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The effect of wortmannin on the localisation of lysosomal type I integral membrane glycoproteins suggests a role for phosphoinositide 3-kinase activity in regulating membrane traffic late in the endocytic pathway

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SUMMARY

Addition of wortmannin to normal rat kidney cells caused a redistribution of the lysosomal type I integral membrane proteins lgp 110 and lgp120 to a swollen vacuolar compartment. This compartment did not contain the cation independent mannose 6-phosphate receptor and was depleted in acid hydrolases. It was distinct from another swollen vacuolar compartment containing the cation independent mannose 6-phosphate receptor. The swollen lgp110-positive compartment was accessible to a monoclonal antibody against lgp120 added extracellularly, showing that it had the characteristics of an endosomal compartment. Wortmannin had no gross morphological effect on the *trans*-Golgi network or lysosomes nor any effect on the delivery to the *trans*-Golgi network of endocytosed antibodies against the type I membrane protein TGN38. We propose that the observed effects of wortmannin were due to inhibition of membrane traffic between cation independent mannose 6-phosphate receptor-positive late endosomes and the *trans*-Golgi network and to inhibition of membrane traffic between a novel lgp120-positive, cation independent mannose 6-phosphate receptor-negative late endosomal compartment and lysosomes. The effects of wortmannin suggest a function for a phosphatidylinositol 3-kinase(s) in regulating membrane traffic in the late endocytic pathway.

Key words: lgp120, Wortmannin, PI 3-kinase

INTRODUCTION

The endosomal compartment plays a central role in the intracellular transport and sorting of endocytosed ligands, membrane proteins and lysosomal enzymes from both the cell surface and the *trans*-Golgi network (TGN). The precise intracellular pathways and mechanisms underlying transport events from the cell surface to different destinations within the endocytic pathway have yet to be firmly established. Indeed, there remains a debate as to whether vesicular transport (Griffiths and Gruenberg, 1991) or endosome maturation (Murphy, 1991) is responsible for the passage of ligands from peripheral, early endosomes through deep lying, late endosomes en route to lysosome degradation. Although late endosomes have been identified morphologically as multivesicular bodies (Courtoy, 1991) and been shown in a cell-free system to be capable of content mixing with pre-existing lysosomes (Mullock et al., 1994), little is known about the molecular basis of their structure and function. To date, no resident late endosomal membrane proteins have been cloned and sequenced and it is not clear whether late endosomes are structurally and functionally homogeneous organelles. In addition to their role in transferring endocytosed ligands to lysosomes, late endosomes have also been identified as the prelysosomal compartment (PLC) through which newly synthesised lysosomal hydrolases pass during movement from the TGN to lysosomes (Griffiths et al., 1988). Mannose 6-phosphate receptors are responsible for targeting many such hydrolases to lysosomes (Kornfeld, 1986; Kornfeld and Mellman, 1989). Immunoelectron microscopic studies have shown that the cation independent mannose 6-phosphate receptor (M6PR) is not present in lysosomes but is enriched in the PLC (Griffiths et al., 1988). This compartment has also been shown to contain a proportion of lysosomal membrane glycoproteins including the type I integral membrane protein lgp120 (Geuze et al., 1988; Griffiths et al., 1990).

Recently, an approach that has yielded considerable information concerning the molecules which may be involved in directing proteins to lysosomes has been the generation of yeast mutants which are defective in sorting to the vacuole (Stack and Emr, 1993). One protein identified as playing a role in vacuolar sorting is the product of the *VPS34* gene (Herman and Emr, 1990). It has significant homology to the catalytic subunit of the mammalian phosphatidylinositide 3-kinase (PI3-K) thus implicating a function for a lipid kinase in intracellular membrane traffic as well as in signal transduction. Two recent studies (Brown et al., 1995; Davidson, 1995) have investigated the role of PI3-K in M6PR-dependent targeting of newly synthesized cathepsin D to lysosomes in mammalian cells using the microbial metabolite wortmannin, which at nM

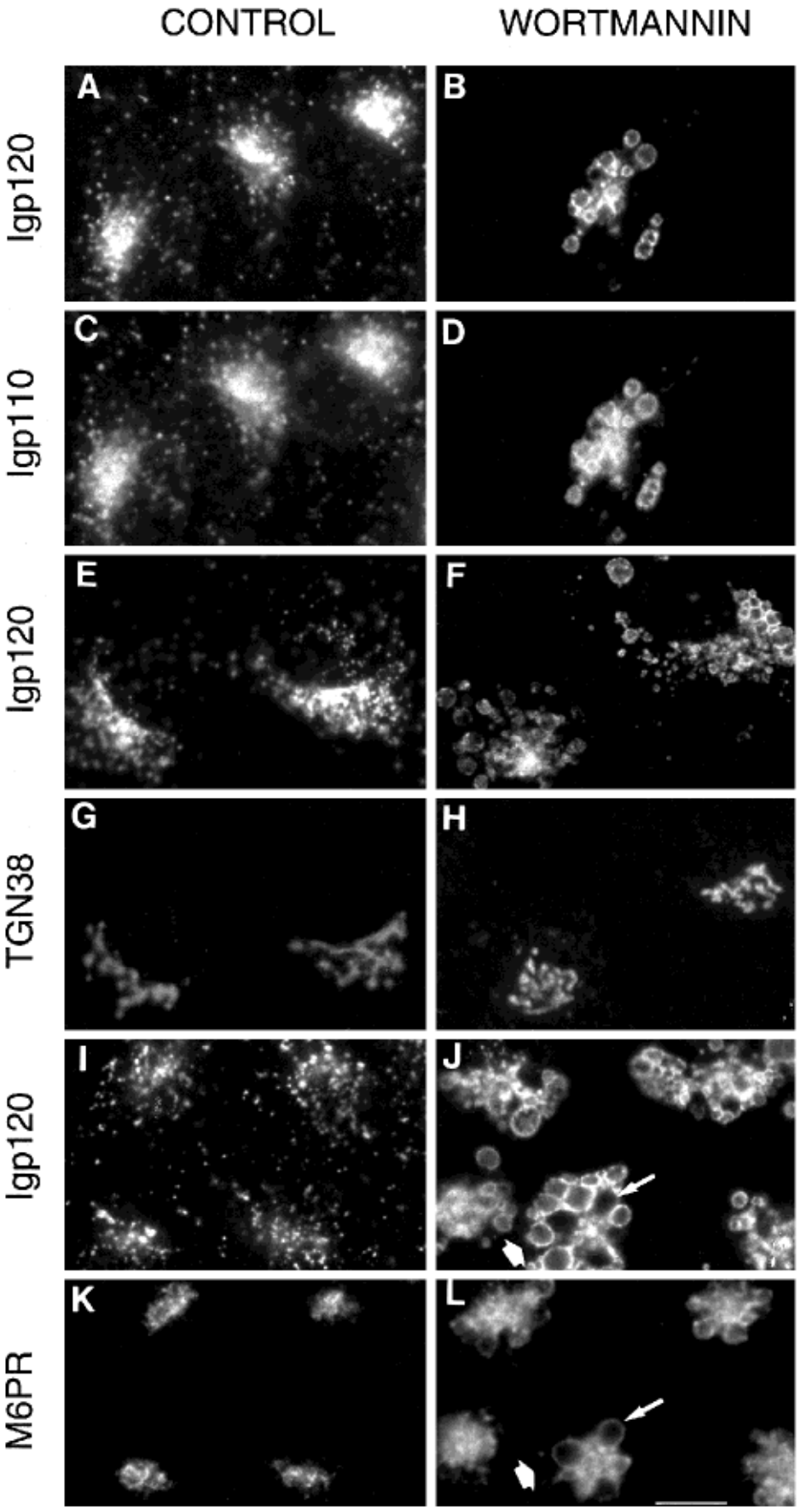

Fig. 1. Steady-state distribution of lysosomal type I integral membrane glycoproteins, TGN38 and M6PR in control and wortmannin-treated cells. NRK cells were incubated in the absence (A,C,E,G,I and K) or presence (B,D,F,H,J and L) of 100 nM wortmannin for 1 hour prior to methanol fixation and immunofluorescence microscopy. Cells were dual-labelled with a mouse anti-lgp120 antibody (A,B,E,F,I,J) and rabbit antibodies to lgp110 (C and D), TGN38 (G and H) and M6PR (K and L). The large arrowheads in J and L indicate lgp120-positive, M6PR-negative structures and the small arrows indicate lgp120-negative, M6PR-positive structures. Bar, 10 μm.

concentrations is a relatively specific inhibitor of PI3-K (Okada et al., 1994). In the presence of wortmannin, unprocessed cathepsin D was found to be secreted into the medium and there was a dramatic intracellular redistribution of M6PR into swollen vacuoles.

