Inhibition of Calcium-independent Mannose 6-Phosphate Receptor Incorporation into trans-Golgi Network-derived Clathrin-coated Vesicles by Wortmannin*

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The transport of pro-cathepsin D from the trans-Golgi network (TGN) to the endosomal pathway is dependent on binding to the calcium-independent mannose 6-phosphate receptor (ci-M6PR), which is incorporated into TGN-derived clathrin-coated transport vesicles (CCVs). Inhibition of this transport step by wortmannin has led to the proposal that it is dependent upon a phosphoinositide 3-kinase activity necessary for ci-M6PR recruitment into TGN-derived CCVs or in the formation of those vesicles (Brown, W. J., DeWald, D. B., Emr, S. D., Plutner, H., and Balch, W. E. (1995) J. Cell Biol. 130, 781–796; Davidson, H. W. (1995) J. Cell Biol. 130, 797–806). In this study we have addressed the effect of wortmannin on the TGN step of the ci-M6PR cycle. CCVs from K562 cells, pretreated or not with 250 nM wortmannin, were purified on equilibrium density gradients. Quantification of TGN-derivative CCVs, assessed by γ-adaptin content in purified vesicle fractions, showed that the formation of the vesicles was only marginally decreased after 20 min of treatment with the drug, while for the same wortmannin treatment, the amount of ci-M6PR recruited into those vesicles was decreased by 70% compared with control. At a later time point (2 h), a reduction in the amount of γ-adaptin in CCV fractions was also observed. These findings demonstrate that inhibition of ci-M6PR recruitment into CCVs but not of vesicle formation is the primary reason for the observed defect in cathepsin D transport following wortmannin treatment.

For many years, intracellular membrane traffic has been considered to comprise protein-regulated events governing vesicle formation and fusion, while a passive role has been generally assumed for phospholipids. During the same period, phosphoinositides have been widely studied for their participation in transducing inputs received by cell surface receptors. For example, activation of G-protein-coupled receptors may result in stimulation of phospholipase C hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP2), leading to the production of the second messengers inositol 1,4,5-trisphosphate and diacylglycerol. Phosphoinositide 3-kinases (PI3Ks) have also been extensively studied for their role in cellular proliferation and differentiation resulting from their interaction with growth factor receptors (1). More recently, different types of PI3K have been isolated and cloned, which may play other roles in the cell, including the regulation of membrane traffic (2). Together with separate observations linking the small GTPase ADP-ribosylation factor, which is involved in budding of coated vesicles, to activation of phospholipase D, these studies have promoted widespread interest in the role of lipids in governing aspects of membrane traffic (3).

The use of wortmannin, a specific inhibitor of PI3K in the low nanomolar range (4, 5), has allowed the requirement for PI3K activity in many cellular processes to be tested. Many aspects of membrane traffic are influenced by application of the drug, including fluid phase endocytosis (6–8), early endosome fusion (7, 9, 10), transferrin receptor recycling (10, 11), platelet-derived growth factor (PDGF) receptor down-regulation (12, 13), and delivery of pro-cathepsin D from the trans-Golgi network (TGN) to the endocytic pathway (14, 15).

Two major PI3K classes have now been characterized. The first class includes the wortmannin-sensitive heterodimeric mammalian PI3Ks represented by the p85/p110 kinases, which link to receptors with tyrosine kinase activity (1), and the p101/p117 or p101/p120 PI3Ks, which are activated by heterotrimeric G-protein βγ-subunits (16). These heterodimeric PI3Ks exhibit an in vivo substrate preference for PIP2 (17). The second class, specific for phosphatidylinositol (PtdIns), is exemplified by the wortmannin-resistant Vps34p from Saccharomyces cerevisiae (18) and a recently identified wortmannin-sensitive mammalian PtdIns 3-kinase (19).

