Cloning and Characterization of a Wortmannin-sensitive Human Phosphatidylinositol 4-Kinase*

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Phosphatidylinositol (PtdIns) 4-kinases catalyze the synthesis of PtdIns-4-P, the immediate precursor of PtdIns-4,5-P₂. Here we report the cloning of a novel, ubiquitously expressed PtdIns 4-kinase (PI4Kβ). The 2.4-kilobase pair cDNA encodes a putative translation product of 801 amino acids which shows greatest homology to the yeast PIK1 gene. The recombinant protein exhibits lipid kinase activity when expressed in Escherichia coli, and specific antibodies recognize a 110-kDa PtdIns 4-kinase in cell lysates. The biochemical properties of PI4Kβ are characteristic of a type III enzyme. Interestingly, both recombinant PI4Kβ and the endogenous protein are inhibited by 150 nM wortmannin, suggesting that we have cloned the previously described PtdIns 4-kinase that is responsible for regulating the synthesis of agonist-sensitive pools of polyphosphoinositides (Nakanishi, S., Catt, J. K., and Balla, T. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 5317–5321).

The metabolism of phosphoinositides has long been acknowledged to play a central role in the transduction of signals triggered by a variety of growth factors and hormones. Both the enzymes and their product phosphoinositides are present in virtually all eukaryotic organisms and tissues that have been studied. Over the past several years the complexity of phosphoinositide metabolism has become better appreciated. In the classically defined phosphatidylinositol (PtdIns) turnover pathway, sequential phosphorylation of the 4 and 5 positions yields PtdIns-4-P and PtdIns-4,5-P₂, the latter of which acts as a substrate for phospholipase C producing inositol 1,4,5-trisphosphate, a stimulator of intracellular Ca²⁺ release (2), and diacylglycerol, a stimulator of certain protein kinase C isoforms (3). More recently PtdIns-4-P and PtdIns-4,5-P₂ have been shown to regulate cytoskeletal rearrangement through the association with a variety of actin binding proteins (4, 5). PtdIns-4,5-P₂ has also been shown to stimulate both phospholipase D (6, 7) and β-adrenergic receptor kinase (8). Finally, all of these lipids are substrates of PtdIns 3-kinase, yielding an array of 3-phosphorylated products (9). It is now clear that the synthesis of a variety of polyphosphoinositides from the starting substrate PtdIns is catalyzed by at least three types of PtdIns kinases (10, 11).

PtdIns 3-kinase (a type I enzyme) catalyzes the phosphorylation of PtdIns at the D3 position of the inositol ring. This enzyme was initially identified through its association with viral oncoproteins and a number of growth factor receptors (12). More recently several additional classes of PtdIns 3-kinases have been identified including a G protein-activated enzyme (13) and VPS 34p, a protein involved in protein trafficking in yeast (14).

PtdIns 4-kinases catalyze the phosphorylation of PtdIns at the D4 position of the inositol ring and have been divided into two types (II and III) based on their size and sensitivity to various compounds (11). The type II enzymes were initially characterized as membrane-associated 55-kDa proteins whose lipid kinase activity is highly stimulated by detergent and inhibited by both adenosine and the monoclonal antibody 4C5G (11, 15). The type III enzymes are membrane-associated proteins predicted to be >200 kDa in size that are less stimulated by detergent and are not inhibited by adenosine or 4C5G antibodies. The PtdIns 4-kinases are highly abundant and have been identified in a large number of membrane structures (reviewed Ref. 16).

