells grown in the presence (A, C, E, G) or absence (B, D, F, H) of leupeptin. Cells were grown on coverslips and incubated for 7 h (C, D), overnight (E, F) and for 2 days in medium (±) 150 μg/ml leupeptin. Cells were fixed and permeabilized using paraformaldehyde and Triton X-100. Ii molecules were labelled using the monoclonal N-terminal antibody Vic Y1 (A, C, E, G) and the monoclonal C-terminal antibody LN2 (B, D, F, H). The primary antibodies were visualized using FITC-conjugated second antibody. Bar, 18.5 μm.

only small amounts of MHC class II molecules could be immunoprecipitated directly after the pulse labelling (Fig. 1). After a 2 h chase, MHC class II α (35 kDa) and β (29 kDa) chains, the associated Ii (33 kDa) and its processed forms P22, P18 and P12 were immunoprecipitated (Pieters et al. 1991). For chase times up to 17 h in the presence of the protease inhibitor an accumulation of complexes of MHC class II with associated P22 was observed.

Characterization of the effect of leupeptin on the localization of Ii by light microscopy

Light microscopy was used to investigate the accumulation of Ii and P22 in cells treated with leupeptin for different times. Mel Juso cells were cultured both with and without leupeptin for the times indicated in Fig. 2. For localizing Ii and P22 two different antibodies were used. VIC Y1 recognizes an N-terminal, cytoplasmic determinant (anti-IiN) while LN2 recognizes a C-terminal, luminal epitope of Ii, which is not present in P22 (anti-IiC) (Wraight et al., 1990; Pieters et al. 1991). The labelling patterns observed with the two antibodies were quite different (Fig. 2). In the absence of leupeptin both antibodies gave a strong labelling in the perinuclear region as well as of vesicular structures in the peripheral cytoplasm, with the latter more pronounced. Following leupeptin treatment this pattern did not significantly change with respect to the anti-IiC. Starting at 7 h and becoming more pronounced after overnight and two days of treatment with leupeptin, there was an increased labelling with anti-IiN (Fig. 2C, E and G). A strong labelling for Ii was detected in distinct vesicular structures both in the nuclear region and in the peripheral cytoplasm. As expected, since P22 is not recognized by anti-IiC (Pieters et al., 1991), the P22-containing vesicles which accumulated after leupeptin treatment could not be labelled with this antibody.

Characterization of the effect of leupeptin on the localization of Ii by electron microscopy

The effect of leupeptin on the distribution of Ii was followed using thawed cryosections and immunogold labelling. Our previous study using this approach had shown that, in addition to the Golgi complex, three distinct endocytic organelles could be labelled with the two anti-Ii antibodies (Pieters et al., 1991). These are the early endosome, the multivesicular body-like, endosome carrier vesicles (ECV) and the MPR-enriched PLC. In the present study we examine the effects of leupeptin on the distribution of Ii with respect to these compartments.

In order to facilitate identification of the various endocytic compartments BSA-gold conjugates were added to the culture medium of the cells prior to fixation. For late endocytic structures (PLC or lysosomes) 16 nm gold-BSA was pulsed for 4 h and chased overnight. For early endosomes 5 nm gold BSA was internalized for 5 min while a 30 min continuous internalization with this conjugate was used to label all endosomal compartments up to the PLC. These cells were then prepared for cryo-sectioning and labelled with anti-IiN.

The labelling pattern seen with the anti-IiN was essentially the same in both untreated and leupeptin-treated cells. As observed in our earlier study (Pieters et al., 1991), there was significant labelling of early endosomes, ECV (Fig. 3) and the PLC (not shown). In both conditions the labelling of the ECV with the anti-IiN was predominantly on the outer membrane of these vesicles rather than on the inner, predominantly tubular membranes (Fig. 3). The quantitative data presented in Table 1 show that the concentration of the antigen in the ECV was the same in the presence or absence of the inhibitor. Further, in both cases an analysis of the distribution of the gold particles indicated that between 70 and 75% of the label was associated with the outer membrane (within 20 nm) of the ECV.

In these cryosection preparations, a striking difference in the leupeptin-treated cells was that there appeared to be many more ECV when compared with untreated cells. To investigate this further, conventional plastic embedding studies were done. For this, cells were first incubated with BSA-16 nm gold for 4 h, followed by a 10 h chase to label both PLC and lysosomes (Griffiths et al., 1990). The chase medium contained HRP to label all endocytic structures. After incubation with DAB and embedding in Epon, the HRP-labelled endocytic structures were visualized at low

