Overexpression of a rat kinase-deficient phosphoinositide 3-kinase, Vps34p, inhibits cathepsin D maturation

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Lipid kinases and their phosphorylated products are important regulators of many cellular processes, including intracellular membrane traffic. The best example of this is provided by the class III phosphoinositide 3-kinase (PI-3K), Vps34p, which is required for correct targeting of newly synthesized carboxypeptidase Y to the yeast vacuole. A probable mammalian Vps34p orthologue has been previously identified, but its function in the trafficking of lysosomal enzymes has not been resolved. To investigate the possible role(s) of mammalian Vps34p in protein targeting to lysosomes, we have cloned the rat orthologue and overexpressed a kinase-deficient mutant in HeLa cells. Expression of the mutant protein inhibited both maturation of procathepsin D and basal secretion of the precursor. In contrast wortmannin, which also inhibited maturation, caused hypersecretion of the precursor. We propose that mammalian Vps34p plays a direct role in targeting lysosomal enzyme precursors to the endocytic pathway in an analogous fashion to its role in the fusion of early endocytic vesicles with endosomes. We further suggest that inhibition of a wortmannin-sensitive enzyme, other than mammalian Vps34p, is responsible for the failure to recycle unoccupied mannose 6-phosphate receptors to the *trans*-Golgi network, and consequent hypersecretion of lysosomal enzyme precursors observed in the presence of this drug.

Key words: endosome, lysosome, phosphoinositide 3-kinase, *trans*-Golgi network, wortmannin.

INTRODUCTION

There is now abundant evidence to indicate that, in addition to their roles in cell signalling, lipid kinases and their phosphorylated products are important regulators of intracellular membrane traffic (reviewed in [1–3]). Perhaps the best example of this is provided by the class III phosphoinositide 3-kinase (PI-3K), Vps34p. This enzyme was first identified as a component of the yeast vacuolar protein sorting machinery, which, when inactivated, causes aberrant secretion of newly synthesized carboxypeptidase Y (CPY) from the yeast Golgi [4,5]. In addition to its role in biosynthetic traffic, there is also considerable evidence to support a requirement for Vps34p in the endocytic pathway. For example, yeast expressing *end12* (a mutant allele of *VPS34*) are defective in transferring endocytosed α-factor to the vacuole [6], and Vac1p/Vps19p, which is required for fusion of transport vesicles to yeast endosomes, binds the lipid product of Vps34p [7,8]. Vac1p is one member of a family of proteins that contain the FYVE domain [3], which is implicated in PtdIns(3)*P* binding [9,10]. Other yeast family members include Vps27p and the PtdIns(3)*P* 5-kinase Fab1p. Deletion of *FAB1* causes abnormally large vacuoles containing fewer internal vesicles than normal to form, suggesting that the lipid product is required for correct sorting in the yeast multivesicular body [11]. Vps34p is the sole PI-3K in *Saccharomyces cerevisiae* [5], which implies that this enzyme has multiple roles throughout the yeast endocytic system.

In general, the membrane trafficking machinery appears highly conserved between all eukaryotes. Hence the involvement of a Vps34p orthologue in targeting newly synthesized lysosomal hydrolases has been postulated on the basis that PI-3K inhibitors, such as wortmannin and LY294002, cause aberrant secretion of the precursor forms from cultured mammalian cells [12,13], analogous to the mis-targeting of proCPY. Similarly, the observation that treatment of mammalian cells with wortmannin and LY294002 drastically changed the morphology of endosomal compartments [13–15] suggested that a mammalian orthologue of Vps34p might be involved in trafficking to lysosomes, the presumed mammalian equivalent of yeast vacuoles. However, in contrast with yeast, mammalian cells contain multiple PI-3K isoforms, many of which are sensitive to wortmannin and LY294002 at the doses used to perturb membrane transport [1,2], and so the involvement of an individual PI-3K isoform in any particular cellular event cannot be determined on the basis of inhibitor sensitivities alone.

