Wortmannin Causes Mistargeting of Procathepsin D. Evidence for the Involvement of a Phosphatidylinositol 3-Kinase in Vesicular Transport to Lysosomes

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Abstract. At present little is known of the biochemical machinery controlling transport of newly synthesized lysosomal hydrolases from the *trans*-Golgi network (TGN) to endosomes. The demonstration that Vps34p (a protein required for targeting soluble hydrolases to the vacuole in *Saccharomyces cerevisiae*) is a phosphatidylinositol 3-kinase (PI3-K) suggested the possibility that a homologous enzyme might be involved in the equivalent step in mammalian cells. Using the PI3-K inhibitors wortmannin and LY294002, I provide evidence to support this hypothesis.

Treatment of K-562 cells with wortmannin induced secretion of procathepsin D, with half-maximal inhibition of accurate targeting to lysosomes at 10–20 nM. Kinetic analysis indicated that a late Golgi (TGN) step was affected, and that other constitutive vesicular transport events were not. The M6P recognition signal was still generated in the presence of wortmannin suggesting that the drug was directly inhibiting export of the receptor-ligand complex from the TGN, while removal of the drug led to a rapid restoration of accurate sorting. At the concentrations used, wortmannin and LY294002 are presently accepted to be specific inhibitors of PI3-K. I conclude that these data implicate such an enzyme in the trafficking of M6P-receptor-ligand complexes from the TGN towards lysosomes.

I N most mammalian cells, transport of newly synthesized soluble acid hydrolases to lysosomes depends upon their recognition by receptors specific for mannose 6-phosphate (M6P)[1] residues, and their subsequent sequestration into clathrin-coated regions of the *trans*-Golgi Network (TGN) (for reviews see von Figura and Hasilik, 1986; Kornfeld and Mellman, 1989). The M6P modification appears unique to lysosomal hydrolases, and is formed by the concerted action of two enzymes, the first of which (UDP-GlcNAc: lysosomal enzyme N-acetylglucosamine-1-phosphotransferase) recognizes conformation-dependent protein determinants in the lysosomal enzyme (Baranski et al., 1991). Phosphorylation begins before export of the hydrolase from the endoplasmic reticulum, but continues during the passage of the protein through the Golgi apparatus (Lazzarino and Gabel, 1988, 1989). Subsequently N-acetylglucosamine-1-phosphate α-N-acetylglucosaminidase exposes the M6P recognition signal, allowing sorting

to occur, most probably in the TGN (Duncan and Kornfeld, 1988).

Two distinct M6P receptors have been identified, characterized, and cDNAs encoding them cloned and sequenced. Both receptors are integral membrane glycoproteins and have similar binding specificities for M6P containing ligands, but vary in their subunit size, ability to endocytose exogenous ligands, and their relative importance in delivering particular acid hydrolases (Kornfeld and Mellman, 1989; Ludwig et al., 1994). The larger of the two, the 300-kD cation-independent M6P receptor (ci-M6PR), is also the receptor for insulin-like growth factor II (Morgan et al., 1987), and is the more widely studied. Its distribution at steady state appears to vary between individual cell types, but in most cases it is concentrated in the TGN and prelysosomal late endocytic compartments (PLCs) (Griffiths et al., 1988; Kornfeld and Mellman, 1989), consistent with a role in transporting ligands between these two compartments, although the initial site of delivery may be an earlier endocytic compartment (Ludwig et al., 1991). Within PLCs the acidic pH will induce dissociation of receptor-ligand complexes allowing the receptors to be recycled to the TGN where they can bind additional hydrolases (Brown et al., 1986; Duncan and Kornfeld, 1988; Goda and Pfeffer, 1988). Consistent with this itinerary, both immunocytochemical and biochemical analyses indicate that dense lysosomes are devoid of ci-M6PRs (Griffiths et al.,

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1. *Abbreviations used in this paper:* ci-M6PR, cation-independent M6P receptor; M6P, mannose 6-phosphate; PI3-K, phosphatidylinositol 3-kinase; PLC, prelysosomal late endocytic compartment.

1988; Sahagian and Neufeld, 1983), while treatment of cells with permeant weak bases causes hypersecretion of acid hydrolases due to the recycling of occupied M6P receptors to the TGN (von Figura and Hasilik, 1986).

A small proportion (typically ∼10%) of the total population of ci-M6PRs in most cells is located at the cell surface. These receptors can endocytose extracellular M6P containing ligands, deliver them via PLCs to lysosomes (von Figura and Hasilik, 1986; Kornfeld and Mellman, 1989), and subsequently also recycle to the TGN (Duncan and Kornfeld, 1988). Approximately 10% of receptors recycled to the TGN from the cell surface subsequently reappear at the plasma membrane (Jin et al., 1989), suggesting that all ci-M6PRs belong to a common pool which mediates both endogenous and exogenous delivery to lysosomes. However, as growth of cells in M6P containing media does not inhibit delivery of newly synthesized acid hydrolases to lysosomes, it is unlikely that the majority of lysosomal enzymes are delivered via the plasma membrane (von Figura and Hasilik, 1986).

In the TGN, M6P receptors are sequestered into clathrin-coated regions associated with the AP1 adaptor complex, which subsequently bud to form clathrin-coated vesicles (von Figura and Hasilik, 1986; Kornfeld and Mellman, 1989; Pearse and Robinson, 1990). Consistent with their role in mediating the transport of newly synthesized lysosomal hydrolases, isolated clathrin-coated vesicles also contain these proteins, which are present exclusively as the proenzymes and, at least to some extent, membrane associated in a M6P-dependent fashion (Marquardt et al., 1987; Schulze-Lohoff et al., 1985). The formation of TGN-derived clathrin-coated vesicles appears constitutive. It does not require the presence of cargo (Braulke et al., 1987), and may also not require the presence of the receptor. In vitro, ci-M6PR cytoplasmic tails can directly interact with isolated AP1 adaptors (Glickman et al., 1989) suggesting that they may be directly involved in the recruitment of coat proteins to the TGN. However, the interaction is of relatively low affinity, and recent evidence has implicated other TGN membrane proteins in the recruitment of AP1 adaptors (Mallet, W. G., and F. M. Brodsky. 1994. Membrane protein binding to the clathrin coat protein AP1. Mol. Biol. Cell. 5:S:329a; Seaman, 1995). In addition, M6P-dependent targeting of lysosomal enzymes occurs in ci-M6PR deficient fibroblasts (Ludwig et al., 1994), implying that this receptor is not essential for the formation of clathrin-coated vesicles from the TGN.