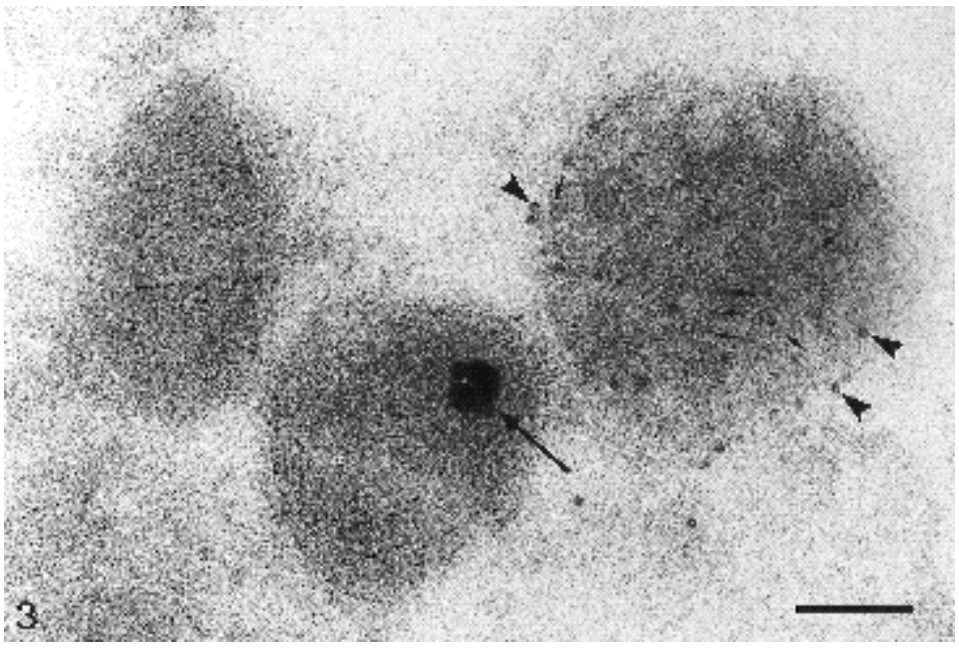


Fig. 3. Localization of Ii in ECVs in Mel Juso cells that were treated with leupeptin. Cells were allowed to internalize BSA-16 nm gold for 4 h, followed by an overnight chase in gold-free medium that contained 150 μg/ml leupeptin to label PLCs and lysosomes. Before preparation of cryosections, cells were incubated in medium (+ leupeptin) enriched with BSA-5 nm gold for 20 min to label early endosomes and ECV. Sections were labelled with anti-IiN antiserum and Protein A-9 nm gold (large arrowhead). Colocalization of 5 nm gold (small arrows) and Ii is found in the ECV. Note that the labelling for Ii is predominantly associated with the outer membrane of ECV. Adjacent to the ECV is a structure containing the 16 nm gold (large arrow) that is devoid of Ii or small gold. From its morphological appearance and complete lack of Ii this structure is probably a lysosome. Bar, 100 nm.

Table 1. *Quantification of the effect of leupeptin on Ii labelling in ECV*

Ii labelling	− Leupeptin	+Leupeptin
No. gold particles per ECV membrane outer profile (gold/μm)	0.225 ± 0.042	0.224 ± 0.039
No. gold particles per ECV area (gold/μm^2)	0.76 ± 0.29	0.84 ± 0.20

Number of micrographs: − leupeptin, 34; + leupeptin, 18. Background: unspecific labelling was estimated by counting the gold particles over the nucleus (0.29 particle/μm^2).

magnification by electron microscopy. In these preparations the ECV are easily identified both by their morphological features and, in this experiment, by the presence of reaction product for HRP. Comparison of Fig. 4 (− leupeptin) and 5 (+ leupeptin) shows that leupeptin treatment does appear to increase the number of ECVs per cell. The typical multivesicular body-like morphology of these spherical endocytic vesicles in these plastic sections is shown in Fig. 6. In Fig. 7 three examples are shown of the structure of the ECV in cryosections either unlabelled (7A) or following labelling with the anti-IiN (7B) or anti-IiC (7C). The tubular appearance of the internal membranes of the ECV is especially evident in these images as well as apparent continuities between the outer and inner membranes (Fig. 7A).

Quantification of the number of ECV

We next quantified the number of ECVs per cell in leupeptin-treated and untreated cells using a stereological approach (see Materials and methods). For this, we first estimated the mean cell volume of both sets of cells. This parameter provided a reference for the subsequent determination of the volume density of ECVs. As shown in Table 2, leupeptin had no significant effect on mean cell volume. Using a point counting method the volume fraction of ECV