In the present study we have investigated the effects of wortmannin on the steady state distribution and endocytosis from the cell surface of the lysosomal type I integral membrane glycoproteins lgp120 (Lewis et al., 1985; Howe et al., 1988; Himeno et al., 1989; Akasaki et al., 1990) and lgp110 (Noguchi et al., 1989; Granger et al., 1990). We have observed a redistribution of both lgp120 and lgp110 into M6PR-negative and lysosomal hydrolase-negative swollen vacuoles. We interpret this in terms of wortmannin inhibition of a PI3-K involved in membrane traffic between a novel late endosomal compartment and lysosomes.

MATERIALS AND METHODS

Materials

Wortmannin was purchased from Calbiochem (Nottingham, UK) and was aliquoted and kept at −20°C as a 1 mM stock in DMSO. LY294002 was kindly provided by Dr P. Shepherd (University of Cambridge), aliquoted and kept at −20°C as a 10 mg/ml stock in DMSO. Nordihydroguaiaretic acid (NDGA) was purchased from Affiniti Research Products (Nottingham, UK) and made up fresh as a 1 mM solution in methanol. Lucifer Yellow, BSA, gold chloride, tannic acid and tri-sodium citrate were purchased from Sigma Chemicals (Poole, Dorset, UK).

The mouse monoclonal antibody to lgp120, designated GM10 (Grimaldi et al., 1987), was originally raised against rat insulinoma secretory granule membranes and was a gift from Professor K. Siddle and Dr J. C. Hutton. Its specificity for lgp120 was demonstrated by the ability to immunoblot a single band of M_r 120,000 after SDS-PAGE of rat liver lysosome preparations and by immunofluorescence of COS-7 cells transfected with a cDNA encoding lgp120 in the eukaryotic expression vector pMEP4 (data not shown). The rabbit polyclonal anti-rat lgp110 antiserum was prepared by immunisation with a Triton X-114 detergent extract of purified rat liver lysosomes. At dilutions of 1:1,000 and greater this antiserum reacted exclusively with a single band of M_r 110,000 on immunoblots after SDS-PAGE of rat liver lysosomes (data not shown). Its specificity was further demonstrated by immunofluorescence of COS-7 cells transfected with a cDNA encoding lgp110 in the eukaryotic expression vector pMEP4 (data not shown). The rabbit polyclonal anti-rat TGN38 antiserum (Luzio et al., 1990) and the mouse monoclonal anti-rat TGN38 N-terminal domain antibody (Horn and Banting, 1994) have been previously described. The rabbit polyclonal anti-rat M6PR antibody was raised to a fusion protein encoding glutathione S-transferase coupled to a portion of the cytoplasmic tail of rat M6PR equivalent to amino acids 2,347 to 2,487 of the human M6PR sequence (Oshima et al., 1988). The rat cDNA encoding this fragment of the M6PR cytoplasmic tail was selected and amplified using the polymerase chain reaction with appropriate primers and was ligated into pGEX-3X. The rabbit polyclonal anti-bovine M6PR antibody used for the immunoelectron microscopy studies was kindly provided by Dr Suzanne Pfeffer (Stanford University, Stanford, CA) (Pfeffer, 1987). The rabbit polyclonal anti-mouse cathepsin L antibody has been shown previously to cross-react with rat fibroblasts (Punnonen et al., 1994) and was a generous gift of Dr Michael Gottesman (National Cancer Institute, Bethesda, MD). Mouse anti-rat transferrin receptor antiserum was purchased from Chemicon International (Harrow, UK). FITC-labelled goat anti-mouse IgG and Texas Red-labelled donkey anti-rabbit IgG were obtained from Amersham (Little Chalfont, Bucks., UK). Goat anti-mouse IgG labelled with 10 nm gold and goat anti-rabbit IgG labelled with 15 nm colloidal gold were purchased from BioCell (Cardiff, UK). Protein A labelled with 15 nm or 10 nm gold was from the Department of Cell Biology, University of Utrecht.

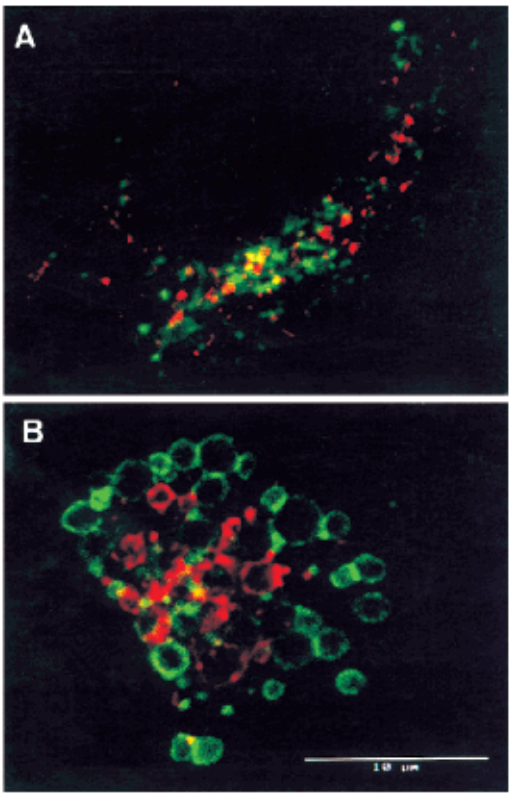

Fig. 2. Distribution of lgp120 and M6PR in control and wortmannin-treated cells. Confocal microscopy of control (A) and wortmannin-treated (B) NRK cells double-labelled with mouse anti-lgp120 (green) and and rabbit anti-M6PR (red) showing that lgp120 and M6PR are distributed in separate large vacuolar structures after wortmannin treatment. Bar, 10 μm.

Cell culture

Normal rat kidney (NRK) fibroblastic cells were grown in tissue culture flasks or on glass coverslips in DME, 10% FCS. Prior to treatment with wortmannin or other pharmacological agents cells were rinsed with PBS and the DME replaced by RPMI±1% FCS.

Fluorescence microscopy

Indirect immunofluorescence microscopy was performed as previously described (Reaves and Banting, 1992; Reaves et al., 1993), using NRK cells grown on glass coverslips. Cells were treated with wortmannin or other pharmacological agents as required, rinsed in PBS and fixed for 5 minutes in methanol at −20°C. Following incubation with antibodies and mounting, the cells were examined using a Planapochromat 63×, 1.4 lens on a Zeiss Axiophot microscope, or for confocal microscopy, a Nikon Optiphot-2 epi-fluorescence microscope equipped with a Bio-Rad MRC 1000 confocal laser scanning attachment (Bio-Rad, Hemel Hempstead, UK). In double-labelling experiments using the mouse monoclonal anti-lgp120 antibody and the rabbit anti-mouse cathepsin L antibody, coverslips were first fixed with 4% paraformaldehyde in PBS for 45 minutes prior to permeabilizing with methanol.

To examine Lucifer Yellow uptake, control and wortmannin treated cells were incubated in the presence of 1 mg/ml Lucifer Yellow for 3 minutes at 37°C prior to rinsing with ice-cold PBS, 3× 50 ml, mounting and examining as above.

Transmission electron microscopy of endocytosed gold

BSA was conjugated to monodisperse 10 nm gold particles prepared by tannic acid/ tri-sodium citrate reduction of gold chloride at pH 7 (Slot and Geuze, 1985). NRK cells were incubated with the conjugate for 4 hours at 37°C followed by incubation in conjugate-free medium for 20 hours at 37°C (Griffiths et al., 1988). Where appropriate cells were treated with 100 nM wortmannin for 1 hour at 37°C prior to processing for electron microscopy. The localisation of BSA-gold was determined using a Philips CM 100 transmission electron microscope.

Immunoelectron microscopy

Cells grown in tissue culture flasks were prepared for ultrastructural immunocytochemistry essentially as described by Griffiths (1993). Cells were fixed with 8% paraformaldehyde in 250 mM Hepes, pH 7.2, at room temperature for 1 hour. Frozen ultrathin sections were cut using a Reichert Ultracut S cryoultramicrotome equipped with a cryochamber attachment (Leica, Milton Keynes, UK), and immunolabelling was performed using the Protein A-gold technique (Slot and Geuze, 1983), as previously described (Bright and Ockleford, 1995). Dual-labeling of M6PR and lgp120 was accomplished by first labelling rabbit anti-M6PR antibodies with Protein A:15 nm gold, washing and fixing the complexes with 1% glutaraldehyde in PBS for 10 minutes. Unreacted aldehydes were quenched with 50 mM NH_4Cl in PBS and the sections incubated with mouse anti-lgp120 followed by goat anti-mouse Ig conjugated to 10 nm gold. The sections were rinsed with distilled water and embedded in 1.8% methyl cellulose/0.3% uranyl acetate (Tokuyasu, 1978). Grids were then air dried and observed in a Philips CM 100 transmission electron microscope. Localisations achieved using the double labelling technique were verified using goat anti-rabbit Ig conjugated to 15 nm gold and goat anti-mouse Ig conjugated to 10 nm colloidal gold.