Despite the considerable amount of knowledge relating to the intracellular itineraries of newly synthesized acid hydrolases and M6P receptors, and the structure and assembly of the clathrin coat, much less is known of the molecular machinery governing vesicular transport between the TGN and endosomes. For other vesicular transport events, the biochemical machinery regulating movement between compartments of the eukaryotic cell appears highly conserved, with many examples of homologous proteins acting at equivalent steps in both yeast and mammalian cells (for review see Pryer et al., 1992). Although yeast lack highly acidic endosomes and lysosomes, the vacuole performs a similar role to mammalian lysosomes (for review see Klionsky et al., 1990), and may be functionally equivalent. Transport of soluble hydrolases to the vacuole

is also receptor mediated and involves delivery to prevacuolar endocytic compartments (Vida et al., 1993), raising the possibility that it may occur by a similar mechanism to lysosomal transport, and that related proteins may govern movement of soluble hydrolases between the late Golgi and endosomes in all eukaryotes. Genetic studies have implicated at least 50 gene products in vacuolar protein sorting (Klionsky et al., 1990) but in most cases mammalian homologues remain to be identified, or have yet to be shown to have a role in lysosomal enzyme transport.

Recently, following the demonstration that the product of the *VPS34* gene is a phosphatidylinositol 3-kinase (PI3-K) (Herman and Emr, 1990; Schu et al., 1993), attention has been focussed upon the potential involvement of lipid kinases in vesicular trafficking events. The *vps34* mutant aberrantly secretes vacuolar hydrolases such as carboxypeptidase Y in their proenzyme forms, indicating that it selectively blocks movement between the Golgi and vacuole without affecting secretion of proteins destined for the extracellular space. Consequently, if a homologous protein is involved in transport to lysosomes, a likely phenotype would be hypersecretion of acid hydrolase proenzymes. I now report that the fungal metabolite wortmannin, and the structurally unrelated compound LY294002, at doses compatible with the inhibition of PI3-K activity, cause hypersecretion of procathepsin D from human K-562 cells, consistent with the hypothesis that transport to lysosomes in mammalian cells requires the action of a lipid kinase.

Materials and Methods

Reagents

Tissue culture media and supplements were purchased from GIBCO-BRL (Paisley, Scotland). Wortmannin was purchased from Sigma (Poole, UK) reconstituted to 1 mM in DMSO and stored as aliquots at −20°C. LY294002 was generously provided by Dr. P. Shepherd (University of Cambridge) and reconstituted at 10 mg/ml in DMSO. All other reagents were obtained from Sigma except where indicated.

Cells and Antibodies

K-562 cells (Lozzio and Lozzio, 1975) were obtained from Mrs. A. Murrell, Department of Haematology (University of Cambridge, Cambridge, UK) and maintained in RPMI 1640 supplemented with 10% vol/vol FCS, 2 mM glutamine, 20 mM Na pyruvate, 50 U/ml penicillin, 50 μg/ml streptomycin, and 1% minimal essential medium nonessential amino acids in a 95% air, 5% CO_2 humidified incubator at 37°C.

A rabbit polyclonal antiserum recognizing all forms of human cathepsin D was obtained from Dako (High Wycombe, UK).

Biosynthetic Radiolabeling

Cells were collected from culture by centrifugation (250 g, 4 min at room temperature) and washed once with PBS containing 5 mg/ml BSA (PBS/BSA). After resuspension in methionine free α-MEM (Sigma) containing 2.2 g/l Na bicarbonate and 5% vol/vol dialyzed new-born calf serum (1.5 × 10^6 cells/ml) they were incubated for 1 h at 37°C in a gassed incubator to deplete endogenous methionine. They were then collected, washed once with PBS and resuspended in methionine free α-MEM containing 20 mM Na Hepes, pH 7.4 (5 × 10^7 cells/ml), and incubated at 37°C for 5 min with 250 μCi Trans[35]S-Label (ICN Biomedicals Ltd., Thame, UK). The cells were then transferred to ice, washed twice with ice cold PBS/BSA, and finally resuspended at 2.5 × 10^6 cells/ml in RPMI 1640 containing 20 mM Na Hepes, pH 7.4, 3 mM methionine, and 1% vol/vol undialyzed serum, with or without 5 mM M6P and inhibitors as indicated. Aliquots (2.5 ×10^6 cells) were incubated at 37°C for various times, the cells and medium collected by centrifugation, and radiolabeled cathepsin D immunoprecipitated as described below.

Immunoprecipitation

Cells were lysed in 500 μl of 50 mM Tris-HCl, pH 7.4, containing 150 mM NaCl, 1% (wt/vol) Triton X-100, 1 mM EDTA, 1 mM PMSF, 50 μM leupeptin, 50 μM E-64, 1 μM pepstatin A, and 10 μg/ml chymostatin for 10 min at 0°C. After centrifugation (25,000 g; 10 min at 4°C) 5 μl of rabbit anti-(human cathepsin D) was added to the supernate, and the mixture incubated overnight at 4°C. Immune complexes were collected using 20 μl protein A–agarose (Sigma), washed three times with 50 mM Tris-HCl, pH 7.5, containing 150 mM NaCl, 1% (wt/vol) Triton X-100, 1% (wt/vol) Na deoxycholate, 0.1% SDS, and 5 mM EDTA, once with 10 mM Tris-HCl, pH 6.8, and eluted with 50 μl of 1.1-fold concentrated SDS-PAGE sample buffer lacking reducing agents by heating to 100°C for 10 min. Eluates were analyzed by SDS-PAGE using 10% gels, fixed for 10 min in 45%(vol/vol) methanol, 10%(vol/vol) acetic acid, dried, and radioactivity determined using a Fuji Bas2000 phosphoimager.