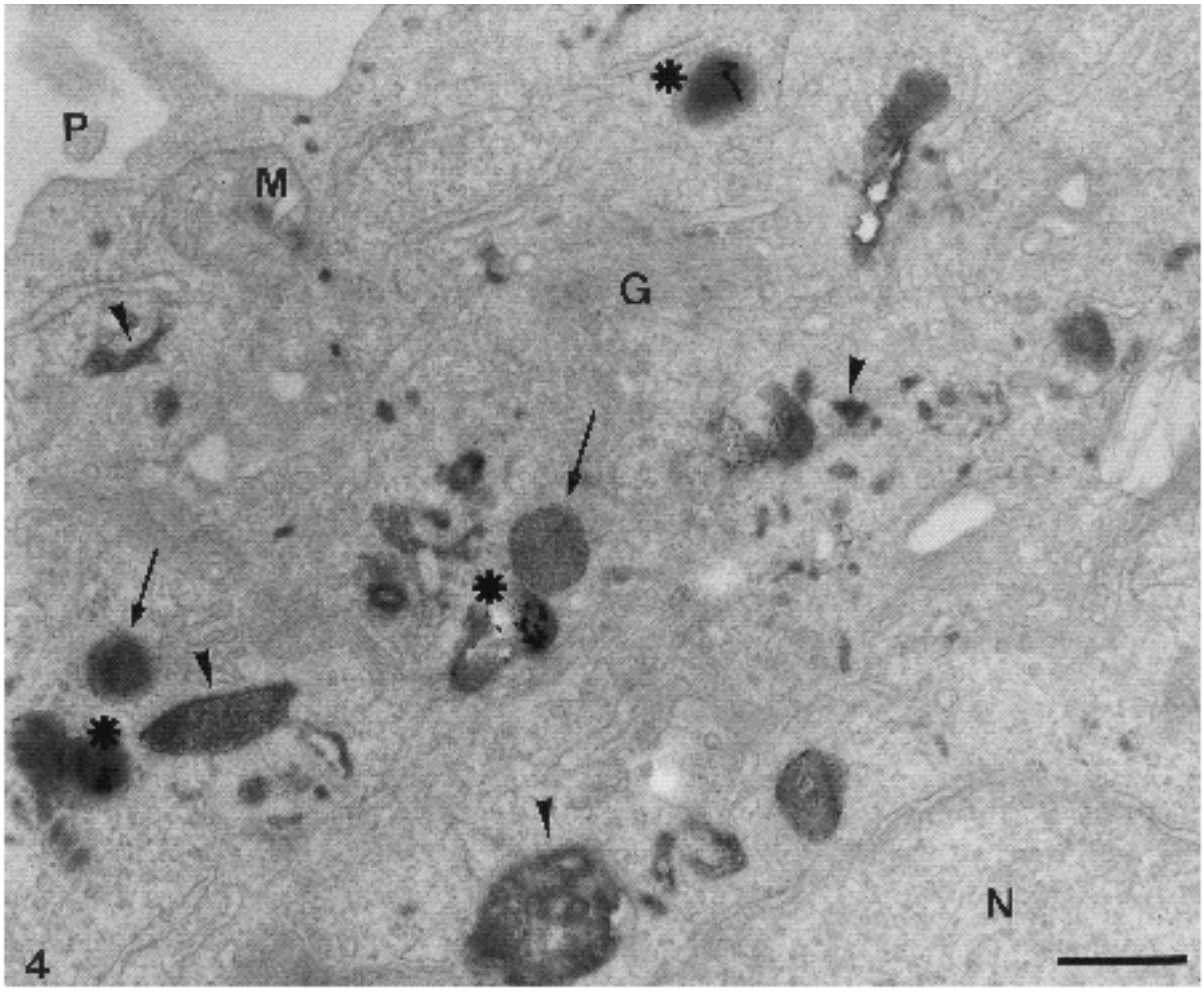
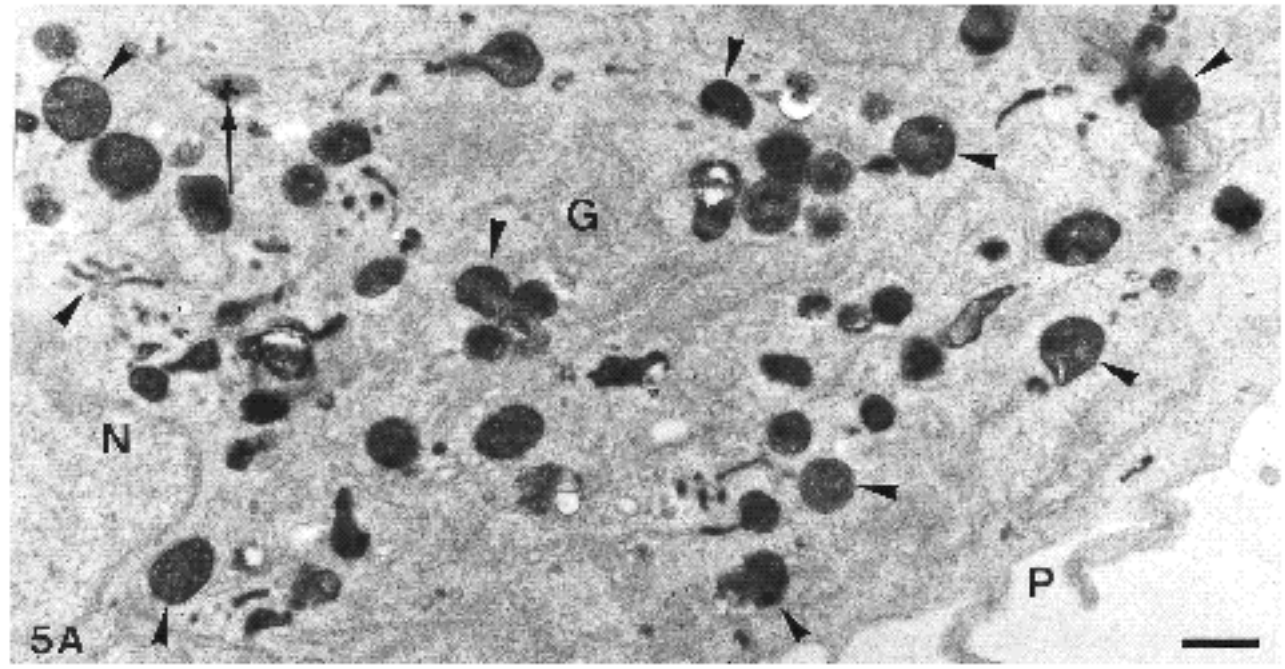