Among membrane traffic pathways involving the phosphoinositide machinery, the transport of vacuolar enzymes from the TGN to the yeast vacuole was the first in which a PI3K activity was implicated. This result was based on the observation of abnormal secretion of carboxypeptidase Y in a S. cerevisiae strain mutated for a gene encoding Vps34p, subsequently identified as the yeast PtdIns 3-kinase (18, 20, 21). In the wild type strain, the enzyme is routed to the vacuolar compartment. On the basis of wortmannin sensitivity, a role for a PI3K was reported in mammalian cells for the calcium-independent mannose 6-phosphate receptor (ci-M6PR)-mediated transport of lysosomal protease cathepsin D (14, 15). This pathway is analogous to the TGN-vacuole pathway in yeast. In these studies the pro-cathepsin D was observed to be secreted into the extracellu-
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EXPERIMENTAL PROCEDURES

Materials—Tissue culture media and supplements were purchased from Life Technologies, Inc. (Paisley, Scotland). Ribonuclease A (≥3,000 units/mg) was purchased from Worthington Biochemicals, Plainsboro, New Jersey (USA), diethiothreitol from Melford Laboratory Ltd (Ipswich, UK), and Picoll 400 from Pharmacia Biotech (Herts, UK). G-215I-Protein A (81 mCi/mg) and Tran-35S-Label (1,200 Ci/mmole) were purchased from ICN Biomedicals Ltd (Thame, UK). Wortmannin was purchased from Sigma (Poole, UK), made up to 1 μM in dimethyl sulfoxide and stored at 20 °C. Polyclonal rabbit anti-(mouse α-ci-M6PR) antibody was kindly provided by Dr. Sharon Tooze (Imperial Cancer Research Fund, London, UK), polyclonal rabbit anti-(human cathepsin D) antibody was purchased from Dako Ltd ( Bucks, UK). Mouse anti-bovine brain α-adaptin (clone 100/2), mouse anti-bovine brain γ-adaptin antibody (clone 100/3), and goat HRP-coupled secondary antibodies were obtained from Sigma. All other reagents were obtained from Sigma.

Cell Culture—K562 cells were grown from 2 × 10^6 cells/ml in 37 °C RPMI 1640 medium supplemented with 10% (v/v) fetal calf serum (FCS), penicillin (100 units/ml)/streptomycin (100 μg/ml) (Pen/Strep) in a 95% air, 5% CO2 humidified incubator.

Isolation of CCVs from K562 Cells—CCVs were isolated from K562 cells by a modification of the method of Woodman and Warren (24). 1.6 × 10^6 cells were washed twice (700 × g for 6 min) with 37 °C phosphate-buffered saline (PBS) and incubated for 3 h in 20 ml of MEM supplemented with Pen/Strep, 2% (v/v) 1 M Hepes, pH 7.3, 5% (v/v) FBS, and 0.5 μCi of Tran-35S-Label. Approximately 1.3 × 10^10 nonlabeled cells were then combined with 35S-labeled cells, washed with 37 °C PBS, and resuspended at 2.5 × 10^6 cells/ml in 37 °C RPMI 1640 medium supplemented with Pen/Strep, 1% (v/v) FBS, and 2% (v/v) 1 M Hepes, pH 7.3. Cells were then folded into three flasklets and centrifuged at 37 °C for 60 min with or without 250 mM wortmannin. Finally, cell suspensions were centrifuged for 5 min at 250 × g. The cells (pellets) were resuspended in lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 1% (w/v) Triton X-100, 1 mM EDTA, pH 7.4) and cells and media (super- nates) were supplemented with protease inhibitors (1 mM phenyl methylsulfonyl fluoride, 50 μM leupeptin, 1 μM pepstatin A, 50 μM aprotinin). After 10 min on ice, cells and half of the media were centrifuged for 10 min at 25,000 × g and 5 μl of rabbit anti-chimpanzee cathepsin D antibody was added to supernatants for an overnight incubation at 4 °C. Immune complexes were collected using 20 μl of protein A-agarose, washed three times with wash buffer (50 mM Tris-HCl, 150 mM NaCl, 1% (w/v) Triton X-100, 5 mM EDTA, 0.1% sodium deoxycholate, 1% (w/v) sodium deoxycholate, pH 7.5), washed once with 10 mM Tris-HCl, pH 8.8, and resuspended in 50 μl of 1.1-fold concentrated SDS-PAGE nonreducing sample buffer. After 10 min at 90 °C, samples were loaded on a 12% SDS-PAGE gel, which was then dried, autoradiographed, and quantified using a PhosphorImager.