Subcellular fractionation and western blotting

Tissue culture flasks (150 cm^2) were innoculated with ~2×10^6 NRK cells and grown to confluency prior to processing. Cells were incubated with or without 100 nM wortmannin for 1 hour at 37°C, removed from the flasks by trypsinization and suspended in 10 ml cold homogenization buffer (0.25 M sucrose, 10 mM Tris-HCl, pH 7.4, 1 mM EDTA). Homogenates were obtained by passing the cells through a stainless steel ball homogeniser (Balch and Rothman, 1985), 12 times at room temperature using a ball bearing 8.012 mm in diameter. Homogenates were centrifuged at 800 ***g*** for 10 minutes at 4°C to obtain a post-nuclear supernatant. Aliquots (0.5 ml) of the post-nuclear supernatant were loaded onto 4.5 ml, linear 0.25 M sucrose-25% Nycodenz gradients with a 0.3 ml 45% Nycodenz cushion and centrifuged to equilibrium essentially as previously described (Mullock et al., 1994). Fractions (0.15 ml) were collected and assayed for β-hexosaminidase (N-acetyl-β-glucosaminidasesaminidase) as previously described (Mullock et al., 1994). SDS-PAGE and electrophoretic transfer of samples onto nitrocellulose were as previously described (Luzio et al., 1990). Incubation of the nitrocellulose with mouse anti-lgp120 was followed by washing and sequential incubation with a rabbit anti-mouse IgG and ^{125}I-labelled Protein A. After washing and autoradiography, quantitative analysis was done using an LKB densitometer or a Fuji Bas2000 Bio-imaging Analyzer System.

RESULTS

Lysosomal membrane glycoproteins and M6PR, but not TGN38, appear in swollen vacuolar structures after wortmannin treatment

To assess the effect of wortmannin on the steady state distribution of lgp120, NRK cells grown on coverslips were treated with 100 nM wortmannin for 1 hour at 37°C prior to fixation and processing for immunofluorescence microscopy. The pattern of staining obtained with the mouse monoclonal anti-lgp120 antibody differed dramatically in control (Fig. 1A), and wortmannin treated cells (Fig. 1B). In contrast to the small punctate structures found throughout the cytoplasm and concentrated in the perinuclear region in untreated cells, anti-lgp120 labelled large vacuolar structures in the perinuclear region in wortmannin treated cells. Peripheral punctate staining was diminished after wortmannin treatment. Results from dose response curves ranging from 25 nM to 1.6 μM indicated that this effect could be seen in ~20% of cells at 50 nM but was apparent in the majority of cells at 100 nM and did not change to any great extent above this concentration. Consequently, in further experiments the dose of wortmannin used was 100 nM. The redistribution also occurred after treatment of cells with another very specific inhibitor of PI3-K, LY294002 (Vlahos et al., 1994), but at a 1,000-fold higher concentration (100 μM in contrast to 100 nM for wortmannin). In view of reports that wortmannin inhibits activated phospholipases (Bonser et al., 1991; Cross et al., 1995), and that inhibitors of phospholipase A_2 prevent intracellular membrane traffic events including early endosome fusion (Mayorga et al., 1993) and intra-Golgi protein transport (Tagaya et al., 1993), the effects of the membrane permeable phospholipase A_2 inhibitor NDGA (Tagaya et al., 1993), were examined. NDGA inhibits intra-Golgi protein transport with an IC_{50} of 9 μM, but in the present study no unusual staining pattern of lgp120 was observed after treatment with 40 μM NDGA for 30 minutes at 37°C and no intracellular vacuolar structures were seen in the treated cells (data not shown).

To determine whether the effect of wortmannin on lgp120 was observed for other lysosomal membrane glycoproteins, double-labelling experiments were carried out using a polyclonal antiserum which recognizes lgp110. Colocalization of lgp120 and lgp110 was demonstrated not only in control cells (Fig. 1A,C) but also in the swollen structures in wortmannin treated cells (Fig. 1B,D) indicating that the staining pattern observed was not unique to lgp120. Wortmannin had no obvious effect on the steady state distribution of TGN38 (Fig. 1H) showing that it did not affect the morphology of all organelles in the endocytic/exocytic system.

It has recently been demonstrated that treatment of rat clone 9 hepatocytes with 1 μM wortmannin results in the appearance of large swollen vacuoles that are M6PR positive (Brown et al., 1995). It seemed a likely possibility that the vacuoles containing lgp120 may arise from the M6PR-positive PLC. Cells were incubated in the presence or absence of wortmannin and subjected to double-label immunofluorescence analysis using the mouse anti-lgp120 monoclonal antibody and a rabbit anti-rat M6PR polyclonal antiserum. In control cells the M6PR (Fig. 1K) was concentrated in small punctate structures in the perinuclear region of the cell. Surprisingly, although both M6PR and lgp120 appeared in structures with similar swollen vacuolar morphology after wortmannin treatment, there was little overlap in distribution (see arrows in Fig. 1J,L). The lack of colocalisation of the swollen vacuoles was confirmed in individual optical sections taken on the confocal microscope (Fig. 2).

In both lgp120-positive and M6PR-positive swollen vacuoles, spots of intense staining were seen around the periphery and occasionally within the lumen of the vacuole. In order to investigate the localisation of these proteins further