Fig. 4. Epon sections of control (untreated) Mel Juso cells that had internalized BSA-gold and HRP to identify endocytic compartments. Cells were incubated with BSA-16 nm gold for 4 h, followed by a 10 h chase in medium containing 5 μg/ml HRP. By this procedure the gold-marker enriches in PLCs and lysosomes and all endocytic compartments are labelled with HRP. The HRP reaction product fills a number of vesicles close to the Golgi stack (G). ECVs can be identified by their typical morphology (arrows). Other HRP-positive vesicles with a more irregular shape are probably early and late endosomes (arrowheads). Compartments that are HRP-positive and



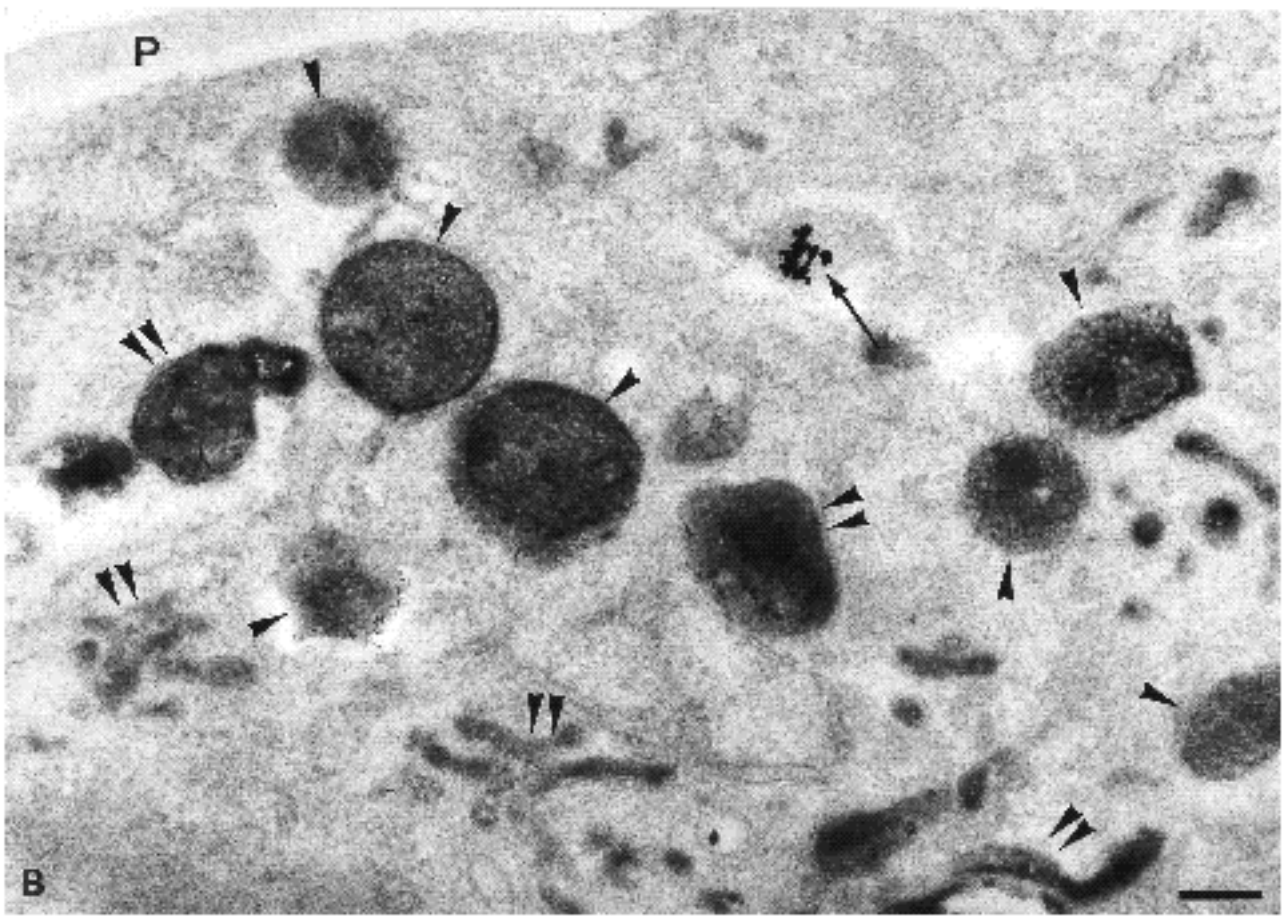


Fig. 5. Epon sections of leupeptin-treated Mel Juso cells that had internalized BSA-gold and HRP to identify endocytic compartments. Cells were incubated for 4 h with BSA-16 nm gold and chased for 10 h in medium that contained 150 μg/ml leupeptin and 5 mg/ml HRP. A low-magnification image of a cell is shown in (A), where the HRP-positive ECVs (arrowheads) are the predominantly reactive structures. One prelysosomal or lysosomal structure (arrow) labelled with BSA-gold is unreactive for HRP. G, Golgi; N, nucleus; P, plasma membrane. Bar, 0.5 μm. (B) A higher magnification image to show details of typical ECVs (single arrowheads) and other endocytic compartments (double arrowheads) that are HRP-positive. One PLC or lysosome (arrow) containing BSA-gold is HRP-