RESULTS

Wortmannin Inhibits ci-M6PR-mediated Transport of Procathepsin D from TGN to Endosomal Compartment—Pro-cathepsin D is synthesized in human cells as a 53-kDa proenzyme, which is modified by the addition of M6P residues that provide the recognition signal for ci-M6PR-mediated sorting at the TGN into CCVs (25). Pro-cathepsin D is then delivered to endosomal compartments, where it is cleaved to form the 47-
kDa intermediate form and then the 31-kDa mature form (26). As shown previously for rat clone 9 hepatocytes, NRK, CHO, MDBK (14), and K562 cells (15), wortmannin treatment triggers a mistargeting of pro-cathepsin D. Following a 10-min pulse radiolabeling with Tran35S-Label, pro-cathepsin D remained associated with cells kept at 4 °C for 120 min and was not processed (Fig. 1, no chase). When cells were incubated for 120 min at 37 °C in the presence of extracellular M6P and in the absence of wortmannin most of the pro-cathepsin D was normally matured and remained associated with the cell, while only a small amount was secreted in the medium (Fig. 1, ctrl). Incubation with 250 nM wortmannin promoted pro-enzyme secretion into the medium, while very few mature or intermediate forms of cathepsin D were found associated with the cell or in the medium (Fig. 1, wort). A minor intracellular band corresponding to the expected molecular weight of the intermediate form is apparent in control and wortmannin-treated cells at similar levels. The significance of this band is unclear, but it is important to note that no processed form is secreted into the medium (Fig. 1, no chase). As shown previously for rat clone 9 hepatocytes, NRK, CHO, MDBK (14), and K562 cells (15), wortmannin treatment triggers a mistargeting of pro-cathepsin D. Following a 10-min pulse radiolabeling with Tran35S-Label, pro-cathepsin D remained associated with cells kept at 4 °C for 120 min and was not processed (Fig. 1, no chase). When cells were incubated for 120 min at 37 °C in the presence of extracellular M6P and in the absence of wortmannin most of the pro-cathepsin D was normally matured and remained associated with the cell, while only a small amount was secreted in the medium (Fig. 1, ctrl). Incubation with 250 nM wortmannin promoted pro-enzyme secretion into the medium, while very few mature or intermediate forms of cathepsin D were found associated with the cell or in the medium (Fig. 1, wort). A minor intracellular band corresponding to the expected molecular weight of the intermediate form is apparent in control and wortmannin-treated cells at similar levels. The significance of this band is unclear, but it is important to note that no processed form is secreted into the medium, indicating that the route taken by the majority of the cathepsin D molecules does not dispose them to processing. Several studies have shown that endocytic trafficking and processing of externally applied cathepsin D is not inhibited by wortmannin (14, 15, 27), so it is highly unlikely that cathepsin D is secreted via an endosomal compartment.