To one half of the total incubation media leupeptin (100 μM), E-64 (50 μM), and PMSF (1 mM) were added from concentrated stock solutions. The resulting mixtures were then centrifuged (25,000 g; 10 min at 4°C), and the supernatants subsequently treated identically to the clarified cell lysates.

Results

Wortmannin Causes Mistargeting of Procathepsin D

Cathepsin D is the major mammalian aspartyl protease and provides the most widely studied example of M6P dependent lysosomal enzyme targeting (for review see Kornfeld and Mellman, 1989). In human cells it is synthesized as an M_r 53-kD proenzyme containing two N-linked oligosaccharides which are modified by the addition of one or more M6P residues to provide the recognition signal (Hasilik and Neufeld, 1980a). After export from the TGN and delivery to endosomal compartments, the propeptide is removed to form a 47-kD intermediate, which is subsequently cleaved in lysosomes to form the mature enzyme comprising noncovalently associated 31-kD and 14-kD polypeptides (Delbrück et al., 1994; Gieselmann et al., 1983; Rijnboutt et al., 1992).

Preliminary experiments demonstrated that the maturation of cathepsin D in erythroleukaemic K-562 cells showed similar kinetics to those observed in other cell lines (Gieselmann et al., 1983; Hasilik and Neufeld, 1980b). Thus after pulse radiolabeling for 5 min, the 53-kD protein was the sole form of cathepsin D detected in the first 20–30 min of chase. Subsequently the 47-kD intermediate was generated, reached a maximum after 90–120 min and thereafter declined, while the mature 31-kD form could not be detected in the first 60 min of chase but became the major form after 2–4 h. To investigate the potential role of PI3-K activity in the transport of lysosomal enzymes K-562 cells were pulse radiolabeled and chased for 4 h in the presence or absence of wortmannin (100 nM). M6P (5 mM) was included in the chase medium to inhibit any endocytosis of secreted proenzyme. As shown in Fig. 1, treatment with this drug almost completely abolished the intracellular maturation of cathepsin D (lanes 2 and 3), resulting instead in almost quantitative recovery of the proenzyme from the chase medium (lanes 4 and 5). A similar result was observed in several other cell lines tested including NRK cells stably transfected with human cathepsin D, and Cos 1 cells, although in these cases a much higher degree of basal procathepsin D secretion in the absence of the drug was also observed (data not shown).
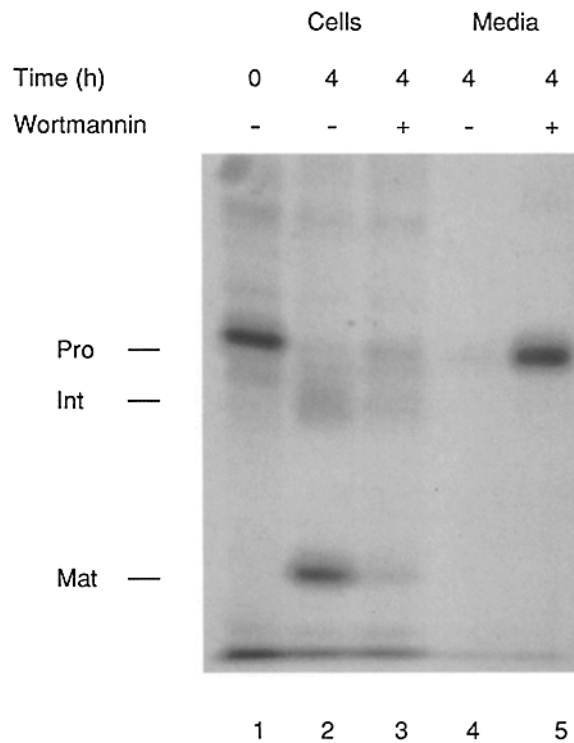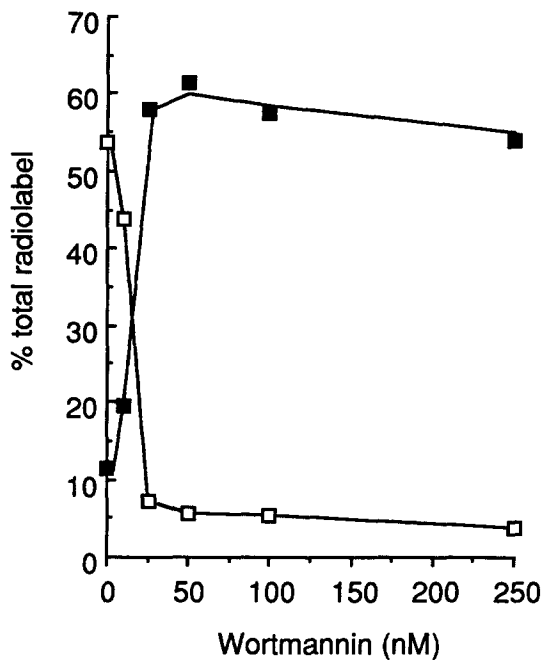

Figure 1. Sorting of procathepsin D in the presence of wortmannin. K-562 cells were pulse radiolabeled and chased for times as indicated in the presence of extracellular M6P (5 mM) and presence or absence of wortmannin (100 nM). Cells and media were collected, and cathepsin D immunoprecipitated and analyzed by SDS-PAGE as described in Materials and Methods. Positions of procathepsin D (Pro), the 47-kD intermediate (Int), and the mature 31-kD heavy chain (Mat) are indicated.