Recently several PtdIns 4-kinases have been cloned and found to be homologous to PtdIns 3-kinases. They all contain both a lipid kinase unique domain and a C-terminal catalytic domain with different homology to protein kinases. In yeast, the PIK1 gene encodes a 125-kDa protein that is indispensable for cell growth and plays a role in cytokinesis (17). It contains the lipid kinase unique domain at its N terminus and the catalytic domain in the characteristic C-terminal position. Although it is intermediate in size, its biochemical properties suggest that it is more similar to the type III enzyme (18). In Dictyostelium discoideum, a putative PtdIns 4-kinase has recently been cloned, whose domain structure is similar to PIK1, extending the identification of these proteins across several species (19). A second yeast gene, STT4, encodes a 200-kDa protein that is dispensable for growth in the presence of osmotic stabilizers and has been implicated in the protein kinase C pathway through its isolation in a screen for mutants sensitive to the protein kinase C inhibitor staurosporine (20). Finally, the first PtdIns 4-kinase from higher eukaryotes, PI4Kα, was cloned and shown to encode a 100-kDa protein with significant homology to STT4 and biochemical properties of a type II enzyme (21). This protein, as well as STT4, contains adjacent lipid kinase unique and catalytic domains at its C terminus. An alternative splice of the PI4Kα gene that generates a 230-kDa protein has also been recently reported (22).

These three types of PtdIns kinases all show homology to an ever expanding family of protein kinases whose substrates have not yet been identified. This family includes the TOR/FRAP proteins that are the cellular targets of the FK506-
binding protein-rapamycin complex and are involved in cellular signaling and cell cycle control (23–27). It is interesting to note that although yeast TOR2 and mammalian FRAP/RAFT1 have associated PtdIns 4-kinase activities, these activities are probably not endogenous to the protein kinase catalytic site (27). Other members of this extended family include the ATM/MECl/DNA-PK proteins that are involved in both cell cycle progression and checkpoint control and chromosomal maintenance and repair (28–30). All these proteins share a conserved C-terminal catalytic domain found in both lipid and protein kinases.

Within this conserved domain are specific amino acid stretches that distinguish the subfamily of PtdIns 4-kinases from PtdIns 3-kinases and the other family members. We have taken advantage of this subfamily specificity to detect degenerate PCR primers for the use in cloning novel PtdIns 4-kinases. We have identified and cloned one such gene and analyzed the biochemical properties of the encoded protein, which we call PI4Kβ. Interestingly, PI4Kβ is wortmannin-sensitive and shows great similarity to a recently described wortmannin-inhibitable PtdIns 4-kinase that was partially purified from bovine adrenal cortex (1). Nakanishi et al. (1) demonstrate that this enzyme is responsible for regulating the hormone-sensitive pools of inositol phospholipids. Recent studies in which we have used this effect of 100 nM to 1 μM wortmannin have been used to implicate phosphatidylinositol 3-kinase in membrane trafficking, cytoskeletal rearrangement, and signal transduction but must be reconsidered in view of the nearly ubiquitous expression of wortmannin-sensitive PI4Kβ.

EXPERIMENTAL PROCEDURES

Materials—Human placenta and heart cDNA libraries and the TA cloning kit were purchased from Clontech. T4g polymerase was purchased from Perkin-Elmer. Expand PCR kit was purchased from Boehringer Mannheim. PtdIns was purchased from Avanti, silica plates from Merck, and wortmannin was purchased from Sigma. Random prime labeling kit was purchased from Pharmacia Biotech Inc.

Cloning and Sequencing—A 32-fold degenerate primer (G/GGA/T/CGAC/T/CGAC/TGCA/A/GACA/A/G/GGA/AGC), corresponding to the sense orientation of the conserved sequence GDD/C/I/RQD/E, as well as a 64-fold degenerate primer (AT/G/T/TICA/A/G/GTG/CTGGGA/T/G/CT/GTCT/CTT) corresponding to the antisense orientation of the conserved sequence KDHRNG1 were used in PCR reactions containing ~1 × 106 plaque-forming units of a human placenta cDNA library in AGT16. Reaction conditions were 30 cycles of 94 °C for 1 min, 55 °C for 1 min, and then a 1:18 extension at 72 °C for 1 min. PCR product was digested with SmaI to eliminate PI4K to eliminate PI4Kα clones from the population of PCR products, reamplified as indicated above, and redigested with SmaI. Individual clones were sequenced following subcloning into a TA cloning vector (Clontech) using M13 forward and reverse primers.