Analysis of Rate Sedimentation and Equilibrium Gradients—The presence of proteins coating the vesicle lipid bilayers confers upon vesicles a high density, which can be exploited for the preparation of highly enriched fractions of CCVs on Ficoll/2H2O equilibrium density gradients. Post-mitochondrial supernatants containing −35% of total radioactivity incorporated in cells (data not shown) were first applied to 10-ml 9–90% 2H2O velocity gradients (see “Experimental Procedures”). Immunoblot analysis of gradient fractions for γ-adaptin (Fig. 2a) showed that membrane associated γ-adaptin was concentrated in fractions 2–7 (1 ml, fraction 1 = top). This is in agreement with the ATP-dependent immunoprecipitation profile of 125I-transferrin observed previously by Woodman and Warren (24) in the same gradient. Moreover, quantification of γ-adaptin immunoblots showed no significant difference in γ-adaptin distribution regardless of wortmannin treatment (Fig. 2b). Fractions 2–6, representing −6% of total radioactivity in cells (data not shown) were pooled for further purification. Fig. 3a demonstrates typical radioactivity profiles after fractions 2–6 were applied on 10-ml Ficoll/2H2O equilibrium density gradients. Similar radioactivity profiles were obtained through the gradient regardless of wortmannin treatment. Two peaks were observed accounting for contaminating membranes (lightest peak, fractions 1–4) and for CCVs (heaviest peak, fractions 6–8) as demonstrated previously on the basis of their fusion activity (23). CCV peak in control cells represented −1% of total radioactivity associated with cells as estimated previously by Pearse (28). Fig. 3b shows a typical autoradiography of fractions collected from the Ficoll2H2O gradient and analyzed by SDS-PAGE. Fractions 6–8 contained a major band at 180 kDa and other bands at −100 kDa and 45–50 kDa consistent with the presence of clathrin heavy chain and the adaptor complexes, respectively (28).

Wortmannin Causes a Decrease in ci-M6PR Content of CCVs Formed at the TGN—To determine if wortmannin treatment was inhibiting CCV formation at the TGN or ci-M6PR recruitment in those vesicles, we analyzed γ-adaptin and ci-M6PR contents in fractions collected from the Ficoll2H2O gradients. The level of γ-adaptin is the critical indicator of TGN-derived clathrin-coated vesicles and the ratio of γ-adaptin over total Tran35S-Label radioactivity (γ-adaptin to total 35S) in the gradient is the appropriately normalized ratio for judging their relative abundance. The ratio of ci-M6PR/γ-adaptin is the critical indicator of receptor content in TGN-derived CCVs. Clathrin heavy chain, accounting for both plasma membrane- and TGN-derived CCVs and α-adaptin (only plasma membrane-derived CCVs), has also been analyzed in those fractions to provide accessory information regarding the effect of wortmannin on CCV formation in K562 cells (Fig. 5). Fig. 4 shows results obtained from the analysis of a typical experiment. γ-Adaptin (Fig. 4a) and ci-M6PR (Fig. 4b) in fractions 5 to 8 were detected from immunoblots using 125I-protein A affinity binding and subsequent phosphorimaging. α-Adaptin (Fig. 4c) was detected from immunoblots using a SuperSignal CL-HRP substrate system. Clathrin heavy chain content was directly determined by PhosphorImager analysis of the 35S signal (Fig. 4d). Intensity measurements of immunoblots (Fig. 4, a–c) or 35S label (Fig. 4d) were normalized by ratioing over total Tran35S-Label radioactivity measured in fractions 1–10 of the Ficoll2H2O gradients (Fig. 3a). Normalized results obtained...
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Previous studies (14, 15) have shown that the ci-M6PR-mediated transport of lysosomal pro-cathepsin D from the TGN to the endosomal compartment is inhibited in cells treated with the PI3K specific inhibitor wortmannin. In wortmannin-treated cells, the pro-enzyme is abnormally secreted into the extracellular medium instead of being delivered to endosomal compartments, where it would be processed (see also Fig. 1). It has also been shown that wortmannin does not affect the addition of the M6P recognition signal to the pro-cathepsin D, the binding of M6P-modified pro-cathepsin D to the ci-M6PR in the Golgi compartment (15), or the transport of the ci-M6PR through the endocytic pathway (14, 15). It was therefore concluded that wortmannin inhibits a PI3K involved in ci-M6PR dependent traffic from the prelysosomal compartment (PLC) to the early endosome via the TGN. However, it remained unresolved, precisely which function is affected by wortmannin.

Four models can be proposed (Fig. 6, i–iv) for sorting of the ci-M6PR into TGN-derived CCVs, (ii) directly in CCV formation at the TGN, (iii) for fusion of TGN-derived CCVs with endosomes, or (iv) for recycling of the ci-M6PR from the PLC to the TGN.