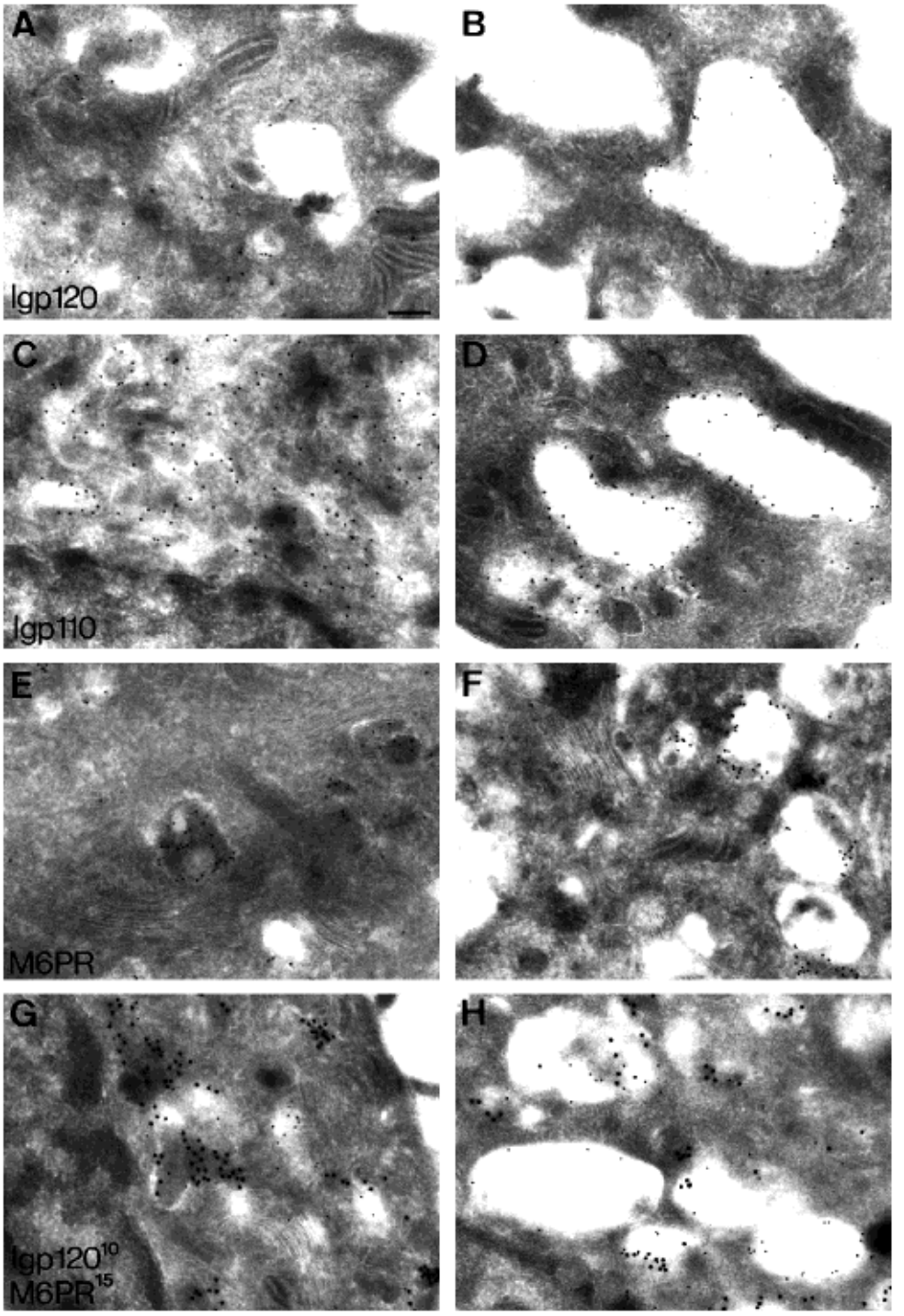

Fig. 3. Immunoelectron microscopic analysis of lgp110, lgp120 and M6PR in control and wortmannin-treated cells. Ultrathin frozen sections of control (A,C,E,G) and wortmannin-treated (B,D,F,H) cells were either single-labelled with anti-lgp120 (A,B), anti-lgp110 (C,D), anti-M6PR (E,F) or dual-labelled (G,H) with anti-lgp120 (10 nm gold) and anti-M6PR (15 nm gold). Note some vacuolar structures apparent in wortmannin-treated cells are not labelled in the sections processed for single-labelling studies (B,D,F). Bar, 200 nm.

and to confirm the localisation found by light microscopy, cells were processed for immunoelectron microscopy. In single-labelling experiments both lgp110 and lgp120 labelling was found in small heterogeneous structures throughout the cytoplasm in control cells (Fig. 3A,C). Occasionally, some labelling was observed in vacuolar structures in control cells. M6PR labelling was found in multi-lamellar bodies and tubular elements close to the stack of Golgi cisternae (Fig. 3E). After treatment with wortmannin large vacuolar structures were apparent. Although approximately half of these vacuoles were labelled with lgp120 (Fig. 3B) or lgp110 (Fig. 3D), some structures were devoid of any labelling. M6PR was also found in swollen vacuoles but these were often smaller than the lgp110 or lgp120 positive structures (Fig. 3F). In dual-labelling experiments in both control and wortmannin treated cells, M6PR-positive structures containing a small amount of lgp120 labelling were observed (Fig. 3G,H). In contrast many lgp120-positive structures showed no M6PR labelling. After wortmannin treatment, labelling of both M6PR-positive and lgp120-positive structures was seen along the peripheral membrane of swollen vacuoles and concentrated over dense structures within the vacuoles (Fig. 3H).

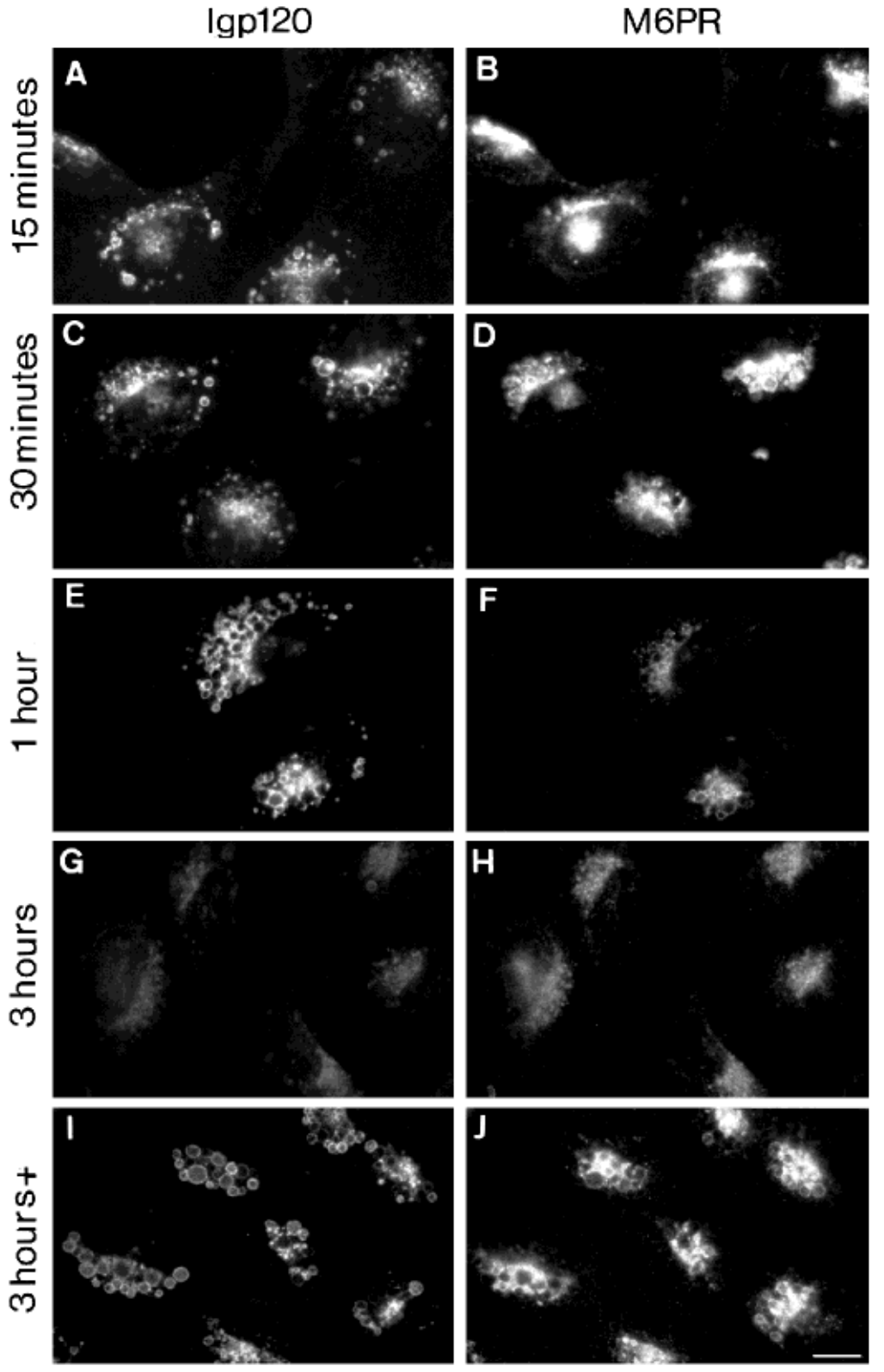

Fig. 4. Time course of wortmannin-induced redistribution of lgp120 and M6PR. Cells were fixed and double-labelled for lgp120 (A,C,E,G and I) and M6PR (B,D,F,H and J) after 15 minutes (A,B), 30 minutes (C,D), 1 hour (E,F) and 3 hours (G,H) in RPMI, 1% FCS containing 100 nM wortmannin. To test whether the loss of vacuoles was due to inactivation of the wortmannin, medium was replaced with fresh wortmannin every hour up to 3 hours and the cells processed as above (I,J; 3 hours +). Bar, 10 μm.

Time course of redistribution and recovery of M6PR and lgp120

Although the lgp120- and M6PR-labelled compartments in wortmannin-treated cells appeared to be quite distinct after 1 hour of wortmannin treatment, there remained the possibility that the kinetics of redistribution could be different. After the addition of wortmannin for 15 minutes lgp120-positive swollen vacuoles were clearly visible in some cells and compared to control cells there was a loss of punctate staining in the cell periphery (Fig. 4A; compare with Fig.