The fragment, corresponding to a novel putative PtdIns 4-kinase, was random prime-labeled with [α-32P]dCTP and used to screen a human heart cDNA library in AGT10, under standard procedures (32). ~5 × 106 plaques were screened and 8 positive clones were obtained. Each was subcloned into Bluescript pKS-(Strategene) at the SmaI end of the sequences obtained was labeled and used to rescreen the heart cDNA library in AGT10. The longest clone (3.2) was 1.5 kb, containing a KpnI/HindIII site, digested, and ligated into the 3.2 vector prepared above, yielding pKS4K. pKS4K was then digested and cloned into pGEMX-2. Recombinant clones were screened by SDS-polyacrylamide gel electrophoresis and the fusion proteins were purified as described above. To obtain reasonable amounts of active proteins, we used lower concentrations of isopropyl-1-thio-galactopyranoside (0.1 mM) and overnight induction at 25 °C.

Immunoprecipitation and Western Analysis—Frozen pelleted J774A.1 cells were resuspended in cold lysis buffer (0.3 M NaCl, 20 mM Hepes, pH 7.5, 1.5 mM MgCl2, 0.2 mM EDTA, 1 mM dithiothreitol, 0.2% Triton X-100, and 500 μl of a 2 mg/ml GST affinity column, and specifically bound antibodies were eluted under both acidic and basic conditions as described elsewhere (34). To prepare GST-cleared blotting antibodies, a 1:10,000 dilution of crude serum in TBST was blotted against a membrane containing 40 μg of GST, and the supernatant was collected. The removal of GST antibodies was confirmed by Western blotting the supernatant against untagged GST fusion proteins as described below.

The N-terminally deleted 4Kβ and the full-length 4Kβ cDNAs were prepared by fusing three DNA fragments together as follows. First the backbone was prepared by digesting clone 3.2 in pKS+ with StuI/HindIII. Second, clone 8.1 was PCR-amplified using a 5' sense primer containing a HindIII site and a 3' antisense primer containing a StuI site, digested, and ligated into the 3.2 vector prepared above, yielding pk524Kβ. pk524Kβ was then fused to 5'–RACE products to yield 4Kβ and 4Kβ as follows. 5'-RACE product p3 was amplified using Clontech sense adapter primer (AP1) and an antisense primer recognizing aa 274–280, yielding a 1.5-kb fragment. pKS4Kβ was PCR-amplified using a sense primer recognizing aa 239–245 and a T7 antisense primer, yielding a 2.5-kb fragment. To generate 4Kβ, these two fragments were PCR-amplified using a sense primer recognizing aa 48–87 and an antisense primer recognizing aa 797–801 that contain the appropriate restriction site for subcloning. To generate 4Kβ, these two fragments were PCR-amplified using a sense primer recognizing aa 1–5 and the same antisense primer. The 2.5-kb (pK4Kβ) and 2.5-kb (p4Kβ) amplified products were digested and subcloned into pGEMX-2. Recombinant clones were screened by SDS-polyacrylamide gel electrophoresis and the fusion proteins were purified as described above.

To obtain reasonable amounts of active proteins, we used lower concentrations of isopropyl-1-thio-galactopyranoside (0.1 mM) and overnight induction at 25 °C.

Northern Analysis—A multiple human tissue blot (Clontech) was probed with the randomly primed 321-nucleotide PCR product from the original placenta cDNA library, as per manufacturer’s instructions.