A probable mammalian orthologue of Vps34p has been identified [16], although to date its function has not been fully determined. Experiments using antibodies towards the mammalian protein, which significantly reduced its enzymic activity, caused defects in endosomal function in microinjected cells [17], and inhibited an endosome–endosome fusion assay *in vitro* [18]. These effects were probably due to a failure of EEA1 (the probable mammalian orthologue of Vac1p) to bind to endosomal membranes. However, these studies did not address the involvement of mammalian Vps34p in the biosynthetic pathway. In the present study we report that overexpression of a dominant negative form of rat Vps34p in HeLa cells does indeed inhibit the trafficking of procathepsin D to lysosomes. In contrast with the effects of the PI-3K inhibitor wortmannin, the mutant Vps34p did not cause hypersecretion of the precursor protein. This strongly suggests that multiple PI-3Ks are involved in trafficking

Abbreviations used: CPY, carboxypeptidase Y; αMEM, alpha modification of minimal essential medium Eagle; PI-3K, phosphoinositide 3-kinase; TGN, *trans*-Golgi network.

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of newly synthesized lysosomal hydrolases between the *trans*-Golgi network (TGN) and lysosomes, and that a wortmannin-sensitive enzyme other than mammalian Vps34p is responsible for the hypersecretion which we and others have previously observed.

MATERIALS AND METHODS

Cloning of rat *VPS34*

Rat liver cDNA (ClonTech, Cambridge, U.K.) was amplified by PCR with degenerate oligonucleotides encoding the sense sequence GDDLRQD (5′-GGNGAYGAYYTNMGNCARGA-3′) and the complement of FHADFG (5′-NCCRAARTCNG-CRTGRAAN-3′). The 350–450 bp products were ligated into pBluescript (Stratagene, Cambridge, U.K.) and inserts from 4 positive clones were sequenced. Two were 99 % identical to the catalytic domain of mouse p110α. Another had 58 % identity to *VPS34*. This insert was used to probe a rat insulinoma cDNA expression library [19]. Three positive clones having identical 2.2 kb inserts were isolated and sequenced. Each contained a 2059 bp open reading frame, a stop codon and 3′ untranslated material. To obtain the 5′ end, a PCR primer (5′-TTAGATG-CAGGAGGAGGGGAGGCC-3′) complementary to a region 900 bp from the 5′ end of the partial clones was used to probe a 'Marathon-Ready' rat liver cDNA library (ClonTech) by 5′ rapid amplification of cDNA ends. The resulting 1400 bp PCR product comprised 50 bp of 5′ untranslated sequence upstream of a Kozak consensus sequence, an initial ATG codon and an additional 598 bp 5′ to the overlap with the previous clones. The complete cDNA was then assembled using a unique *Nde*I restriction site, and re-sequenced in both directions using the service provided by the Department of Biochemistry, University of Cambridge. The contiguous clone contained a 2661 bp open reading frame encoding a predicted 887 residue protein (submitted to EMBL/GenBank® as AJ006710).

Mutagenesis

cDNA clones encoding mutations corresponding to $Asp^{743} \rightarrow$ Ala and $Asn^{748} \rightarrow$ Ile were constructed as follows: PCR fragments were generated using a primer containing the start codon, 5′-TGTGCCGAGCTTGTCATGGGGGA-3′, and a mutagenic primer, 5′-CTTTGTTAACAGAAGGATATCTAGATGCCG-GGCTC-3′, containing both of the point mutations described above, as well as two silent mutations which generated an *Xba*I site to provide a diagnostic tool for isolating mutant clones (mutations are shown in bold, an *Hpa*I site is underlined). The PCR fragment was isolated, purified and digested with *Hpa*I generating a 1241 bp fragment. The wild-type sequence was then replaced by the mutated fragment following *Hpa*I digestion of the original cDNA. Sequencing of both strands confirmed the presence of the intended mutations, and the absence of any additional changes. For expression studies in mammalian cells, a *Bam*H1 fragment containing the entire coding sequence and approximately 300 bp of the 3′ untranslated region of the wild-type and mutant rat *VPS34* was cloned into *Bam*H1-cut pCDNA3-puro (a gift from J. Karttunen, University of Cambridge).