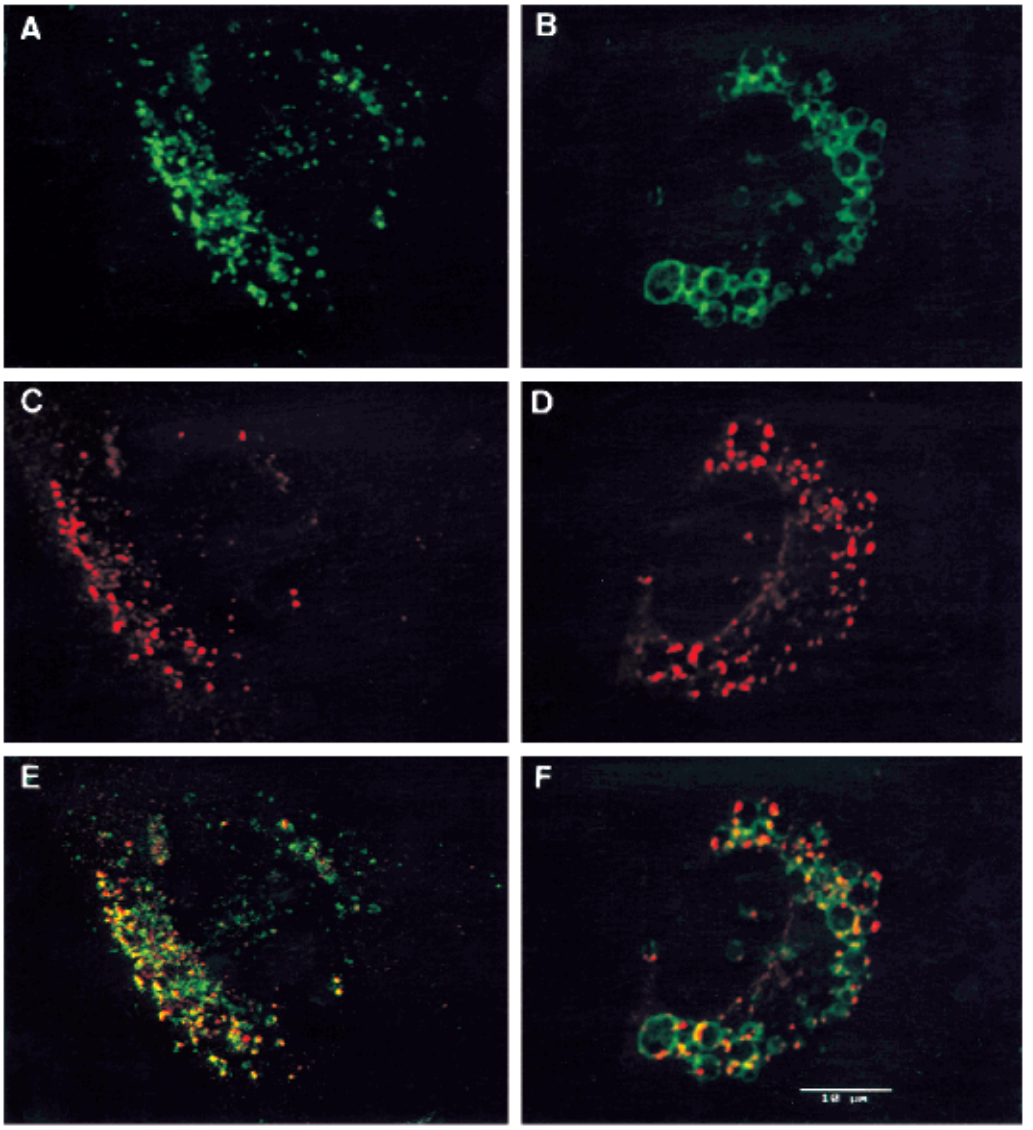

Fig. 5. Cathepsin L does not appear in wortmannin-induced swollen vacuoles. Mouse anti-rat lgp120 detected with goat anti-mouse FITC (A,B) and rabbit anti-mouse cathepsin L detected with donkey anti-rabbit TRITC (C,D) was visualized using confocal microscopy. In control cells (A,C) co-localisation of lgp120 and cathepsin L is seen as shown in the merged image of A and C (E). In wortmannin-treated cells (B,D) there is a lack of co-localisation of cathepsin L with the lgp120 positive vacuoles as shown in the merged image of B and D (F). Bar, 10 μm.

1A). The effect of wortmannin on lgp120-positive structures was maximal between 30 minutes and 1 hour (Fig. 4C,E) by which time the swollen vacuolar structures had reached their maximum diameter. However, by 3 hours (Fig. 4G) there was a dramatic loss of the large immunolabelled vacuolar structures and reappearance of some punctate lgp120 staining at the cell periphery. The time course of M6PR redistribution into swollen vacuolar structures was similar (Fig. 4B,D,F). By 3 hours after addition of wortmannin there was also a loss of M6PR-positive vacuolar structures (Fig. 4H). To test whether the loss of both lgp120-positive and M6PR-positive vacuolar structures after 3 hours of treatment was due merely to an inactivation of the wortmannin over time, medium from wortmannin-treated cells was replaced with fresh medium containing the same concentration of wortmannin every hour up to 3 hours. As can be seen in Fig. 4I and J (3 hours +), immunolabelled vacuolar structures were still apparent suggesting that over the time course of the experiment shown in Fig. 4A-H there was a loss of activity of the wortmannin.

Wortmannin treatment causes redistribution of lysosomal glycoproteins but not lysosomal content markers

To investigate whether the vacuoles containing lgp120 simply corresponded to enlarged lysosomes, the distribution of lgp 120 and the soluble lysosomal enzyme cathepsin L was determined by confocal microscopy on double-labelled control and wortmannin treated cells. In control cells, the majority of cathepsin L in the perinuclear area colocalised with lgp120 (Fig. 5A,C,E). After wortmannin treatment the cathepsin L staining remained in discrete punctate structures and did not co-localise with the large vacuolar lgp120-positive structures (Fig. 5B,D,F). Similar results were obtained using COS-7 cells transfected with cDNA encoding rat lgp120 and stained for expressed rat lgp120 together with endogenous cathepsin D (data not shown).

These experiments suggested that the large vacuolar structures containing lgp120 were not derived from dense core lysosomes. To substantiate these findings we used uptake of BSA-gold to identify lysosomal structures in plastic embedded thin sections (Griffiths et al., 1988). After incubating NRK cells

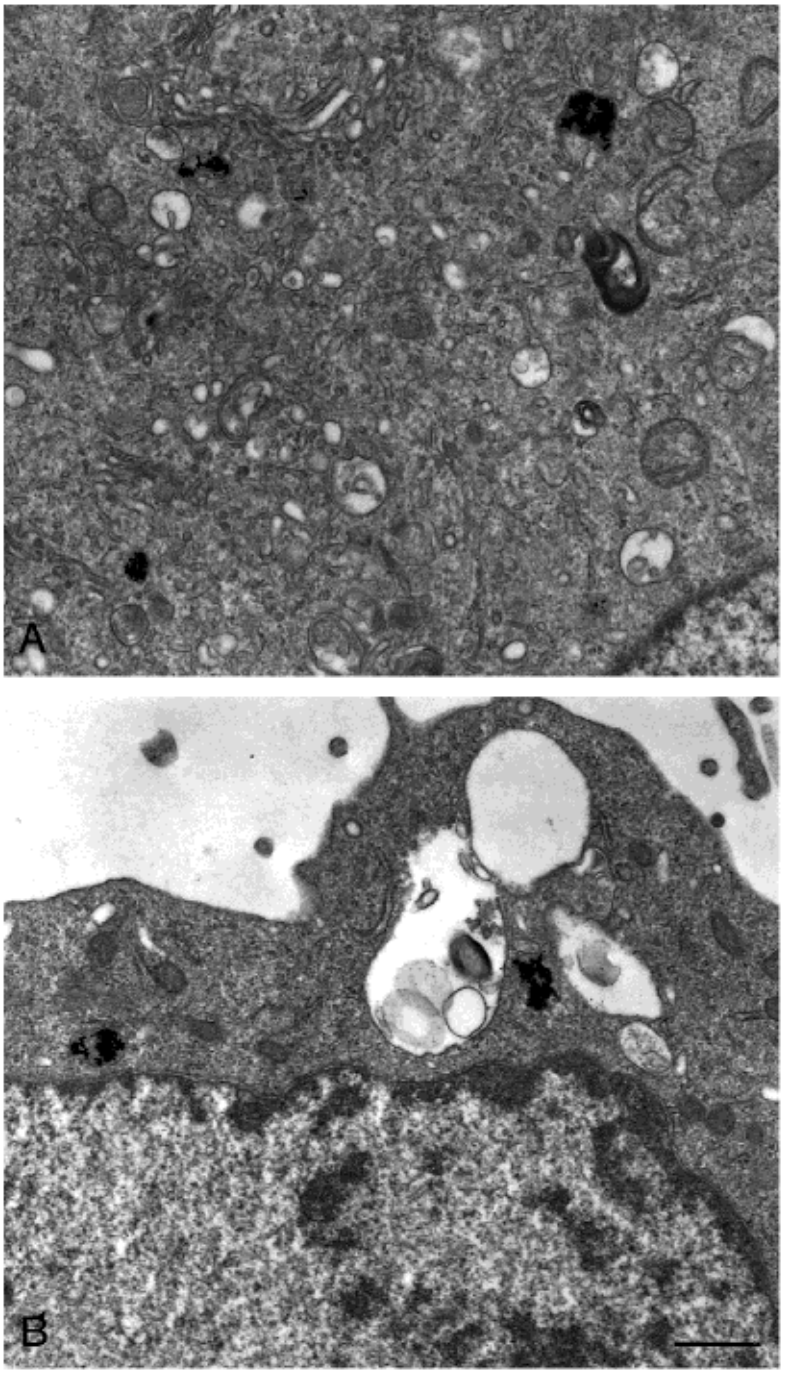

Fig. 6. Transmission electron microscopy of control and wortmannin-treated NRK cells after uptake of BSA:10 nm gold. Uptake of BSA coated 10 nm gold was performed as described in Material and Methods. In control cells (A) the BSA coated 10 nm gold is concentrated in the lumen of dense core lysosomes. After treatment with wortmannin these structures are unaffected (B) but are in close proximity to large vacuoles. Note the diminished appearance of the smaller vesicular structures found in the control cells. Bar, 500 nm.