In this study we have first repeated an experiment of Davidson (15) and obtained similar results in terms of pro-cathepsin D secretion into the extracellular medium following wortmannin treatment of K562 cells, indicating that in our hands the cells are responding similarly (Fig. 1). We have then per-
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Formed experiments to discriminate the four models proposed above by preparing CCVs from wortmannin-treated and untreated cells and then quantifying their γ-adaptin and ci-M6PR contents. By rationing the quantified protein values for each condition, we are able to obtain internally consistent values independent of variation in the load applied to the final gradient, much as ratiometric fluorescence measurements reflect ion concentrations independent of fluorophore loading.

Model ii was originally proposed by Emr and co-workers (20), on the grounds that phosphorylation of lipid headgroups would increase the average headgroup area relative to the acyl chain area and promote membrane curvature necessary for budding according to the bilayer couple hypothesis of Sheetz and Singer (29). This model could also be proposed from another point of view, following the recent observation that binding of Golgi adapter EGFL/35 to the TGN occurs shortly after application of wortmannin. We have found that the activation of this kinase forms a complex with Vps15p, a serine/threonine kinase, which participates in transport, to the yeast lysosome-like vacuole (33). It has also been shown that the active form of this kinase forms a complex with Vps15p, a serine/threonine kinase, which participates in the recruitment of ci-M6PR to the TGN membrane and to its activation (20, 34). Vps34p appears to be the only PI3K present in yeast strain mutated in the VPS34 gene encoding for the yeast, wortmannin-insensitive PtdIns 3-kinase, Vps34p (18). A defect in that gene was accompanied by an abnormal secretion of carboxypeptidase Y in the extracellular medium instead of being delivered, through receptor-mediated transport, to the yeast lysosome-like vacuole (33). It has also been shown that the active form of this kinase forms a complex with Vps15p, a serine/threonine kinase, which participates in the recruitment of Vps34p to the TGN membrane and to its activation (20, 34). Vps34p appears to be the only PI3K present in yeast, and can only use PtdIns as substrate. Waterfield and co-workers have recently cloned a mammalian PtdIns 3-kinase (19, 35), which represents the best candidate for a role in ci-M6PR sorting. This kinase is 1) a PtdIns 3-kinase sharing relatively high homology with Vps34p, 2) associated with and

in ci-M6PR, which is inconsistent with a simultaneous shut-down in vesicle formation and vesicle consumption.

Finally, the fact that the depletion of ci-M6PR from CCVs occurred only after 20 min of treatment with wortmannin argues against an inhibition of ci-M6PR recycling from the PLC to the TGN (model iv) because the half-time for recycling has been estimated to be between 1 and 2 h in K562 cells (30). In most cell lines, the steady state distribution of ci-M6PR shows accumulation in the TGN, so exit from the TGN should have a relatively long half-time. Most importantly, a recent study by Nakajima and Pfeffer (27) has shown that wortmannin fails to inhibit the recycling of ci-M6PR in K562 cells under conditions where pro-cathepsin D processing is inhibited. In addition, the drug had no effect on an in vitro assay that reconstitutes the transport step between late endosomes and the trans-Golgi network (27). Brown et al. have reported that the PLC compartment was depleted in ci-M6PR after long exposure to wortmannin while ci-M6PR was still present in the TGN (14). They have also shown that the block to cathepsin D transport from the TGN occurs shortly after application of wortmannin. We have found that the distribution of ci-M6PR on a 0.7–1.3 M sucrose equilibrium gradient was unchanged by wortmannin treatment and co-distributed with a TGN46 (31) peak (data not shown). Taking all these observations together, we consider model iv unlikely at least for K562 cells.

Our data on K562 cells are therefore most consistent with model i, in which the wortmannin treatment inhibits the recruitment of the ci-M6PR into CCVs derived from the TGN. This result is in agreement with the observations by Sphetner et al. (32), who have shown by immunofluorescence that γ-adaptin distribution in HepG2 cells was not affected by 10 min of wortmannin treatment. We have also made the same observation with HeLa cells after more prolonged treatment (data not shown).