Cells and antibodies

BHK-21 [20], Cos-7 [21] and HeLa [22] cells were obtained from the European Collection of Cell Cultures (Porton Down, Salisbury, Wilts., U.K.), and maintained as recommended by the suppliers.

Polyclonal rabbit anti-(human cathepsin D) serum was obtained from Dako (High Wycombe, Bucks., U.K.). To generate antisera towards rat Vps34p a *Kpn*I–*Bam*HI fragment comprising amino acids 789–887 (see Figure 1, top panel) was fused in frame to glutathione S-transferase (pGEX; Pharmacia, Milton Keynes, U.K.). The fusion protein was purified from *Escherichia coli* lysates according to the methods recommended by the manufacturers, and polyclonal antisera were raised in guinea pigs.

Transfection of cell lines

For analysis of lipid kinase activity BHK-21 cells were transiently transfected by lipofection (LIPOFECTAMINE™; Gibco BRL, Paisley, Renfrewshire, Scotland, U.K.) with the empty pCDNA3-puro vector, or vector containing wild-type or mutant rat *VPS34*. Following incubation for 24 h cells were harvested, and PI-3K activity was assayed in guinea pig anti-(rat Vps34p) immunoprecipitates essentially as described by Volinia et al. [16]. Expression of rat Vps34p in the transfected cells was monitored by Western blotting [23], using a horseradish peroxidase-conjugated rabbit anti-(guinea pig Ig) secondary antiserum (Sigma) and enhanced chemiluminescence detection (Pierce & Warriner, Chester, U.K.).

For analysis of rat Vps34p–p150 association, Cos-7 cells were cotransfected by lipofection with pMT2-p150 [24] and either the empty pCDNA3-puro vector, or vector containing wild-type or mutant rat *VPS34*. After 24 h the culture medium was replaced with methionine- and cysteine-free Dulbecco's modified Eagle's medium (Sigma) containing 5 % (v/v) dialysed newborn-calf serum, and the cells were preincubated at 37 °C for 1 h. They were then labelled in fresh methionine- and cysteine-free medium supplemented with 83 μCi/ml ProMix (Amersham, Little Chalfont, Bucks., U.K.) for an additional 2 h. After extensive washing with ice-cold PBS the cells were solubilized as described previously [12]. Vps34p and associated proteins were recovered from the clarified lysates using the polyclonal guinea pig sera, and were analysed by SDS/PAGE and fluorography [25].

For analysis of mammalian Vps34p function, HeLa cells were transfected with either the empty pCDNA3-puro vector, or vector containing mutant rat *VPS34*, using SuperFect (Qiagen, Crawley, West Sussex, U.K.) according to the manufacturer's instructions. After 24 h the culture medium was supplemented with 5 μg/ml puromycin (Sigma), and the surviving cells were analysed 72 h post-transfection. Trafficking of (pro)cathepsin D was assayed essentially as described previously [12], modified to accommodate the use of adherent rather than suspension cells. Briefly, the cells were washed twice with PBS, then incubated in methionine-free alpha modification of minimal essential medium Eagle (αMEM) (Sigma) containing 2.2 g/l sodium bicarbonate, and 5 % (v/v) dialysed newborn-calf serum, for 1 h at 37 °C in an incubator with a CO_2/air atmosphere (1:19). After washing twice with PBS, methionine-free medium containing 20 mM Hepes and 100 μCi/ml ProMix was added, and the cells were incubated at 37 °C for 15 min. They were then transferred to ice, washed three times with ice cold PBS/5 mg/ml BSA and chased for up to 4 h in αMEM containing 20 mM Hepes, 1 mg/ml BSA and 1 mM methionine. In some experiments the chase medium also contained wortmannin (100 nM), which was replenished every hour of the chase period. Labelled (pro)cathepsin D was immunoprecipitated, and analysed by SDS/PAGE and PhosphoImaging (Fuji).