with BSA-gold for 4 hours followed by a 20 hour chase period, the majority (>95%) of the BSA-gold was localized in organelles with the classical appearance of dense core lysosomes in both control and wortmannin treated cells (Fig. 6A,B). In the wortmannin treated cells (Fig. 6B), however, there was a pronounced decrease in small vesicular structures throughout the cytoplasm and a concommitant increase in large vacuolar structures in close proximity to the BSA-gold labelled organelles.

Data from subcellular fractionation studies were also consistent with a shift of lgp120 from lysosomes to a different compartment after wortmannin treatment. The distribution of β-hexosaminidase on isopycnic Nycodenz gradients was only slightly altered after wortmannin treatment (Fig. 7), but there was a decrease of lgp120 associated with the major β-hexosaminidase peak and a concommitant increase in a less dense region of the gradient (Fig. 7).

Wortmannin does not inhibit endocytic routes from the cell surface via early endosomes to the TGN and late endosomal compartments

It has previously been shown that antibodies directed to the lumenal domain of TGN38 may be added to the outside of intact cells to follow the endocytic route taken by TGN38 from the plasma membrane to the TGN (Ladinsky and Howell, 1992; Reaves et al., 1993). In order to test whether there was any gross inhibition of endocytosis and intracellular targeting by wort-

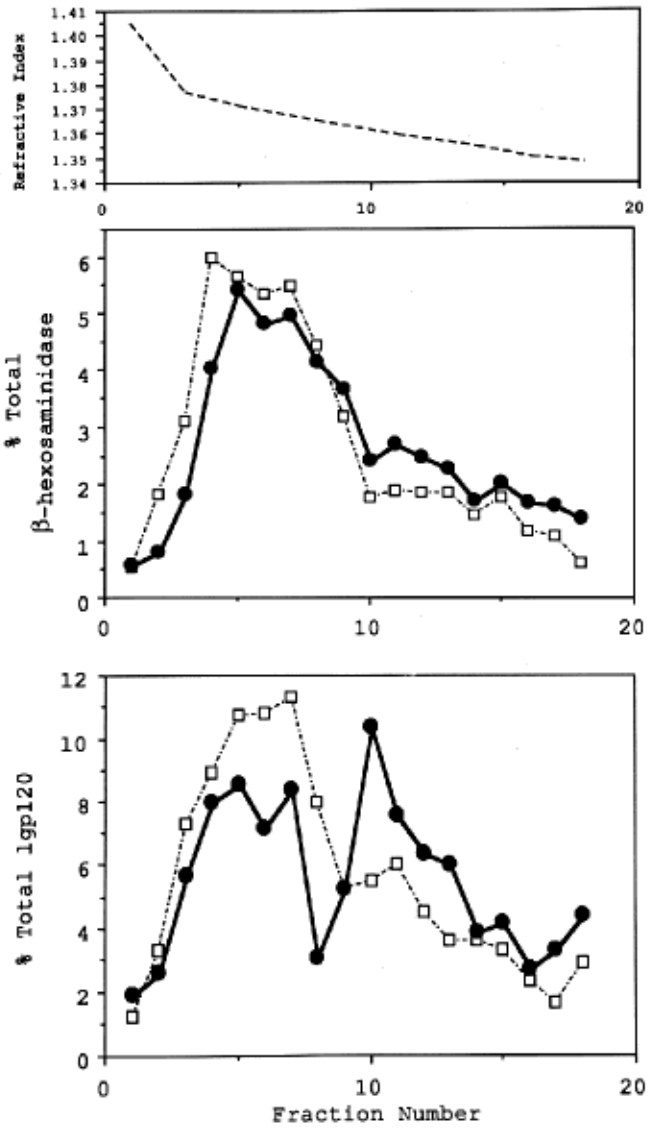

Fig. 7. Distribution of lgp120 and β-hexosaminidase after isopycnic density gradient centrifugation. Post-nuclear supernatants from control (□) and wortmannin (●)-treated cells were fractionated on continuous 1-25% Nycodenz gradients. Fractions were assayed for β-hexosaminidase activity and lgp120 immunoreactivity. Data are expressed as the percentage of total recovered activity in each case.

mannin, mouse monoclonal antibodies to the lumenal domains of lgp120 and TGN38 were added on the outside of cells for 2 hours in the presence or absence of wortmannin, prior to fixation. Cells were double labelled to identify the intracellular localisation of the endocytosed monoclonal antibodies and to compare this with the steady state localisation of lgp110 and TGN38 identified with rabbit polyclonal antibodies. Endocytosed anti-lgp120 antibodies were delivered to lgp110-positive punctate structures concentrated in the perinuclear region in control cells (Fig. 8A and C). After wortmannin treatment, endocytosed anti-lgp120 antibodies were still delivered to lgp110-positive compartments, but as in the previous experiments, these were mostly swollen vacuolar structures (Fig. 8B and D). The accumulation of endocytosed anti-lgp120 antibodies in the swollen lgp110-positive structures after wortmannin treatment indicated proper targetting but an inability to either reach and/or remain in dense lysosomes. The delivery of endocytosed anti-TGN38 antibodies to the TGN was not affected by wortmannin treatment (Fig. 8E-H).

The experiments described above demonstrated that endocytosed material could be delivered to lgp-positive large vacuoles, but not whether they corresponded to early or late endosomes. Lucifer Yellow uptake was examined to distinguish between endocytic compartments labelled at early versus late time points. It was found that in control cells, Lucifer Yellow endocytosed for short periods of time (<3 minutes) principally labelled peripheral punctate structures (Fig. 9A), consistent with labelling of early endosomes. Labelled structures had a similar appearance in wortmannin treated cells at early time points, but the intensity of labelling was diminished (Fig. 9B). After longer periods of uptake (>30 minutes) into wortmannin treated cells (Fig. 9D), Lucifer Yellow progressively labelled swollen vacuoles consistent with them being a late endosomal compartment. To test whether the peri-centriolar recycling compartment (Mayor et al., 1993) was also affected by wortmannin treatment, the steady-state distribution of the transferrin receptor was determined in control and treated cells. In control cells a diffuse pattern of punctate staining was seen in the perinuclear area. After wortmannin treatment, however, some transferrin receptor was found in M6PR-positive, lgp120-negative swollen vacuoles (data not shown).

DISCUSSION

The present experiments have demonstrated a dramatic redistribution of the type I lysosomal integral membrane glycoproteins lgp120 and lgp110 from lysosomes to swollen, M6PR-negative, late endosomal structures after treatment of intact NRK cells with wortmannin. A separate complement of swollen M6PR-positive, lgp120-negative structures was also observed subsequent to wortmannin treatment. The data presented raise two issues: the identity of the swollen compartments and the molecular mechanism of the wortmannin effect. The swollen compartments may be formed from the PLC, either as a result of inhibition of vesicle budding and outgoing membrane traffic without a concommitant inhibition of incoming traffic, elevated homotypic fusion, swelling, or a combination of these events.

A likely explanation for the appearance of the swollen M6PR-positive compartment subsequent to wortmannin treatment is the inhibition of retrograde transport to the TGN from the PLC. In control cells, vesicular transport in this direction is known to occur in order to recycle the M6PR for re-use and is dependent on the small GTP binding protein rab 9 (Riederer et al., 1994). Inhibition of this pathway may be sufficient to explain the effect of wortmannin in preventing delivery to lysosomes of newly synthesised pro-cathepsin D (Brown et al., 1995; Davidson, 1995). At steady state in NRK cells ~90% of M6PR is present in the PLC (Griffiths et al., 1990), so that inhibition of recycling to the TGN would be expected to have a rapid and dramatic effect on lysosomal delivery pathways utilising this receptor. However, we cannot rule out a separate and additional effect at the level of the TGN which prevents correct sorting of M6P tagged proteins.