Bacterial Expression and Antibody Production—A GST-fusion protein was generated by PCR using oligonucleotide primers recognizing amino acids 143–438 (aa 410–414 [XTRSH]) in the sense orientation of the amplified 534–538 gene (PYGHL) in the antisense orientation, both tailed with appropriate restriction enzyme recognition sites for subcloning into pGEX4T2 (Pharmacia). Recombinant clones were screened by SDS-polyacrylamide gel electrophoresis of Ercherichia coli protein lysates after isopropyl-1-thio-β-galactopyranoside induction, and the fusion protein (GST-PI4Kβ) was purified using glutathione-agarose affinity chromatography using standard procedures (33). The purified 45-kDa GST fusion protein was injected into rabbits, and antiserum was collected using standard procedures (Charles River PharmServices). Affinity purified antibodies were prepared by first incubating 5 ml of crude serum (diluted 1:10 in 10 mM Tris, pH 7.5), with 500 μl of a 2 mg/ml GST affinity column, for 2 h at 4 °C. Unbound antibody was then chromatographed over a 2 ml/g GST4Kβ affinity column, and specifically bound antibodies were eluted under both acidic and basic conditions as described elsewhere (34). To prepare GST-cleared blotting antibodies, a 1:10,000 dilution of crude serum in TBST was blotted against a membrane containing 40 μg of GST, and the supernatant was collected. The removal of GST antibodies was confirmed by Western blotting the supernatant against untagged GST fusion proteins as described below.

Western blots were probed under standard procedures (Promega) using TBST+ milk (10 mM Tris, pH 7.5, 150 mM NaCl, 0.05% Tween 20, 50 mM NaCl, 0.05% nonfat dry milk) for blocking and TBST+ for antibody incubations and washes. Signal was detected using horseradish peroxidase-coupled secondary antibodies and chemiluminescence as described by the manufacturer (DuPont NEN).

PtdIns Kinase Assay and HPLC Analysis—PtdIns kinase assays were performed essentially as described elsewhere (35). Briefly, GSH beads or Protein A beads containing PI4Kβ were incubated in 50-μl reactions containing 0.3% Triton X-100, 50 μl ATP, 20 mM Hepes, pH 7.5, 10 mM MgCl2, 0.2 mM mg/ml sonicated PtdIns, and 50 μl [γ32P]ATP
(300 Ci/mmol DuPont NEN) at 37°C for 20 min. Reactions were stopped with 105 μl of 1 N HCl and extracted with 160 ml of 1:1 (v/v) mixture of CHCl₃:MeOH. The organic layer was collected and analyzed by both thin layer chromatography and HPLC, as described elsewhere (36).

RESULTS

Cloning of PI4K₃c cDNA and Relationship to the PtdIns 4-Kinase Family—To isolate novel PtdIns 4-kinases, DNA from a human placenta cDNA library was used as a template in PCRs primed with degenerate oligonucleotides derived from two regions highly conserved among PI4Kα, PIK1, and STT4. The 312-bp product generated by PCR was SmaI-digested to eliminate PI4Kα products from the population, and the amplified PCR product was subcloned and sequenced to reveal a novel DNA fragment with homology to the family of PtdIns 4-kinases. This fragment was random prime-labeled and used for Northern blot analysis of human tissues (see below). Based on tissue distribution, it was used to screen a human heart cDNA library. A number of overlapping clones were isolated, and the longest clone (3.2) was further analyzed. The 1.5-kb insert contained a 1.2-kb open reading frame, a 3′ stop, and 0.3-kb of 3′-untranslated region. Since no 5′ stop was detected, the library was re-screened with a probe from the 5′ end of clone 3.2 yielding clone 8.1 which contained 750 bp of additional open reading frame and still no 5′ stop codon. To obtain the remainder of the open reading frame 5′-RACE was performed on human placenta cDNA, yielding a number of overlapping clones, five of which were analyzed and shown to be identical over >80% of their length. The clones differed slightly at the 5′ end but all contained an identical open reading frame contiguously with that of clone 8.1 and all had identical stop codons in all three reading frames 5′ of a potential initiating methionine. The 2.4-kb full-length cDNA predicts a protein of 801 aa with a predicted size of ~90-kDa, starting from an initiation codon with a favorable Kozak consensus sequence for translation initiation (Fig. 1) (37). A second potential initiating methionine would result in a protein 104 amino acids shorter of predicted size; ~80 kDa. Both initiating methionines are followed by glycines suggesting myristoylation of the N terminus (Fig. 1).