(*a*)

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CTTGTCATGGGGGAGGCGGAGAAGTTCCACTACATCTACAGCTGTGACCTGGACATCAACGTGCAGCTGAAGATAGGG   78
      M  G  E  A  E  K  F  H  Y  I  Y  S  C  D  L  D  I  N  V  Q  L  K  I  G     24

AGCTTGGAAGGGAAGAGAGAACAAAAGAGCTACAAAGCTGTCCTAGAAGATCCCATGCTAAAGTTTTCCGGACTATAC  156
S  L  E  G  K  R  E  Q  K  S  Y  K  A  V  L  E  D  P  M  L  K  F  S  G  L  Y     50

CAAGAGACATGCTCTGATCTGTATGTGACTTGTCAAGTTTTTGCTGAAGGGAAGCCCTTGGCCTTGCCAGTCAGAACA  234
Q  E  T  C  S  D  L  Y  V  T  C  Q  V  F  A  E  G  K  P  L  A  L  P  V  R  T     76

TCCTACAAACCATTTAGTACACGATGGAATTGGAACGAGTGGCTGAAACTCCCGGTGAAATACCCTGACCTGCCCAGG  312
S  Y  K  P  F  S  T  R  W  N  W  N  E  W  L  K  L  P  V  K  Y  P  D  L  P  R    102

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CTCTTTGGAAAATATGGCATGTTTCGCCAAGGAATGCACGACTTGAAAGTCTGGCCTAACGTGGAGGCAGATGGCTCC  468
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GAACCCACACGAACCCCGGGCAGAACAAGCAGCACACTCTCAGAAGACCAGATGAGCCGCCTTGCCAAGCTTACCAAG  546
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Q  Y  W  R  K  •                                                               887
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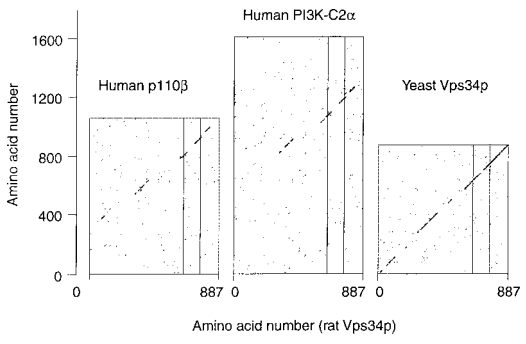
(*b*)



For statistical analysis, the ratios of the amounts of the mature product or secreted precursor recovered after a 4 h chase from control and inhibited cells were calculated for each experiment, and mean values were determined. The null hypothesis that there was no difference between them (mean ratio = 1) was tested using the Student's *t* test.

RESULTS

The components involved in membrane trafficking pathways are often highly conserved between yeast and higher eukaryotes. To test the hypothesis that an enzyme related to the product of the *S. cerevisiae VPS34* gene is required for delivery of lysosomal hydrolases to lysosomes, we used degenerate oligonucleotide primers encoding conserved regions of the consensus lipid kinase domain [26] to amplify cDNA from a rat liver library by PCR. One product was 58 % identical to yeast *VPS34*, and was used as a probe to clone a probable rat orthologue. The predicted protein sequence of rat Vps34p (Figure 1, top panel) was 36.7 %, 50 % and 93 % identical to the *S. cerevisiae* [4], *Drosophila melanogaster* [27], and human [16] orthologues respectively. Comparative analysis of the predicted protein sequence of rat Vps34p with those of a representative of the class I (p110β) or class II (PI-3K-C2α) enzymes revealed four short regions showing greater than 30 % identity (Figure 1, bottom panel). Two of these corresponded to the amino and carboxyl regions of the previously defined lipid kinase domain, which, overall, showed greater than 40 % identity. In contrast, comparison with *S. cerevisiae* Vps34p revealed extensive similarity throughout the primary sequence. The C-terminal third of the molecule was especially well conserved, which may reflect the similar substrate specificities of the two enzymes.

To confirm that the rat protein had enzymic activity we introduced the cDNA into BHK-21 cells. As expected, Vps34p immunoprecipitates from cells overexpressing the wild-type rat Vps34p contained a high level of PI-3K activity (Figure 2), which like yeast Vps34p [5] was specific for phosphoinositide as substrate (results not shown). Unlike yeast Vps34p, but consistent with the human enzyme, rat Vps34p was potently inhibited by wortmannin [16,28]. To further examine the rat enzyme we introduced two point mutations into the lipid kinase domain, analogous to those previously shown to inhibit the activity of yeast Vps34p [5]. As predicted, the mutant protein expressed in BHK-21 cells was kinase inactive (Figure 2). These mutations also abolished lipid kinase activity in human Vps34p (results not shown).