The origin of the swollen lgp120-positive, M6PR-negative

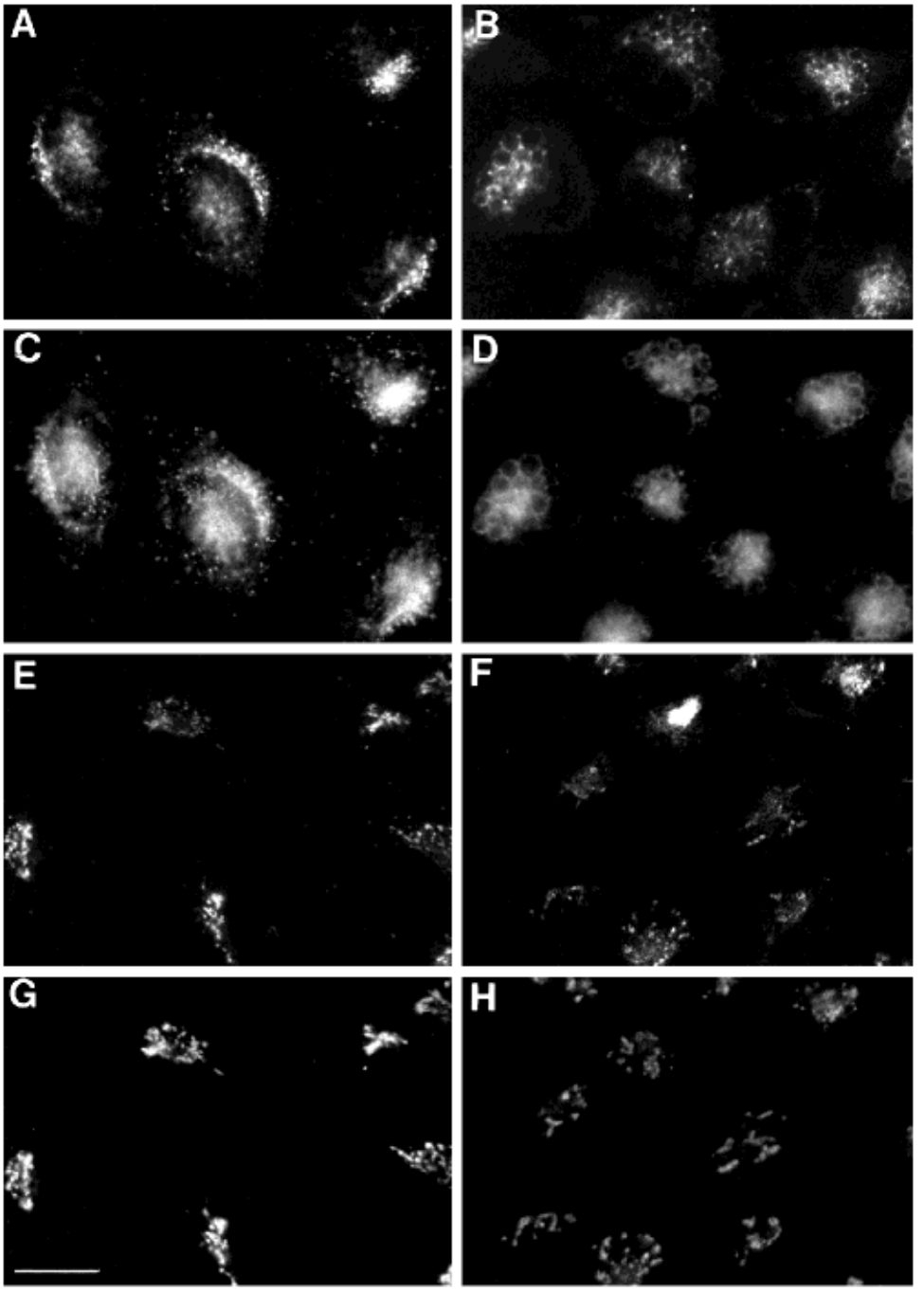

Fig. 8. Uptake and delivery of antibodies to lgp120 and TGN38 is not inhibited in wortmannin-treated cells. Cells were incubated in the absence (A,C,E,G) or presence (B,D,F,H) of wortmannin for 30 minutes prior to removal of the medium and replacement with medium containing monoclonal antibodies to lgp120 (A,B) or TGN38 (E,F) in the absence or presence of fresh wortmannin. After 2 hours at 37°C cells were methanol fixed and incubated with polyclonal antibodies to either lgp110 (C,D) or TGN38 (G,H) followed by Texas-Red labelled donkey anti-rabbit IgG to identify steady state compartments and FITC-labelled goat anti-mouse IgG to identify endocytosed anti-lgp120 and anti-TGN38. Endocytosed lgp120 (A and B) is delivered to lgp110-positive compartments in control cells (C) and swollen lgp110-positive compartments in wortmannin treated cells (D). Endocytosed monoclonal antibody to TGN38 (E and F) is delivered to the TGN, identified with a polyclonal antibody in both control (G) and wortmannin-treated cells (H). The TGN is morphologically unaltered after wortmannin treatment. Bar, 10 μm.

compartment is more difficult to determine. A consensus of data and opinion is consistent with the existence of a late endosomal PLC which is M6PR-positive and acid-hydrolase negative (Geuze et al., 1988; Griffiths et al., 1988, 1990). This compartment is characterised by the presence of intra-organelle membranes which are M6PR-positive by immuno-electron microscopy and give the organelle the appearance of a multivesicular body. Less evidence exists for a separate late endosomal compartment which might correlate with the lgp120-positive structures observed in the present experiments. However, the search for the MHC class II compartment has produced evidence for heterogeneous late endosomal and lysosomal compartments (reviewed by Harding and Geuze, 1993), and infection of epithelial cells with pathogenic strains of *Helicobacter pylori* has suggested the formation of a novel late endosome-derived vacuolar compartment (Papini et al., 1994).

Since dissipation of ionic gradients seems an unlikely explanation for the swelling of these vacuoles based on experiments with acridine orange (Brown et al., 1995), the simplest explanation of our data is that the type I membrane proteins lgp110 and lgp120 normally recycle between lysosomes and an M6PR-negative, late endosomal compartment and that wortmannin treatment results in inhibition of the traffic to lysosomes. Experiments using both wortmannin and LY294002 in a cell-free late endosome-lysosome content

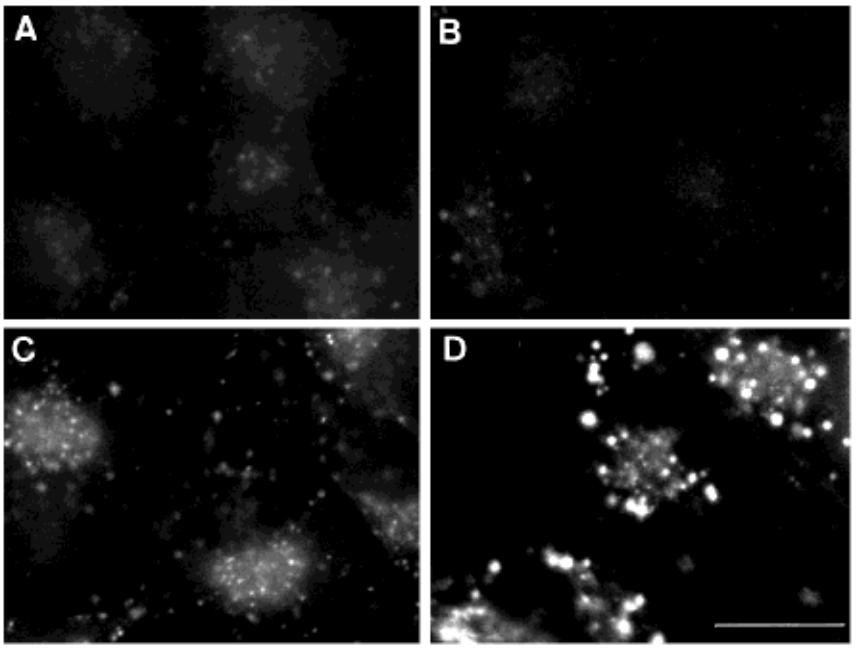

Fig. 9. Effect of wortmannin on uptake of Lucifer Yellow into early endosomes. Lucifer Yellow (1 mg/ml) was taken up into living NRK cells for 2 minutes (A,B) or 40 minutes (C,D) before washing, mounting and microscopic observation. The time from the start of washing to the end of photography was approximately a further 10 minutes. (A and C) Control cells; (B and D) wortmannin-treated cells. The same fields were also viewed by light microscopy to confirm the appearance of swollen vacuoles after wortmannin treatment (data not shown). Bar, 10 μm.