The predicted protein, PI4K₃, contains an N-terminal domain (lipid kinase unique domain) (Fig. 2A) that is shared by members of both the PtdIns 3- and PtdIns 4-kinase families (22). Additionally, a C-terminal catalytic domain (Fig. 2B) defines this protein as a member of a much larger family of protein/lipid kinases that include the PtdIns 3- and PtdIns 4-kinases, the TOR proteins, ATM, DNA-PK, MEC1/RAD3, and MEI4 whose members are involved in such diverse functions as mitogenic signaling, cell cycle regulation, and DNA repair (reviewed in Ref. 38). Interestingly, the functionally related PtdIns 4P5-kinase family appears to share no significant sequence homology in either the lipid kinase unique domain or the catalytic domain (39, 40).

PI4K₃ shares most significant sequence homology with yeast PIK1 (42% identity in the catalytic domain and 17% in the lipid kinase unique domain) and with the newly described D. discoidum gene DdPIK4 (45% in the catalytic domain and 19% in the lipid kinase unique domain). This is consistent with the conserved domain structure among these three proteins.

Lipid Kinase Activity of Recombinant and Endogenous PI4K₃—Wortmannin-sensitive PtdIns 4-Kinase

**Fig. 1.** Sequence analysis of PI4K₃. Nucleotide sequence and predicted amino acid sequence of PI4K₃. The single-letter codes for the deduced amino acid sequence are indicated below the nucleotide sequence. Numbering on the left indicates amino acids, and numbering on the right indicates nucleotides. The two potential initiator methionines and the myristoylation signals are highlighted, and the sequences of the original PCR probes are underlined.
PI4K

To confirm that PI4K\(b\) encodes an active PtdIns 4-kinase, several GST fusion constructs were generated, and recombinant protein was expressed in *E. coli*. One such construct, lacking the N-terminal 82 aa of PI4K\(b\) (GST4K\(b\)L) was expressed, purified, and assayed for lipid kinase activity in reactions containing PtdIns as a substrate (Fig. 4A). In contrast to the control (C) GST fusion protein that lacked the catalytic domain, GST4K\(b\)L generated significant amounts of PtdIns-P at both concentrations tested. The full-length PI4K\(b\) gave identical results (data not shown) confirming that the N-terminal nonconserved portion of PI4K\(b\) was not required for lipid kinase activity. Since *E. coli* lacks endogenous PtdIns kinases, these results confirm that PI4K\(b\) encodes a PtdIns kinase.

To identify the lipid products generated, we performed HPLC analysis on the deacylated products of the PtdIns kinase assay. A single peak, precisely comigrating with \(^{3}H\)glycerophosphorylinositol 4-phosphate standard, was observed (Fig. 4B), supporting the classification of PI4K\(b\) as a PtdIns 4-kinase. We observed only modest inhibition by 500 \(\mu\)M adenosine and the type II-specific inhibitory monoclonal antibody 4C5G (15), suggesting that PI4K\(b\) is not a type II enzyme (data not shown). To further explore the enzymatic properties of PI4K\(b\) we next examined its inhibition by wortmannin, a fungal metabolite that inhibits PtdIns 3-kinase at nanomolar concentrations (41, 42). Although the PI4Ka and PIK1 enzymes were resistant to micromolar concentrations of this drug, a partially purified PtdIns 4-kinase from bovine adrenal cortex was shown to be inhibited by 100 \(n\)M wortmannin (1). We assayed GST4K\(b\) activity in the presence of various concentrations of wortmannin and observed concentration-dependent inhibition with an IC\(_{50}\) of 120 \(n\)M (Fig. 4C). This suggests that PI4K\(b\) may be the same enzyme as was previously shown to be wortmannin-sensitive (1).