In yeast, the introduction of mutations into Vps34p, which abolish lipid kinase activity, creates a dominant-negative phenotype when kinase inactive protein is overproduced in a wild-type strain. This occurs because the mutant protein competes with wild-type Vps34p for binding to the myristoylated membrane protein Vps15p, a protein kinase required for membrane association and activation of the wild-type lipid kinase [29]. A Vps15p orthologue has been isolated in association with the human enzyme [16,24], and so we postulated that kinase-deficient

Figure 1 cDNA and translated protein sequence of rat Vps34p

Top panel: rat cDNA was cloned and sequenced as described in the Materials and methods section. The underlined region shows the original PCR product, and the amino acids in bold are those mutated in the kinase-deficient constructs. Bottom panel: diagon analysis of the predicted protein sequence of rat Vps34p with the sequences of human p110β [42], human PI-3K-C2α [43], and *S. cerevisiae* Vps34p [4] was conducted using the Staden software package [44]. The vertical lines within each plot show the boundaries of the PCR probe.

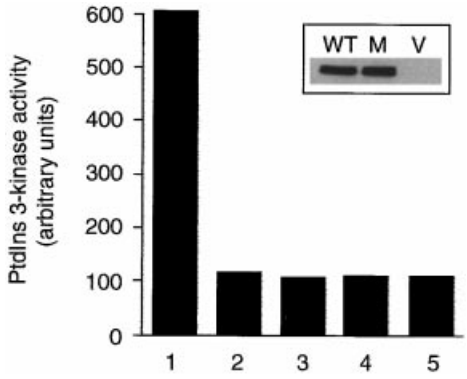


Figure 2 PI-3K activity of wild-type and mutant rat Vps34p

PI-3K activity was determined in immunoprecipitates of BHK-21 cells transfected with pCDNA3 constructs encoding wild-type (columns 1, 4 and 5), mutant rat Vps34p (column 2) or vector alone (column 3) as described in the Materials and methods section. Precipitations used guinea pig anti-(rat Vps34p) (columns 1-4), or pre-immune sera (column 5). Analyses were conducted in the absence (columns 1–3 and 5) or presence (column 4) of 100 nM wortmannin. Results are expressed in arbitrary units following quantification using a Fuji Bas2000 PhosphoImager. The inset shows an immunoblot of the expressed Vps34p from samples transfected with wild-type (WT), mutant (M) or vector alone (V).

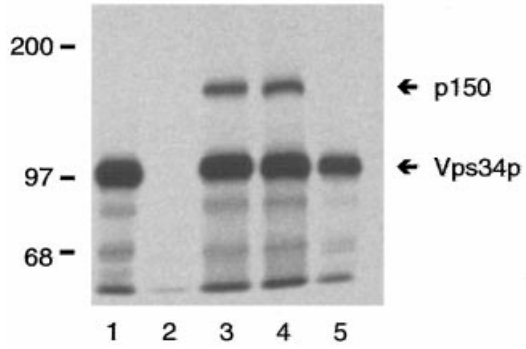


Figure 3 Co-precipitation of rat Vps34p and human p150

Cos-7 cells were transfected with cDNAs encoding wild-type (lanes 1, 3 and 5) or mutant (lane 4) rat Vps34p and human p150 (lanes 2–5) as described in the Materials and methods section. The cells were radiolabelled 24 h post-transfection, and Vps34p and associated proteins were recovered from detergent lysates using guinea-pig anti-(rat Vps34p) and Protein A. Lane 5 contains material recovered from the combined lysates of cells separately transfected with rat Vps34p and human p150.