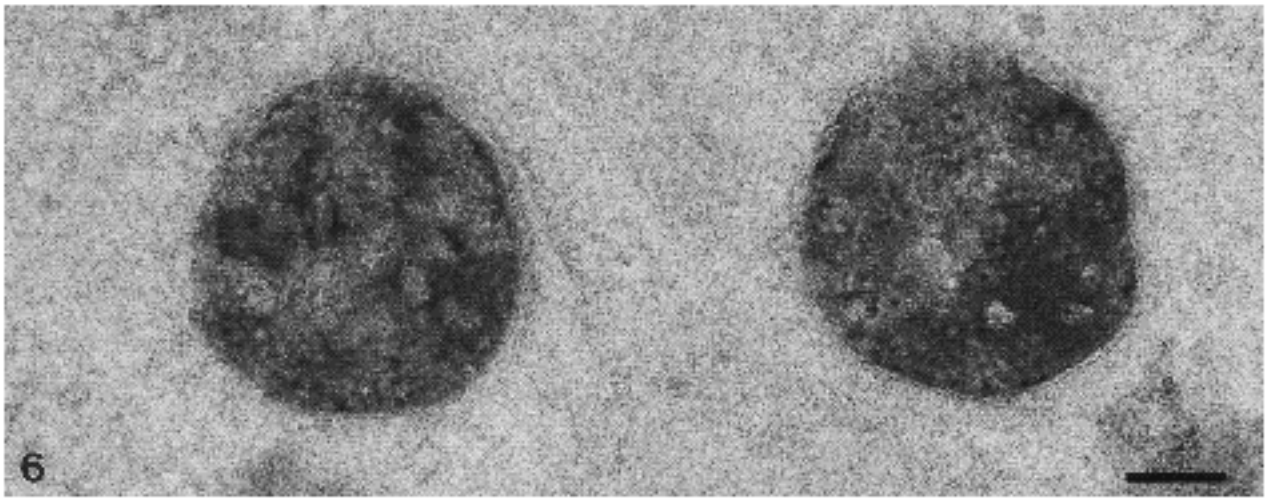


Fig. 6. Structure of ECVs in Epon sections. The spherical multivesicular body-like morphology is evident. Bar, 100 nm.

per cell was next estimated. There was clearly a significant increase in this parameter following leupeptin treatment, which led to a significant increase in their volume (Table 2). There was also a slight increase in the mean diameter of the ECV following leupeptin treatment. Assuming that the ECVs are spherical we can provide an estimate of the average volume of one ECV from the average diameter. The volume fraction of the ECV per cell multiplied by the mean cell volume gives the total volume of these vesicles per cell. This parameter, divided by the mean volume of the average vesicle, gives the average number of ECVs per cell. Leupeptin increases this number from 281 in the control to almost 550 in leupeptin-treated cells (Table 2).

Effect of leupeptin on transport of endocytosed material from ECV to PLC

The experiments described above demonstrate that leupeptin results in a significant increase in the number of ECVs per cell. It was previously argued that the ECVs function to transport endocytosed material from the early endosome to the PLC (see Griffiths and Gruenberg, 1991). We therefore investigated whether an increase in the number of ECVs seen following leupeptin treatment had any effect on the transport of an endocytic marker from the ECV to the PLC. For this, cells were first allowed to internalize HRP for 10 h either with or without leupeptin. PLCs and lysosomes were labelled with BSA-16 nm gold as described