Mistargeting Probably Involves Inhibition of a PI3-K

Although at low nanomolar concentrations wortmannin is presently considered to be a specific inhibitor of PI3-K activity (Arcaro and Wymann, 1993; Powis et al., 1994), at higher concentrations it can also inhibit other enzymes including myosin light chain kinase (Nakanishi et al., 1992). To investigate whether or not the observed mistargeting of procathepsin D was consistent with the involvement of a PI3-K in lysosomal enzyme transport I examined the dose dependence of this effect. As shown in Fig. 2, wortmannin is an extremely potent inhibitor of lysosomal transport in K-562 cells, with a half maximal effect at 10–20 nM. For the same cell line, an identical response was obtained using several different batches of the drug. However the relationship appeared to be cell specific, and some of the other cell lines tested required up to 50–100 nM wortmannin to cause half-maximal mistargeting (data not shown). In these circumstances inhibition was only 70–80% complete at 250 nM, the highest concentration tested in this study.

Nevertheless, the dose respose relationship described above strongly implicates the involvement of a PI3-K in lysosomal trafficking in K-562 cells, although the possibility that an alternative target exists cannot be completely eliminated. Recently a structurally unrelated compound, LY294002 has also been shown to be a selective inhibitor
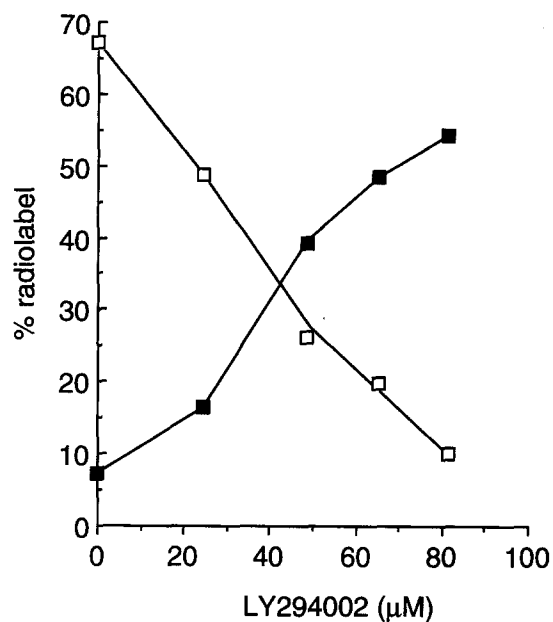
Figure 2. Concentration dependence of wortmannin-induced mistargeting. K-562 cells were pulse radiolabeled and chased for 2 h in the presence of extracellular M6P (5 mM) and wortmannin at the concentrations indicated. In each case the final concentration of DMSO was 0.1%. Cells and media were collected, cathepsin D immunoprecipitated, and analyzed by SDS-PAGE and phosphoimaging as described in Materials and Methods. Results are expressed as the percentage of the total radiolabeled cathepsin D recovered from the medium (■) or in cell-associated processed forms (□).

Figure 3. Inhibition of procathepsin D sorting by LY294002. K-562 cells were pulse radiolabeled and chased for 2 h in the presence of extracellular M6P (5 mM) and LY294002 at the concentrations indicated. In each case the final concentration of DMSO was 0.25%. Cells and media were collected, cathepsin D immunoprecipitated, and analyzed by SDS-PAGE and phosphoimaging as described in Materials and Methods. Results are expressed as the percentage of the total radiolabeled cathepsin D recovered from the medium (■) or in cell-associated processed forms (□).

of PI3-K (Vlahos et al., 1994), albeit at doses in the micromolar range. To further test my hypothesis that a PI3-K is involved in lysosomal enzyme trafficking, I examined the effect of this drug. As shown in Fig. 3, LY294002 also caused mistargeting of procathepsin D with a half-maximal effect at 40–50 μM. At this dose the drug also appears to selectively target PI3-K activities (Vlahos et al., 1995), consistent with the conclusion that inhibition of such an activity is responsible for the drug induced mistargeting of procathepsin D I observe.

Wortmannin Inhibits Segregation of Procathepsin D from the Constitutive Secretory Pathway

In studies of the effect of wortmannin and LY294002 on other cellular events, such as the respiratory burst and glucose transporter translocation, it has been shown that inhibition can readily be observed within 5 min of the addition of the drugs (e.g., Arcaro and Wymann, 1993; Shepherd et al., 1995; Vlahos et al., 1995). Consequently, as addition of these drugs to K-562 cells at a time when virtually all of the radiolabeled cathepsin D was still in the endoplasmic reticulum resulted in hypersecretion of the proenzyme (Figs. 1–3) this strongly suggests that at the concentrations used, they do not inhibit vesicular transport either between the endoplasmic reticulum and Golgi, within the Golgi stack, or between the Golgi and plasma membrane.