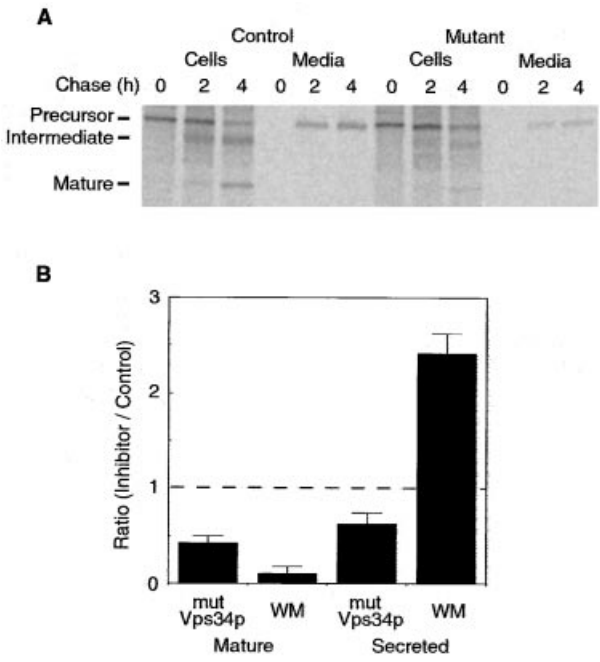


Figure 4 Expression of kinase-deficient rat Vps34p in HeLa cells

(**A**) HeLa cells were transfected with vector alone (control) or vector containing mutant Vps34p cDNA as described in the Materials and methods section. Radiolabelled (pro)cathepsin D was recovered from cell detergent lysates and media respectively and analysed by SDS/PAGE and PhosphoImaging. A representative experiment is shown. (**B**) The ratios of cathepsin D recovered as the fully processed (mature) and secreted forms of cells, either transfected with mutant Vps34p, or treated with 100 nM wortmannin, relative to the matched control cells, were calculated. Columns 1 and 3 show the means $\pm$ S.E.M. ($n = 6$) from experiments involving cells transfected with mutant Vps34p (mut Vps34p), and columns 2 and 4 show the means $\pm$ range ($n = 2$) of wortmannin (WM) treated cells. The broken line shows the expected ratio (1) if there were no difference between the inhibitor-treated cells and the control cells.

rat Vps34p would also act as a dominant-negative inhibitor if expressed in mammalian cells. To confirm that the rat enzyme could associate with the human Vps15p orthologue, we transfected Cos-7 cells with cDNA encoding either wild-type or mutant rat Vps34p with or without human Vps15p cDNA. As shown in Figure 3, significant amounts of the overexpressed human Vps15p could be recovered using antibodies towards rat Vps34p in cells co-transfected with either the wild-type or mutant lipid kinase (lanes 3 and 4). Interestingly the association of Vps15p and Vps34p appeared to be dependent upon co-expression within the same cells, since Vps15p was not efficiently recovered if lysates from separately transfected cells were combined prior to immunoprecipitation (lane 5).

We subsequently examined the effects of expressing the mutant rat enzyme on cathepsin D targeting and maturation. Our initial attempts to isolate stable lines expressing high levels of the mutant protein were unsuccessful, perhaps because of the reported role of Vps34p in growth-factor stimulated mitogenesis [17]. Accordingly we adopted a transient expression protocol, and to overcome problems associated with untransfected cells, utilized a vector conferring resistance to puromycin. This inhibitor killed more than 95% of HeLa cells not expressing the *pac* gene product within 24 h of exposure (results not shown) providing an efficient mechanism to discriminate between transfected and untransfected cells. Indirect immunofluorescence microscopy indicated that there was significant heterogeneity in mutant Vps34p expression within the selected population of cells, but that in all cases staining was essentially cytosolic (results not shown).