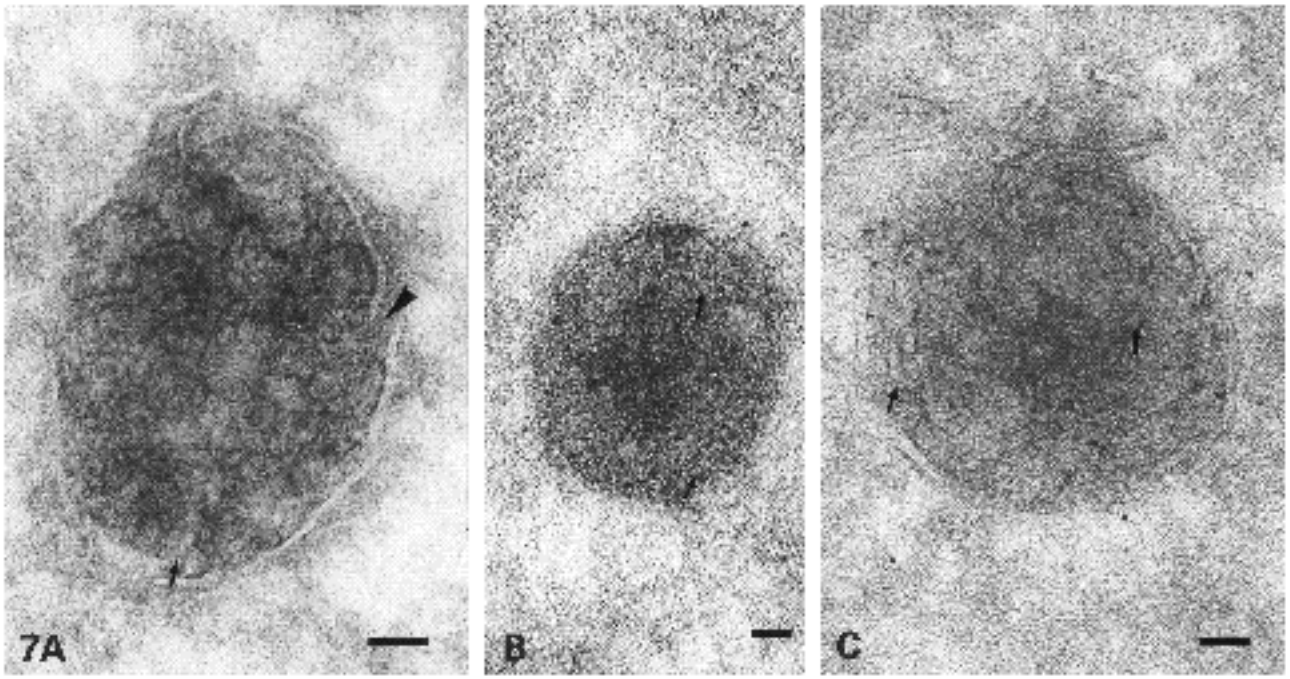


Fig. 7. Examples of ECV images in cryosections. (A) is unlabelled, while (B) and (C) are from cells labelled with anti-IiN and -IiC, respectively, followed by Protein A-gold. The arrows indicate the tubular profiles within the lumen, while the arrowhead in A shows a possible continuity between the outer membrane and an internal membrane profile. Bars, 50 nm.

Table 2. *Stereological ECV estimation of the number of ECVs per cell*

	− Leupeptin	+ Leupeptin
Nuclear volume (μm^3)*	421.46 ± 62.77	507.10 ± 76.10
Volume density, nuclei per cell (% of total cell volume)†	39.67 ± 5.26	42.03 ± 7.38
Cell volume (μm^3)	1062.41 ± 158.91	1206.52 ± 181.10
Cytoplasm volume (μm^3)	640.70 ± 95.40	698.99 ± 104.76
ECV diameter (μm)	0.258 ± 0.038	0.350 ± 0.044
Average volume of one ECV (μm^3)	0.009 ± 0.001	0.0224 ± 0.0028
Volume density ECV‡ (% of cell volume)	0.394 ± 0.072	1.759 ± 0.192
Total volume ECV/cell (μm^3)	2.53 ± 0.357	12.29 ± 1.84
Total number ECV/cell	281.11± 41.69	548.66± 82.35

*Number of nuclear profiles, 275.
†Number of micrographs, 20.
‡Number of micrographs, 30.

Table 3. *Quantification of the effect of leupeptin on endocytosed HRP in ECVs and PLCs*

HRP labelling (gold/μm^2)	− Leupeptin (n = 30)	+ Leupeptin (n = 25)
Gold per area ECV	99.45 ± 10.90	94.88 ± 10.74
Gold per area PLC	84.85 ± 17.52	41.56 ± 10.75

Background: unspecific labelling was estimated by counting the gold particles (6 and 9 nm) over the nucleus (0.33 particles/μm^2). The ECVs were defined on morphological criteria; that is, spherical, multivesicular-body-like vesicles; while the PLC was defined as profiles containing at least 3 gold particles labelling the MPR. *n*, number of micrographs.

above. As shown in Figs 3 and 4, the reaction product of the endocytic marker HRP was seen in typical endocytic structures in both treated and untreated cells. These included the ECVs, which, as expected, appeared more numerous in the cells treated with leupeptin. However, the late endocytic organelles (which include the PLC) that were labelled with 16 nm internalized gold particles showed much less reaction product for HRP in the leupeptin-treated cells. This suggested that in these cells the HRP was being transported less efficiently to the late endocytic structures.

In order to characterize this effect in more detail the following experiment was performed. The cells, with or without leupeptin were allowed to internalize BSA-16 nm gold followed by HRP for 10 h as described above. Cryosections were then prepared and were double-labelled with antibodies specific for HRP and MPR. The latter serves to identify the PLC, which was also labelled with the 16 nm gold. The ECVs were again identified by morphological criteria. The amount of HRP present in the PLC was quantified using anti-HRP and Protein A-gold in untreated and leupeptin-treated cells (Fig. 8). As shown in Table 3, following leupeptin treatment the density of labelling for HRP in the ECVs was not influenced by the protease inhibitor. In untreated cells the density of labelling for HRP in the PLC reached similar values to that seen in the ECV. However, in leupeptin-treated cells the labelling of HRP in the PLC was reduced by 50%. Collectively, these data argue strongly that leupeptin significantly inhibits the delivery of the endocytic marker from the ECV to the PLC.

Discussion

Leupeptin is a protease inhibitor that mainly inhibits thiol proteases. It has been extensively used in both in vitro and in vivo studies to block protein degradation. When added to the culture medium of living cells it is taken into the endocytic pathway, presumably by fluid-phase endocytosis. In principle, it could block thiol proteases anywhere in this pathway. When used in this fashion it can block the degradation of the MHC class II-associated invariant chain. In previous studies this block in degradation coincided with the accumulation of an intermediate form of the Ii, P22 (Pieters et al. 1991). The simplest interpretation of those data was that the protease(s) responsible for the further degradation of P22 to P18 and P12 were inhibited by leupeptin. In the present study an alternative interpretation appears equally possible: that the further degradation of P22 is inhibited because of a transport block proximal to the PLC, a compartment expected to contain relatively high concentrations of proteases.