To examine more closely the subcellular location of the events in cathepsin D maturation sensitive to PI3-K inhib-
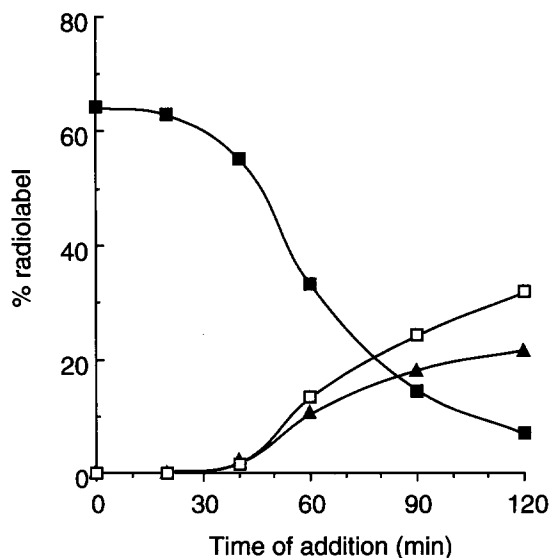
itors, K-562 cells were pulse radiolabeled for 5 min and chased for a total of 2 h. At various times wortmannin was added to a final concentration of 100 nM and the incubation continued for the remainder of the 2-h period. As shown in Fig. 4, complete inhibition of processing was observed when the drug was added within the first 20–30 min of chase, after which there was a progressive decrease in the amount of the proenzyme secreted (*closed squares*) and concoitant increase in the amount of processed intracellular forms (*open squares* and *closed triangles*). Such kinetics are consistent with the inhibition of a late Golgi event, and suggest that the major effect of wortmannin on cathepsin D maturation is to inhibit its segregation from the constitutive secretory pathway in the TGN. At no time point was a selective accumulation of the 47-kD intermediate form of cathepsin D observed (compare open squares with closed triangles) and for the later time points, there was a significant increase in the amount of processed cathepsin D detected at the end of the incubation over that present at the time that the wortmannin was added. This suggests that under the conditions used wortmannin does not inhibit processing of procathepsin D per se, nor prevents movement between endosomes and lysosomes, but rather acts at a single point in the lysosomal trafficking pathway.

### Wortmannin Does Not Prevent the Formation of the M6P Recognition Signal

The data described above strongly suggest that wortman-

Figure 4. Time course of wortmannin-induced mistargeting. K-562 cells were pulse radiolabeled and chased for 2 h in the presence of extracellular M6P (5 mM). At the times indicated wortmannin (100 nM) was added, and the incubation continued for the remainder of the 2-h period. Cells and media were collected, cathepsin D immunoprecipitated, and analyzed by SDS-PAGE and phospho-imaging as described in Materials and Methods. Results are expressed as the percentage of the total radiolabeled cathepsin D recovered from the medium (■) or in cell-associated intermediate (□) and mature (▲) forms.

nin prevents sorting of newly synthesized lysosomal enzymes from the constitutive secretory pathway. Since segregation is receptor mediated and depends upon the formation of the M6P recognition signal (Kornfeld and Mellman, 1989), one possible explanation for my observations would be that this signal is not correctly formed in the presence of the drug. Studies of the phosphorylation of β-glucuronidase in a murine macrophage cell line indicated that this modification could be detected within 15–20 min of the synthesis of the enzyme but continued for the next 40–80 min (Goldberg and Kornfeld, 1981). Thus the kinetic data shown in Fig. 4 do not completely eliminate this possible explanation of the effect of wortmannin on cathepsin D maturation. I therefore decided to directly examine the ability of procathepsin D synthesized in the presence of wortmannin to bind to M6P receptors.
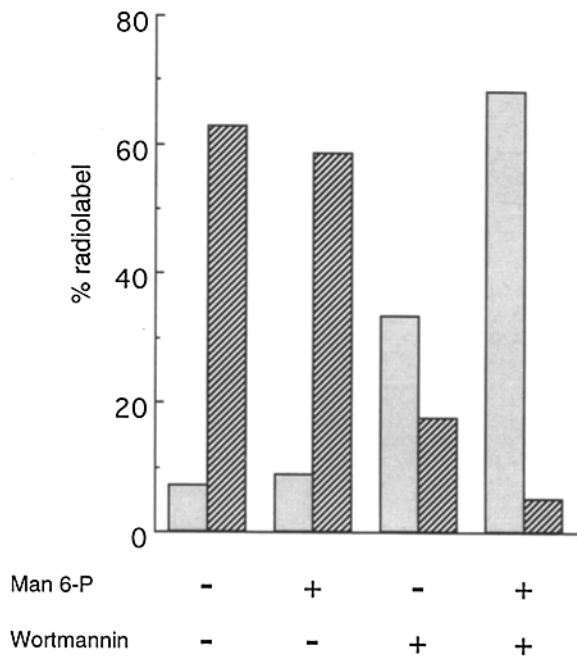
Medium from K-562 cells pulse radiolabeled and chased in the presence of wortmannin but absence of M6P was collected and concentrated fivefold by ultrafiltration. It was then applied to unlabeled cells freshly collected from culture. After incubation overnight at 37°C in the presence or absence of extracellular M6P (5 mM), cells and media were collected and radiolabeled cathepsin D analyzed by immunoprecipitation and SDS-PAGE. Under these conditions, cells incubated in the absence of exogenous M6P endocytosed and processed ~13% of the added procathepsin D (Fig. 5, track 1). This was reduced to 1.4% by inclusion of the phosphorylated sugar (track 2) consistent with the hypothesis that endocytosis was largely dependent upon ci-M6PRs and hence that wortmannin does not prevent the synthesis of the recognition signal. As expected, whereas
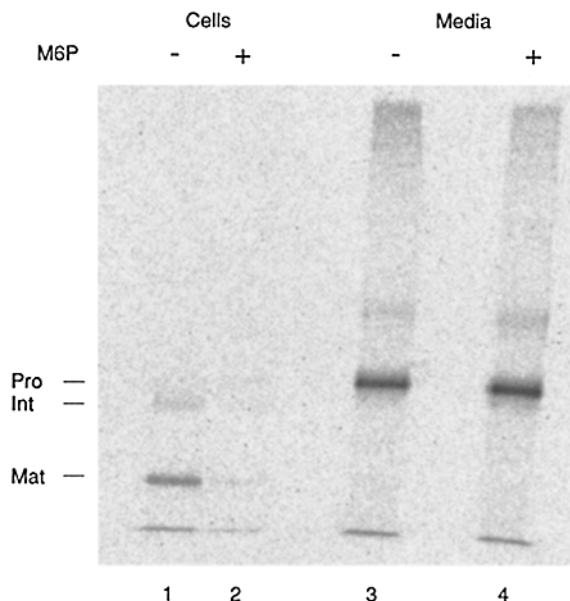
Figure 5. Endocytosis of procathepsin D secreted in the presence of wortmannin. K-562 cells (2.5 x 10⁷) were pulse radiolabeled and chased for 2 h in the presence of wortmannin (100 nM) as described in Materials and Methods, except that the media contained Na bicarbonate instead of Na Hepes, and the chase incubation was conducted in a gassed incubator. The medium was collected, concentrated fivefold by ultra-filtration using a centricon-30 concentrator (Amicon Ltd., Stonehouse, UK), and applied to freshly isolated cells (2.5 x 10⁶) in the absence or presence of M6P (5 mM) as indicated. After incubation in a gassed incubator for 14 h cells and media were collected, cathepsin D immunoprecipitated and analyzed by SDS-PAGE as described in Materials and Methods. Positions of procathepsin D (Pro), the 47-kD intermediate (Int), and the mature 31-kD heavy chain (Mat) are indicated.