In human cells procathepsin D is converted from the 53 kDa precursor form, via a 47 kDa intermediate, into the mature protein, which comprises two chains of 31 kDa and 14 kDa [30] (Figure 4A). Maturation occurs following delivery of the precursor to late endocytic compartments, and is completed in lysosomes [30]. Previously we showed that treatment of K562 cells with PI-3K inhibitors inhibited the maturation of procathepsin D [12], although we were uncertain whether this was due to the inhibition of a Vps34p orthologue, or to inhibition of another lipid kinase. Consistent with our previous observations, treatment of HeLa cells with 100 nM wortmannin also inhibited maturation of procathepsin D (Figure 4B, column 2). In wortmannin-treated cells the inhibition of procathepsin D matur-

ation occurs because the protein is not targeted to the endocytic pathway, but rather is secreted from the cell (Figure 4B, column 4). However although expression of the dominant-negative mutant Vps34p significantly inhibited procathepsin D maturation in the transfected cells (Figure 4B, column 1; $P < 0.001$), its expression did not cause hypersecretion of the precursor, but in fact reduced basal secretion to approximately 80% compared with the control cells (Figure 4B, column 3; $P < 0.025$). A similar effect on procathepsin D maturation was also observed in Cos-7 cells expressing the mutant Vps34p (results not shown).

The effects of the mutant protein on lysosomal enzyme sorting and maturation did not appear to be due to a general disturbance of the early secretory pathway, since constitutive secretion of cotransfected α_1-antitrypsin was essentially identical in control and mutant cells (results not shown). In addition, immunofluorescence microscopy did not reveal any gross differences in the respective subcellular distributions of (pro)cathepsin D, cation-independent mannose 6-phosphate receptors, and the late Golgi marker TGN46, between those cells expressing the mutant Vps34p and those transfected with the empty vector (results not shown).

DISCUSSION

Previously we, and others, have speculated that a mammalian orthologue of yeast Vps34p might be required for delivery of newly synthesized lysosomal hydrolases to lysosomes [12,13]. The data described in the present study provide the first direct proof that this is indeed the case. However, in contrast with our expectation that loss of Vps34p function would result in hypersecretion of lysosomal precursors, as is the case in wortmannin-treated cells, we demonstrated that secretion was actually significantly below basal levels in the presence of the dominant mutant. This strongly suggests that at least one additional wortmannin-sensitive enzyme is required for transport of newly synthesized lysosomal enzymes from the TGN to lysosomes, and that, functionally, this enzyme acts upstream of mammalian Vps34p.

Sorting of procathepsins at the TGN is a receptor-mediated process involving the recognition of mannose 6-phosphate residues [31]. Thus the hypersecretion observed in the presence of PI-3K inhibitors indicates that under these conditions there are insufficient unoccupied receptors in the TGN able to bind newly synthesized precursor molecules. This could occur either if export of receptor-ligand complexes from the TGN were blocked (with the total pool of receptors accumulating in the TGN in an occupied state), or alternatively if recycling of unoccupied receptors to the TGN from endocytic compartments was inhibited. There is still much debate as to which interpretation is correct, with some workers favouring a direct effect upon export of receptor–ligand complexes from the TGN [13,32], and others the perturbation of the recycling of the empty receptors back to the TGN [14,15,33].

The data from the studies referred to above are interpreted mainly in the context of the hypothesis that the PI-3K inhibitors act predominantly at a single transport step. However, the results of the present study support the alternative hypothesis that multiple PI-3Ks are involved in trafficking of lysosomal precursors and their receptors. The export and recycling mechanisms of inhibition are not mutually exclusive. Thus the differences in the conclusions of the various studies may reflect the relative steady state distributions of mannose 6-phosphate receptors (and consequent sensitivity to perturbation of either the export or recycling pathway), or the absolute amounts of the various target enzymes in the cell lines used. It is also interesting to note that the recent study which investigated the effect of microinjecting inhibitory antibodies against human Vps34p demonstrated defects in the early endosomal pathway of human and rodent cells [17], but did not show the large vacuoles often seen in wortmannin-treated cells.