mixing assay (Mullock et al., 1994) support the hypothesis of a block in transport from a late endosomal compartment to lysosomes (data not shown). The swollen M6PR-negative, lgp-positive compartment may be another PLC that lies between the M6PR-positive PLC and the lysosomes (Fig. 10). Careful examination of control cells by immunofluorescence and immunoelectron microscopy has shown the occasional presence of swollen lgp120-positive, M6PR-negative structures, and subcellular fractionation always shows the presence of some lgp120 at an 'endosomal' position in isopycnic Nycodenz density gradients (Fig. 7). The presence of some lgp120 in the M6PR-positive PLC is consistent with our interpretation since retrograde traffic between lysosomes and the M6PR-positive PLC has been demonstrated (Jahraus et al., 1994; Akasaki et al., 1995). There is considerable evidence that lgp120 and its homologues in other species can recycle between lysosomes, endosomes and in some cells, the plasma membrane (Lippincott-Schwartz and Fambrough, 1987; Harter and Mellman, 1992). It has been suggested that cleavage of the two carboxy-terminal cytosolic tail residues, Thr and Ile, results in locking the protein into the lysosome (Guarnieri et al., 1993). The existence of a locking mechanism may explain why complete redistribution of lgp120 into swollen vacuolar structures after wortmannin treatment was not observed by immunofluorescence or subcellular fractionation in the present experiments. An alternative explanation is that wortmannin has differential effects on more than one membrane traffic event such that the observed alteration of steady state distribution of lgps is the net result of these effects.

Several types of PI3-K with different substrate specificities have been identified in mammalian cells. The best studied is the p85/p110 complex involved in hormone/growth factor signalling (reviewed by Stephens et al., 1993) which phosphorylates phosphatidylinositol (PtdsIns), PtdIns(4)P and PtdIns(4,5)P2. This enzyme is also known to be involved in at least one membrane traffic event, the recruitment of GLUT4 glucose transporters to the plasma membrane in response to insulin stimulation (Kanai et al., 1993; Clarke et al., 1994; Hara et al., 1994). Since mutations in the *VPS34* gene affect sorting of carboxypeptidase Y from the TGN to the vacuole in yeast it seems likely that a mammalian homologue(s) of vps34p was the target for wortmannin in the present experiments. Recently, two examples of a mammalian PI3-K with a substrate specificity similar to yeast vps34p, phosphorylating only PtdIns, have been described. The first was partially purified from bovine cells (Stephens et al., 1994) and is similar to vps34p in its relative insensitivity to wortmannin having an IC_{50} in the μM range. Secondly, a human PtdIns specific PI3-K has been cloned which has an IC_{50} for wortmannin in the low nM range (Volinia et al., 1995). Concentrations of wortmannin used in the present experiments were consistent with an effect on the latter PI3-K. The structurally unrelated compound LY294002 which is a specific inhibitor of PI3-K (Vlahos et al., 1994), had similar effects.

Interpretation of our data suggests that at least two membrane traffic events are inhibited: recycling from M6PR-positive late endosomes to the TGN and recycling from the novel lgp120-positive late endosomal compartment to lysosomes (Fig. 10). Several other inhibitory effects of wortmannin on membrane traffic pathways have been reported. These include delivery of activated platelet-derived growth factor receptor to a degradative compartment in the endocytic pathway (Joly et al., 1994, 1995), insulin stimulated recruitment of transferrin receptors to the plasma membrane (Shepherd et al., 1995), transcytosis (Hansen et al., 1995), fluid phase endocytosis and early endosome fusion (Clague et al., 1995; Li et al., 1995; Jones and Clague, 1995). A role for PI3-K within the endocytic system is also supported by the isolation of a *vps34* yeast mutant designated *end12*, which is deficient in delivery of α-factor to the vacuole but not internalisation from the cell surface (Munn and Riezman, 1994; Horazdovsky et al., 1995). In the present study the steady-state distribution of transferrin receptors was altered in the presence of wortmannin with some appearing in a swollen M6PR-positive, lgp120-negative vacuolar compartment (data not

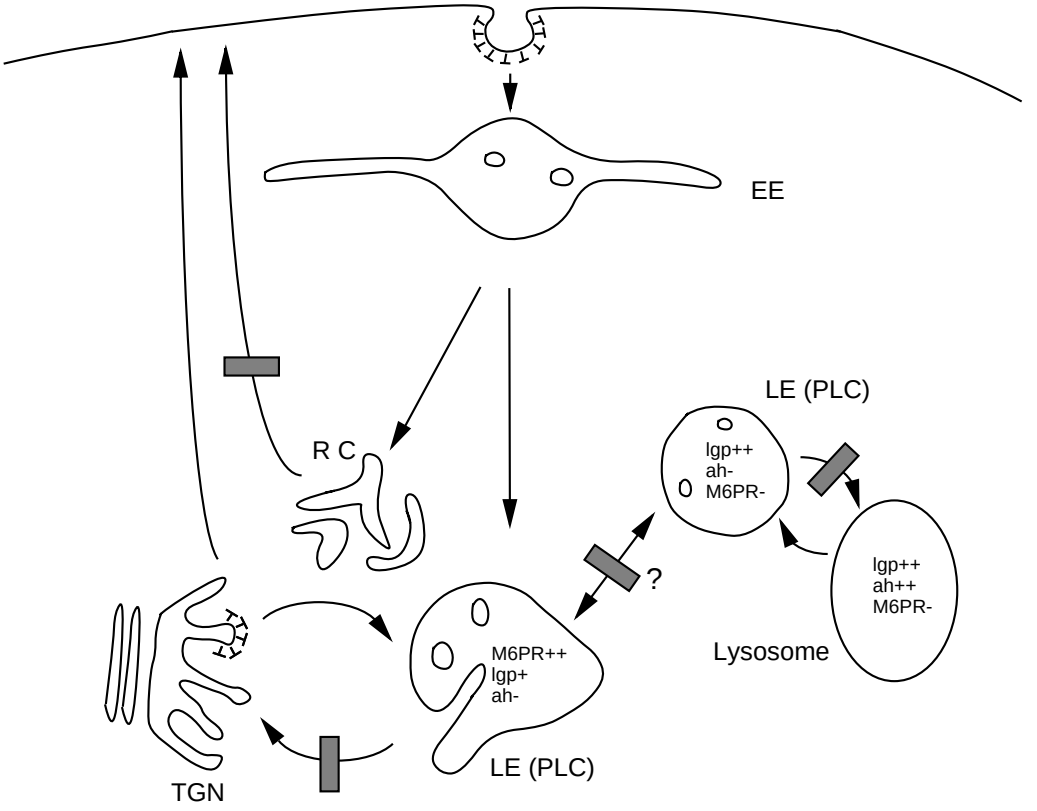

Fig. 10. Inhibition of membrane traffic pathways by wortmannin. Two late endosomal (LE) PLCs are shown with blocks in membrane traffic indicated by stippled bars. The distribution of M6PR, lgps and acid hydrolase (ah) is shown with ++ indicating major sites of localisation, + indicating presence and – indicating depletion. The early endosome (EE) and recycling compartment (RC) are shown, the stippled bar indicating inhibition of membrane traffic back to the plasma membrane as has been suggested by Shepherd et al. (1995).

shown). Since the Lucifer Yellow uptake experiments suggested that peripheral early endosomes did not swell after wortmannin treatment, the effect on the transferrin receptor is most likely due to an effect of wortmannin on the recycling compartment described by Mayor et al. (1993). The exact mechanism of PI3-K regulation of these membrane traffic events remains unclear. However, evidence for an effect on nucleotide exchange on rab proteins has been provided by experiments using a cell-free endosome fusion assay system in which addition of rab5 stabilised in the GTP bound form overcame wortmannin effects (Li et al., 1995).

There is now good evidence for the effects of wortmannin and therefore involvement of PI3-K at several distinct steps on endocytic membrane traffic pathways (Fig 10). A common theme seems to be emerging suggesting a wortmannin-induced inhibition of membrane traffic out of the endosomal system (GLUT4 translocation, transferrin receptor recycling, PDGF receptor degradation, lgp120 and M6PR recycling). In contrast, membrane traffic into the endosomal system from the TGN, the plasma membrane or the lysosome is relatively unaffected, and possibly not affected at all as a primary event. The consequence of the inhibition of membrane traffic out of the endosomal system is the swelling of the late endosomal compartments. The present experiments suggest that wortmannin inhibition may well prove a useful starting point to isolate and characterise these compartments. The identification of which PI3-K's function at which membrane traffic steps, and how, will clearly also serve as a focus for much future work.

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