The immunofluorescence data show that with increasing times after adding leupeptin to the culture medium the Mel Juso cells showed a large increase in the number of vesicles that were labelled with the anti-IiN terminus, but not the anti-IiC terminus, antibody. This argues that these vesicles are enriched in molecules of Ii which have been proteolytically processed at their luminally orientated C terminus. As seen by electron microscopy these vesicles correspond to the multivesicular body-like ECV. In a previous study these structures could be labelled with antibodies against both the N and C terminus of Ii (Pieters et al., 1991). The accumulation of these vesicles following leupeptin treatment coincided with the accumulation of P22. This suggests that under normal conditions the proteolytic step from the mature Ii to this P22 form either occurs predominantly in the ECV, or at least begins in these structures (or earlier) and continues in the PLC. The finding that the PLC can be significantly labelled with both anti-IiN and anti-IiC antibodies in the absence of leupeptin (Pieters et al., 1991) argues more for the latter possibility.

From a previous study on the endocytic pathway it has been proposed that the ECV bud off the early endosome and are transported in a microtubule-dependent fashion to the perinuclear region of the cell where they fuse with the PLC (see Griffiths and Gruenberg, 1991, for review). The results of the present study are entirely consistent with this view. The doubling in the number of ECVs by leupeptin coincided with a significant decrease in the delivery of HRP into the PLC. This effect of leupeptin is consistent with the data of Tolleshaug and Berg (1981), who showed that pretreatment of rat hepatocytes with leupeptin blocked the degradation of internalized asialofetuin, an effect that correlated with an inhibition in transport from low density vesicles to high density lysosomes.

The simplest interpretation of our data is that leupeptin does not affect the formation of the ECV, but rather some step leading to their fusion with the PLC. It is an intriguing question as to how the presence of a protease inhibitor on the luminal side of a compartment (at least predominantly) could affect an event that occurs on the cytoplasmic face of its membrane. One possibility is that the accu-