the mature enzyme was the major cell associated form of radiolabeled cathepsin D, the proenzyme was the sole form recovered from the media after overnight incubation (Fig. 5, lanes 3 and 4). Additional experiments demonstrated that procathepsin D secreted in response to wortmannin could bind directly to immuno-affinity isolated human ci-M6PRs (data not shown), consistent with the major role of this receptor in the targeting of cathepsin D to lysosomes (Ludwig et al., 1994).

I next examined the effect of excluding M6P from the chase medium. As shown in Fig. 6, the presence of the ligand had only a minor effect upon the amount of proenzyme recovered in the chase medium (stippled bars) or processed intracellularly (striped bars) during a 2-h chase in the absence of wortmannin. In contrast there was an approximate twofold decrease in "secreted" proenzyme and increase in processed cathepsin D between cells treated with the drug in the absence and presence of the competing ligand. The most likely interpretation of this observation is that in the absence of the competing phosphorylated sugar procathepsin D secreted in response to wortmannin can bind to, and be endocytosed by, cell surface ci-M6PRs, and subsequently be transported to lysosomes. Consequently these data again suggest that wortmannin does not prevent the formation of the recognition signal, and also imply that
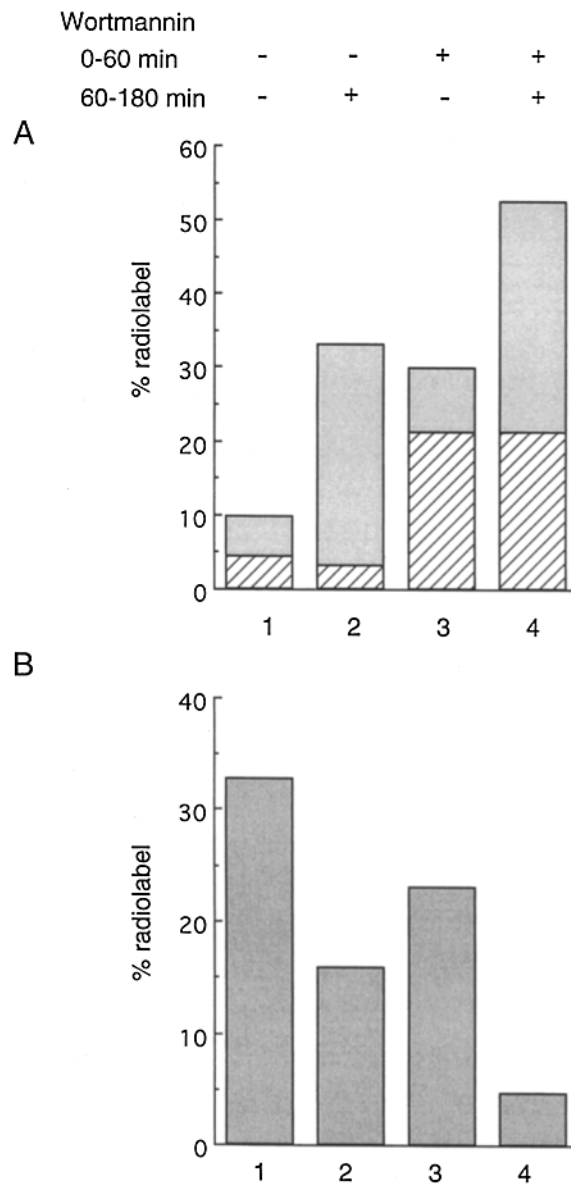
Figure 6. Effect of extracellular M6P on wortmannin-induced mistargeting. K-562 cells were pulse radiolabeled and chased for 2 h in the absence or presence of wortmannin (100 nM) and extracellular M6P (5 mM) as indicated. Cells and media were collected, cathepsin D immunoprecipitated, and analyzed by SDS-PAGE and phospho-imaging as described in Materials and Methods. Results are expressed as the percentage of the total radiolabeled cathepsin D recovered from the medium (■) or in cell-associated processed forms (▨).

Figure 7. Reversal of the inhibition of procathepsin D sorting by wortmannin. K-562 cells were pulse radiolabeled and chased for 1 h in the absence of M6P and the absence or presence of wortmannin (100 nM) as indicated. Cells and media were collected, and the 0–60 min media retained. The cells were washed once with ice-cold PBS/BSA, resuspended in fresh medium containing wortmannin if indicated, and chased for an additional 2 h. (*A*) Cathepsin D was immunoprecipitated from the respective 0–60 min and 60–180 min media, and analyzed by SDS-PAGE, and phospho-imaging as described in Materials and Methods. Results are expressed as the percentage of the total radiolabeled cathepsin D recovered from the cells and media present in the 0–60 min (▨) or 60–180 min (■) media samples. (*B*) Cell-associated cathepsin D was immunoprecipitated and analyzed by SDS-PAGE and phospho-imaging as described in Materials and Methods. Results are expressed as the percentage of the total radiolabeled cathepsin D from the cells and media recovered as mature cathepsin D.

at the doses used the drug does not significantly inhibit binding of procathepsin D to M6P receptors, receptor-mediated endocytosis or trafficking to lysosomes through the endocytic pathway. This latter conclusion is not inconsistent with the observation that there was a decrease in the percentage of cell-associated processed cathepsin D in wortmannin-treated cells incubated in the absence of M6P as compared to control cells, since this is unlikely to result from a direct effect of the drug on trafficking or processing within the endocytic pathway. Instead it is almost certainly a consequence of the kinetic nature of the assay, and the longer time taken for material secreted into the medium to reach processing compartments after receptor-mediated endocytosis as compared to the direct transport to endosomes which occurs in the absence of wortmannin.