The PI3K involved in ci-M6PR sorting is still unidentified. Involvement of a PI3K in protein sorting at the TGN was originally reported in a yeast strain mutated in the VPS34 gene encoding for the yeast, wortmannin-insensitive PtdIns 3-kinase, Vps34p (18). A defect in that gene was accompanied by an abnormal secretion of carboxypeptidase Y in the extracellular medium instead of being delivered, through receptor-mediated transport, to the yeast lysosome-like vacuole (33). It has also been shown that the active form of this kinase forms a complex with Vps15p, a serine/threonine kinase, which participates in the recruitment of Vps34p to the TGN membrane and to its activation (20, 34). Vps34p appears to be the only PI3K present in yeast, and can only use PtdIns as substrate. Waterfield and co-workers have recently cloned a mammalian PtdIns 3-kinase (19, 35), which represents the best candidate for a role in ci-M6PR sorting. This kinase is 1) a PtdIns 3-kinase sharing relatively high homology with Vps34p, 2) associated with and
activated by another protein (p150) with homology to Vps15p, 3) only able to use PtdIns as a substrate, and 4) wortmannin-sensitive. The enzyme is distinct from other mammalian PI3Ks, which couple to activated growth factor receptors or G-protein-coupled receptors and prefer PIP$_3$ as substrate in vivo.

Phosphorylation of the ci-M6PR is tightly associated with its exit from the TGN via the CCV route (36). It is tempting to speculate that the serine/threonine kinase activity of the p150 adaptor subunit of the PtdIns 3-kinase plays a role in this process, although this activity would not be expected to be inhibited directly by wortmannin. 3-Phosphorylated phosphoinositides may themselves regulate this or another crucial serine/threonine kinase, just as phosphatidylinositol 3,4-bisphosphate has recently been shown to specifically activate the c-Akt kinase (37). The fact that CCV formation continues as normal, despite severe depletion of ci-M6PR in the vesicles themselves, suggests that they can form independently of ci-M6PR incorporation, and that other proteins (e.g. LAMP-1; Ref. 38) interacting with γ-adaptin might traverse the TGN to endosome route irrespective of wortmannin treatment. This would be consistent with the observation that alkaline phosphatase in a Vps34 mutant strain of yeast reaches the vacuole as normal (20). Specific sorting of receptors coupled to PI3Ks may be a general feature shared by ci-M6PR and the plasma membrane receptors for PDGF (13) and colony-stimulating factor (39). For each case it remains to be resolved precisely how PI3K activity is translated into a sorting signal.