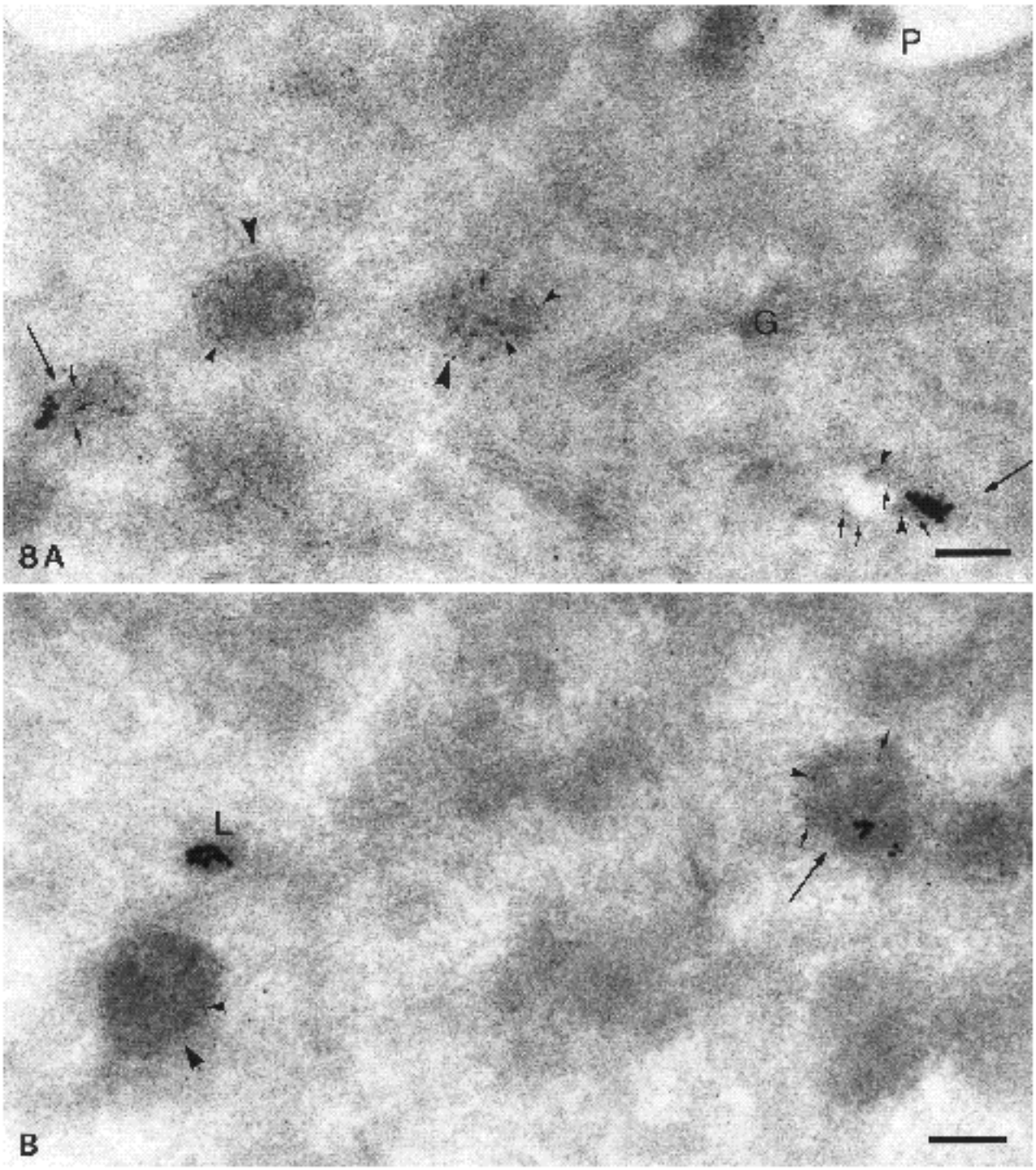


Fig. 8. Examples of images from the triple-labelling of Mel Juso cells, used to quantify the amount of HRP that is transported from ECVs to PLCs. Cells were allowed to internalize BSA-16 nm gold for 4 h, followed by a 10 h chase to label PLC (large arrows) and lysosomes (L). The chase medium contained HRP to label all endocytic structures. (A) Shows the control cell, while (B) indicates the leupeptin-treated cells. Cryosections were prepared and double-labelled with anti-HRP and 9 nm gold (small arrowheads) and anti-MPR and 5 nm gold (small arrows). (A) Shows two vesicular profiles (large arrows) that contain internalized BSA-gold, are also positive for HRP and MPR, and which we classify as PLC. Two ECVs (large arrowhead) that are HRP-positive are located close to a Golgi-stack (G). One of these ECVs contains two gold particles for MPR. P, plasma membrane. In (B) an ECV (large arrowhead) is labelled with HRP but not with MPR and is seen adjacent to a profile of a lysosome (L) that is characteristically amorphous, MPR-negative and contains the internalized BSA-gold. In this example no HRP is seen. On the right a typical PLC profile is seen (large arrow) that is significantly labelled with both HRP and MPR. It also contains the 16 nm internalized gold (unmarked). Bar, 0.2 μm.

mulation of undegraded molecules in the PLC leads to release of a factor(s) into the cytosol, which blocks the binding or fusion of the ECV with the PLC. This would be an example of feedback inhibition which could serve to regulate the entry of material from the endocytic pathway into the PLC. That this step is somehow regulated is suggested by our observations that the number of ECVs appears to vary greatly between different cell types. Thus, while Mel Juso cells have relatively high numbers, even without leupeptin, these structures are very difficult to find in MDCK cells unless their transport is blocked with the microtubule-depolymerizing drug nocodazole (Parton et al., 1989; Bomsel et al., 1990). In a recent study it was also found that the ECV to PLC step was blocked in macrophages that had been allowed to accumulate wheat germ agglutinin (Rabinowitz et al., 1992); in the latter study the lectin responsible for the block was only present on the luminal side of the endocytic organelles. An alternative possibility to explain how leupeptin could inhibit fusion of ECVs with the PLCs is that this process is dependent on a fusion-active transmembrane protein. This protein might be activated on its cytosolic side by being degraded from its luminal side. In leupeptin-treated cells the protease that normally generates the fusion-active protein could be inhibited and the ECVs would accumulate. Further studies are needed to determine the actual mechanism of inhibition.

In this paper we use the term ECV for an endocytic compartment that is reached by the endocytosed marker HRP after having passed early endosomes and before reaching late endosomal structures. The ECVs are often referred to as multivesicular bodies because of the vesicular profiles that fill their lumen. This term usually assumes that vesicular profiles represent free vesicles that have budded into the lumen and that membrane proteins destined for degradation are sorted into these vesicles (Hopkins, 1986; Felder et al., 1990; Hopkins et al., 1990; Warren, 1990). Our data in this and in earlier studies are inconsistent with this view. In the cell types we have studied, using thawed cryosections, most of the internal membranes are tubular rather than vesicular; at least some of the tubules are accessible to externally added markers in preparations in vitro (R. Parton and J. Gruenberg, unpublished data). Further, in some cell types we could detect two recycling receptors, transferrin receptor and MPR, on the internal membranes of these vesicles (Killisch et al., 1992). In contrast, as shown in this, as well as in our previous study (Pieters et al., 1992), the Ii, a molecule predominantly destined for degradation, is preferrentially associated with the outer membrane of the ECV. While the mechanism of biogenesis of the ECV is still unknown, these observations contradict the MVB model in its present form.

Finally, our data have implications for antigen presentation, especially with regard to the intracellular transport of the MHC class II-Ii complex. As P22 accumulates in leupeptin-treated cells in ECVs, further proteolytic degradation of P22 might be obligatory for dissociation of the Ii from the MHC class II molecules and for the expression of MHC class II molecules on the cell surface (Neefjes and Ploegh, 1992). If some of these proteolytic events normally occur in the ECV, these organelles might be one of the sites where foreign antigens are proteolytically processed and bind MHC class II molecules. In fact, it has been shown that leupeptin does inhibit the presentation of some soluble proteins (Takahashi et al., 1989; Streicher et al., 1984). These observations were explained by assuming that leupeptin inhibits antigen processing. Our data show that the leupeptin effect is more complex. This drug not only inhibits proteolysis in the endocytic pathway but also reduces the transport rate from the cell surface to prelysosomal compartments.

Many thanks to Dr. Oddmund Bakke for valuable discussions and advice, and to Ruth Hollinshead and Heinz Horstmann for excellent support during the electron microscopic work. We thank Jean Gruenberg and Bernard Hoflack for kindly providing antibodies. Sabine Zachgo was supported by the DFG, grant DO/199/6-1.

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(Recieved 15 June 1992 - Accepted 23 July 1992)