Inhibition of Cathepsin D Segregation Is Reversible

In previous studies of the effects of wortmannin on cellular processes, conflicting results have been obtained concerning whether or not the inhibition is reversible (Clarke et al., 1994; Thelen et al., 1994; Woscholski et al., 1994). I therefore decided to examine whether or not the inhibition of cathepsin D targeting to lysosomes induced by wortmannin could be reversed by removal of the drug. Cells were first pulse radiolabeled and chased for 1 h in the presence or absence of wortmannin (100 nM). They were then collected, resuspended in fresh medium and incubated for an additional 2 h in the presence or absence of

the drug as appropriate. In the absence of wortmannin 3–4% of the total radiolabel was released into the chase medium during the first 60 min of chase (Fig. 7 *A* columns 1 and 2, *striped bars*), while 22% was released in the same period in the presence of the drug (*A* columns 3 and 4, *striped bars*). An additional 30% of the total-radiolabeled cathepsin D was released during the 60–180-min chase period when wortmannin was included in the second chase medium, irrespective of whether or not it had been included in the first incubation (*A* columns 2 and 4, *stippled bars*), however only 8% was released from cells previously exposed to wortmannin if the second chase medium lacked the inhibitor (*A* column 3, *stippled bar*). A complementary effect on the efficiency of procathepsin D processing was also observed (*B*) consistent with the conclusion that the inhibition was rapidly reversed and the population of newly synthesized molecules, which had not passed beyond the TGN when the drug was removed, were subsequently correctly sorted and delivered. Recovery could not be attributed to release of a cohort of molecules retained in the TGN as no significant pool of cell-associated procathepsin D could be detected after long term incubations either when the drug was added directly (Fig. 1) or later during the chase period (data not shown). Nonetheless, together the data presented above suggest that wortmannin acts selectively in the exocytic pathway to inhibit the M6P dependent sorting of newly synthesized lysosomal hydrolases in the TGN.

Discussion

The results described above demonstrate that treatment of human K-562 cells with low nanomolar concentrations of the fungal metabolite wortmannin causes almost complete mistargeting of procathepsin D. A similar effect was also observed with low micromolar concentrations of the structurally unrelated compond LY294002. At the doses used these drugs are currently believed to be specific inhibitors of PI3-K activity (Arcaro and Wymann, 1993; Powis et al., 1994). Thus the most reasonable explanation of these observations is that they demonstrate the involvement of a PI3-K in the delivery of newly synthesized acid hydrolases to lysosomes. The effect was selective for lysosomal targeting: vesicular transport between the endoplasmic reticulum and Golgi, within the Golgi stack, between the TGN and the cell surface, and within the endocytic pathway appeared to be unaffected. This latter observation does not contradict a recent report implicating a PI3-K in the endocytic trafficking of activated PDGF receptors (Joly et al., 1994) since in this case the target appeared to be p110α and to involve the tyrosine kinase dependent events associated with ligand-activated receptors.The ci-M6PR lacks tyrosine kinase activity and so only constitutive endocytosis was examined in the present study.

Newly synthesized lysosomal enzymes are sorted from the constitutive secretory pathway in the TGN (von Figura and Hasilik, 1986; Kornfeld and Mellman, 1989). The kinetic data, and the fact that only procathepsin D was recovered from the medium, strongly suggest that PI3-K inhibitors disrupt these sorting events and act at the level of the TGN. Substantial wortmannin-induced secretion was observed in the absence of extracellular M6P. This sug-

gests that the majority of procathepsin D was not delivered to the cell surface in association with M6P receptors (if this were the case the high affinity of the ligand for its receptor would likely have ensured that it remained cell associated and was subsequently reendocytosed), and that the main effect of the external M6P was to prevent association of secreted procathepsin D with surface ci-M6PR rather than displace ligand from aberrantly targeted receptors. It also makes it unlikely that the major site of wortmannin action is an early endosomal compartment. Given that under normal circumstances newly synthesized acid hydrolases may be delivered from the TGN to an early endocytic compartment (Ludwig et al., 1991; Rijnboutt et al., 1992) from which procathepsin D might potentially be delivered to the cell surface if sorting to lysosomes were disrupted, early endosomes could be an alternative site of wortmannin action; kinetic analysis would not distinguish this from an effect at the TGN. However, if early endosomes were the primary site of action it is probable that the majority of the proenzyme would have remained cell associated in the absence of extracellular M6P, since delivery to this compartment, unlike movement through the secretory pathway to the TGN, is dependent upon association with M6P receptors. In addition, the apparent failure of wortmannin to prevent transport to lysosomes of procathepsin D endocytosed by the ci-M6PR argues against the primary effect of the drug on lysosomal targeting being on elements of the endocytic pathway, although a minor effect of the drug on movement between the cell surface and lysosomes cannot be completely excluded.