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REFERENCES

1. Kapeller, R., and Cantley, L. C. (1994) BioEssays 16, 565–576
2. Zvelebil, M. J., MacDougall, L., Levers, S., Volinia, S., Vanhaesebroeck, B., Geut, I., Panaretou, G., Domin, J., Stein, R., Pages, F., Koga, H., Salim, K., Linacre, J., Das, P., Panaretou, C., Wetzker, R., and Waterfield, M. (1996) Phil. Trans. R. Soc. Lond. B 351, 217–223
3. Kiistak, N. T., Brown, H. A., Waters, M. G., Sternweiss, P. C., and Roth, M. G. (1996) J. Cell Biol. 134, 295–306
4. Wymann, M. P., Bulgarelli-Leva, G., Zvelebil, M. J., Pirola, L., Vanhaesebroeck, B., Waterfield, M. D., and Panaretou, G. (1996) Mol. Cell Biol. 16, 1722–1733
5. Arcaro, A., and Wymann, M. P. (1993) Biochem. J. 296, 297–301
6. Clague, M. J., Thorpe, C., and Jones, A. T. (1995) FEBS Lett. 367, 272–274
7. Li, G. P., Dzouzaschowy, C., Barbieri, M. A., Roberto, R. L., Klippel, A., Williams, L. T., and Stahl, P. D. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 10207–10211
8. Sato, S. B., Taguchi, T., Yamashina, S., and Toyama, S. (1996) J. Biochem. (Tokyo) 119, 887–897
9. Jones, A. T., and Clague, M. J. (1995) Biochem. J. 311, 31–34
10. Sprio, D. J., Boll, W., Kirchhausen, T., and Wessling-Resnick, M. (1996) Mol. Biol. Cell 7, 355–367
11. Shepherd, P. R., Reeves, B. J., and Davidson, H. W. (1996) Trends Cell Biol. 6, 92–97
12. Joly, M., Kazlauskas, A., Fay, F. S., and Convera, S. (1994) Science 263, 684–687
13. Joly, M., Kazlauskas, A., and Convera, S. (1995) J. Biol. Chem. 270, 13235–13230
14. Brown, W. J., DeWald, D. B., Emr, S. D., Plutner, H., and Balch, W. E. (1995) J. Cell Biol. 130, 781–796
15. Davidson, H. W. (1995) J. Cell Biol. 130, 797–806
16. Stephens, L., Smrcka, A., Cooke, F. T., Jackson, T. R., Sternweiss, P. C., and Hawkins, P. T. (1994) Cell 77, 83–93
17. Stephens, L. (1995) Biochem. Soc. Trans. 23, 207–221
18. Stack, J. H., and Emr, S. D. (1994) J. Biol. Chem. 269, 31552–31562
19. Volinia, S., Dhant, R., Vanhaesebroeck, B., MacDougall, L. K., Stein, R., Zvelebil, M. J., Domin, J., Panaretou, C., and Waterfield, M. D. (1995) EMBO J. 14, 3339–3348
20. Stack, J. H., Horandovskv, B., and Emr, S. D. (1995) Annu. Rev. Cell Dev. Biol. 11, 1–33
21. Schu, P. V., Takegawa, K., Fry, M. J., Stack, J. H., Waterfield, M. D., and Emr, S. D. (1995) Science 260, 88–91
22. Yang, J., Clarke, J. F., Ester, C. J., Young, P. W., Kasuga, M., and Holman, G. D. (1996) Biochem. J. 313, 125–131
23. Woodman, P. G., and Warren, G. (1992) Methods Enzymol. 219, 251–260
24. Woodman, P. G., and Warren, G. (1991) J. Cell Biol. 113, 1133–1141
25. Hasilik, A., and Neufeld, E. F. (1980) J. Cell Biol. 88, 684–687
26. Delbruck, R. C., Desel, C., von Figura, K., and Hille-Rehfeld, A. (1994) Eur. J. Cell Biol. 64, 7–14
27. Nakajima, Y., and Pleffer, S. R. (1997) Mol. Biol. Cell 8, 577–582
28. Pearce, B. M. F. (1983) Methods Enzymol. 98, 320–327
29. Sheetz, M. P., and Singer, S. J. (1974) Proc. Natl. Acad. Sci. U. S. A. 71, 4457–4461
30. Jin, M., Sahagian, G. G., and Snider, M. D. (1989) J. Biol. Chem. 264, 7675–7680
31. Prescott, A. R., Lacoq, J. M., James, J., Lister, J. M., and Ponnambalam, S. (1997) Eur. J. Cell Biol. 72, 238–246
32. Shpetner, H., Joly, M., Hartley, D., and Convera, S. (1996) J. Cell Biol. 132, 595–605
33. Marcusson, E. G., Horazdovskv, B. F., Cereghino, J. L., Gharakhianian, E., and Emr, S. (1994) Cell 77, 579–588
34. Stack, J. H., DeWald, D. B., Takegawa, K., and Emr, S. D. (1995) J. Cell Biol. 129, 321–334
35. Panaretou, C., Domin, J., Cockcroft, S., and Waterfield, M. D. (1997) J. Biol. Chem. 272, 2477–2485
36. Méresse, S., and Hoflack, B. (1993) J. Cell Biol. 120, 67–75
37. Franke, T. F., Kaplan, D. R., Cantley, L. C., and Toker, A. (1997) Science 275, 665–668
38. Honing, S., Griffith, J., Geuze, H. J., and Hunziker, W. (1996) EMBO J. 15, 2320–2329
39. Carlberg, K., Tapley, P., Haystead, C., and Rohrschneider, L. (1991) EMBO J. 10, 877–883