Our study showed that secretion of procathepsin D was not stimulated by the presence of the mutant Vps34p, whereas it was stimulated by the PI-3K inhibitor wortmannin. On this basis we conclude that it is most likely that a wortmannin-sensitive enzyme other than Vps34p is required for the recycling of unoccupied mannose 6-phosphate receptors from endosomes to the TGN, and that Vps34p has a regulatory function in the forward pathway. Our hypothesis predicts that wortmannin inhibits both the forward and recycling pathways, but that the overall effect is that in the presence of the drug the pool of vacant receptors in the TGN is depleted, but is not replenished, and hence hypersecretion occurs. In contrast we suggest that expression of the mutant Vps34p imposes a kinetic block on the movement of occupied receptors between the TGN and the endocytic pathway, but does not prevent subsequent recycling of the empty receptors, and that under these circumstances the pool of free receptors in the TGN is not saturated. At present we cannot be certain of the identity of the enzyme(s) whose inhibition prevents the recycling of unoccupied receptors. Vps34p is the only mammalian class III PI-3K identified [2]. Consequently, assuming that there are no undiscovered class III enzymes, we believe it is likely that either a class I or class II enzyme is the relevant target. In this regard it should be noted that whereas PI-3K-C2α is relatively insensitive to wortmannin, the β-isoform has a similar sensitivity to that of mammalian Vps34p and the class IA enzymes [2].

Our observation that expression of the mutant Vps34p inhibits basal secretion of procathepsin D from HeLa cells suggests that at least a proportion of 'constitutive' secretion is in fact receptor-mediated, as has been proposed previously [34]. There are now several lines of evidence to support the hypothesis that entry of mannose 6-phosphate receptor–procathepsin complexes into the endocytic pathway is at the level of early endosomes (for example see [35,36]). Thus secretion could occur either through dissociation of receptor–ligand complexes in early endosomes, and entry of procathepsin D into the recycling pathway, or through delivery of cation-dependent mannose 6-phosphate receptor–procathepsin D complexes to the plasma membrane (perhaps via early endosomes) and subsequent dissociation. In this regard it should be noted that the cation-dependent mannose 6-phosphate receptor, which is the major form expressed by HeLa cells [37], does not bind extracellular ligand [31]. Nevertheless, whichever the route of secreted procathepsin D, delivery of ligand–receptor complexes to early endosomes raises the possibility that mammalian Vps34p is required for fusion of TGN-derived vesicles, in a similar fashion to its role in the fusion of plasma membrane-derived vesicles, with endosomes [18]. This hypothesis thus predicts that a common mechanism underlies fusion of all vesicles, whether derived from the biosynthetic or endocytic pathway, with early endosomes.

Although we favour a direct role for the lipid product of Vps34p in the fusion of TGN-derived, as well as endocytic, transport vesicles with early endosomes, we cannot exclude the alternative possibility that the enzyme is solely involved in the fusion of endocytic vesicles, and that expression of the mutant enzyme inhibits the biosynthetic pathway to lysosomes indirectly by disrupting the target membranes of biosynthetic vesicles. The results of the present study also do not exclude the possibility that Vps34p is directly involved in the formation of transport vesicles at the TGN, as was originally proposed for yeast Vps34p

[5]. In this regard it should be noted that we were unable to detect any overt redistribution of (pro)cathepsin D immunoreactivity towards a more 'Golgi-like' pattern in the cells expressing the mutant Vps34p. Such a redistribution might have been expected if the mutant protein was indeed inhibiting export from the TGN. However, if, as we believe likely, the mutant protein is imposing a kinetic rather than an absolute block upon transport, we cannot be certain that the inhibition we observe in pulse–chase experiments would necessarily be reflected in a significant change to the steady-state distribution of the marker protein. Nonetheless, there now appear to be better candidates than Vps34p for the role of regulating the formation of transport vesicles at the TGN. Thus two groups have each independently identified distinct TGN-associated PI-3Ks, which differ from mammalian Vps34p in either molecular size and/or wortmannin sensitivity respectively [38,39]. Moreover PI-3K-C2α has now been identified as a component of TGN-derived clathrin-coated vesicles [40,41].

In conclusion the present study directly demonstrates a role for mammalian Vps34p in the transport of lysosomal enzyme precursors to lysosomes, but also suggests the involvement of other wortmannin-sensitive enzymes in this process. In yeast Vps34p is the sole PI-3K [5], and appears to be involved in multiple trafficking steps. In contrast it appears that in mammalian cells distinct enzymes have evolved to perform these multiple tasks which are either all performed by Vps34p, or are not required, in yeast.

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