The most probable primary site of wortmannin-induced mistargeting of lysosomal proenzymes is the TGN. However, at present the precise mechanism by which the drug acts remains to be elucidated. Since lysosomal targeting of procathepsin D in K-562 cells is completely inhibited by ammonium chloride (Davidson, H. W., unpublished observation) and hence M6P dependent, the observed effect would arise if any part of this receptor-mediated process were disrupted. Thus one trivial explanation is that wortmannin interferes with the formation of the recognition signal. The data presented above clearly eliminate this possibility as the proenzyme secreted in the presence of the drug was able to selectively bind to both cell associated and isolated ci-M6PRs. Another possibility is that wortmannin inhibits binding of lysosomal hydrolases to M6P receptors in the TGN. This could occur either following an alteration in the internal environment of the organelle (for example by acidification below pH 5.5), or by the prevention of receptor-ligand dissociation in the PLC (for example by alkalinization of this compartment) and consequent recycling of occupied receptors. Such a conclusion might be supported by the apparent lack of a stable TGN-associated pool of procathepsin D, although it is uncertain whether the experimental conditions used, and total populations of M6P receptors and competing ligands present, would permit detection of such a pool if it occurred. To date there have been no reported indications that PI3-K directly affects ion channels or pumps, or that wortmannin or LY294002 directly influence these molecules at the concentrations used, and I consider it unlikely to be the primary cause of the procathepsin D mistargeting presently

observed. Nevertheless the possibility that the inhibitors act directly or indirectly to prevent receptor ligand association and/or dissociation cannot be completely excluded at present.

However, perhaps a more probable explanation of the observed effects is that wortmannin directly influences M6P receptor trafficking, either by the prevention of its recruitment into clathrin-coated buds on the TGN, inhibition of budding and/or fusion of clathrin-coated vesicles, or by the prevention of recycling from the PLC. A similar conclusion was also reached in a related study concurrent with my work (Brown et al., 1995). In this case the authors showed that treatment of rat clone 9 hepatocytes with wortmannin, albeit at significantly higher concentrations than those used in the present study, dramatically changed the subcellular distribution of ci-M6PRs, causing a depletion from both PLCs and the cell surface, and an inferred accumulation in the TGN. Concurrent with this redistribution of ci-M6PRs induced by wortmannin, or the structurally unrelated PI3-K inhibitor LY294002 (Vlahos et al., 1994), Brown and coworkers also observed aberrant secretion of procathepsin D. Thus their data suggests that inhibition of PI3-K activity inhibits exit of ci-M6PRs from the TGN, but does not affect recycling from the PLC; a possibility which could not be excluded by the results of my study. Consistent with their conclusion, wortmannin does not prevent recycling of TGN38 from the cell surface to the TGN under conditions which cause redistribution of ci-M6PRs (Reaves, B. J., and J. P. Luzio, manuscript in preparation), while the demonstration in the present study that efficient targeting of procathepsin D rapidly resumed after removal of the inhibitor argues against the loss of receptors from the PLC observed by Brown and coworkers being due to their degradation.

Nevertheless, it remains unclear whether mistargeting of procathepsin D results from the selective inhibition of M6P receptor recruitment into coated buds, or from the prevention of budding of clathrin coated membrane from the TGN. Wortmannin does not appear to significantly alter the amount of γ-adaptin associated with the TGN in intact NRK cells, affect AP1 adaptor recruitment to the TGN in vitro (Seaman, 1995), nor alter the gross ultrastructure of the TGN (Brown et al., 1995). However, there is no indication as to how dynamic the pool of TGN-associated clathrin and adaptors is in the presence of PI3-K inhibitors, nor whether clathrin-coated vesicles are formed from this organelle under these conditions.

Another question which remains to be answered is the identity of the PI3-K involved in lysosomal enzyme targeting. In S. cerevisiae, inactivation of Vps34p almost completely abolishes the formation of phosphatidylinositol 3-phosphate (Schu et al., 1993), indicating that it is the major, if not only, PI3-K in these cells. In contrast mammalian cells possess at least four distinct PI3-K catalytic subunits (Hiles et al., 1992; Hu et al., 1993; Stephens et al., 1994a,b). Of these at least three are implicated in events associated with ligand-activation of plasma membrane receptors (Hiles et al., 1992; Hu et al., 1993; Stephens et al., 1994b), and one or more of them may be involved in ligand-activated membrane trafficking events such as glucose transporter translocation and PDGF receptor sorting. However it appears unlikely that such enzymes would also

be involved in constitutive transport events such as lysosomal enzyme trafficking. Indeed the observation in the present study that half-maximal inhibition of lysosomal targeting required a dose of LY294002 6–8-fold greater than that required to half-maximally inhibit insulin stimulated PI3-K activity and glucose transporter translocation (Cheatham et al., 1994) suggests that an enzyme distinct from that implicated in ligand-activated events is involved in lysosomal transport. Like Vps34p, but unlike other mammalian PI3-Ks, one of the enzymes identified by Stephens and coworkers is specific for phosphatidylinositol (Stephens et al., 1994a). Again like Vps34p (Woscholski, et al., 1994), this enzyme is relatively insensitive to wortmannin in vitro, with half-maximal activity at ∼400 nM (Stephens et al., 1994a). However such resistance is clearly incompatible with the observation in the present study, that half-maximal mistargeting of procathepsin D occurred at 10–20 nM wortmannin, casting severe doubt on the hypothesis that it is the enzyme whose inhibition is responsible for the effect.

Recently a cDNA clone encoding part of a protein whose sequence is highly homologous to Vps34p, and more closely related to VPS34 than other mammalian PI3-Ks, has been isolated from a rat insulinoma (Davidson, H. W., unpublished observation). A full-length clone encoding a protein immunologically and structurally similar to the rat "vps34 homologue" has been isolated from a human cDNA library (Volinia et al., 1995; Davidson, H. W., and M. D. Waterfield, unpublished observation). This gene encodes a 95-kD PI-specific PI3-K which is inhibited by wortmannin in the low nanomolar range and by LY294002, consistent with the observations made in the present study. At present it remains to be shown that this gene indeed encodes the PI3-K involved in lysosomal enzyme targeting. However it appears to be the best current candidate, and the probable involvement of a mammalian "vps34 homologue" in the movement of newly synthesized lysosomal hydrolases from the TGN to endosomes further supports the hypothesis that the biochemical machinery governing this event is highly conserved in all eukaryotes.

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