This wortmannin-sensitive enzyme was identified in several cell types including the human Jurkat T-cell line. We therefore generated PI4K\(b\)-specific antibodies to investigate the properties of endogenous PI4K\(b\) immunoprecipitated from Jurkat cells. Antibodies were raised against a GST fusion of a 100-aa partial clone of PI4K\(b\). To demonstrate the specificity of these antibodies for PI4K\(b\), the GST-cleared polyclonal serum was used to blot protein lysates from wildtype DH5\(a\) cells or DH5\(a\) cells expressing GST4K\(b\)L (Fig. 5A). A signal corresponding to GST4K\(b\)L and several breakdown products were detected only in lysates from transformed bacteria (lane 2). Identical results were obtained when protein lysates were blotted with the crude PI4K\(b\) antibodies (data not shown) suggesting that the observed signal was generated by PI4K\(b\)-specific antibodies and not by the GST antibodies also present in this crude serum.

Using either preimmune or affinity purified immune serum, we immunoprecipitated PI4K\(b\) from detergent-solubilized cell lysates of Jurkat cells. The proteins were separated by SDS-
polyacrylamide gel electrophoresis and analyzed by Western blot using the PI4Kβ antibody. A single protein migrating at ~110 kDa was observed only in precipitations using immune serum (Fig. 5B). This protein has a mobility slightly slower than that predicted by the 801-aa PI4Kβ. It is likely that post-translational modification, such as myristoylation (see
Fig. 1), accounts for the decreased mobility. Aliquots of these same immunoprecipitates were also assayed for lipid kinase activity in the presence of PtdIns (Fig. 5C). Whereas very low levels of PtdIns-P could be detected in assays containing preimmune serum (Pre), a significant amount of PtdIns-P (10–90-fold higher than preimmune) was routinely produced in assays containing affinity purified antisera (Im) or crude immune serum (data not shown). When similar assays were performed using either PtdIns-4-P or PtdIns-4,5-P₂ as substrates, no phosphorylated products were generated (data not shown). As expected, HPLC analysis of the lipid products confirmed the immunoprecipitation of a PtdIns 4-kinase (data not shown). Additionally, PI4Kβ was unable to phosphorylate PtdIns-3-P (data not shown) suggesting that it is distinct from the previously characterized PtdIns-3-4 kinase (43, 44). PI4Kβ lipid kinase activity was only modestly affected by non-ionic detergents, adenosine and 4C5G (data not shown), but was strongly inhibited by wortmannin (Fig. 5D), with an IC₅₀ of 140 nM. Taken together, these results strongly suggest that the 110-kDa PtdIns kinase immunoprecipitated from Jurkat cell lysates is PI4Kβ.

**Discussion**

We have identified and characterized PI4Kβ, a novel PtdIns 4-kinase that is widely expressed in a variety of tissues. The cDNA encodes an 801-aa protein that exhibits lipid kinase activity when expressed in *E. coli*. Both the bacterially expressed and the endogenous proteins exhibit properties consistent with the characterization of PI4Kβ as a type III enzyme. Antibodies raised against PI4Kβ detect a ~110-kDa protein in a number of cell types across several species. Interestingly, PI4Kβ is the first cloned PtdIns 4-kinase that is inhibitable by wortmannin, potentially implicating PI4Kβ in a variety of wortmannin-sensitive cellular pathways.

Sequence analysis of PI4Kβ places it within the PtdIns 4-kinase family and more generally places it in the larger family of lipid/protein kinases. It contains a conserved C-terminal catalytic domain with distant homology to protein kinases as well as strong homology to the dual specificity kinases such as PtdIns 3-kinase. Within this conserved domain is lysine 549 which, based on homology to PtdIns 3-kinase, is the likely site of wortmannin reactivity (45). All members of this lipid/protein kinase family contain this conserved lysine, yet many, including PIK1 and PI4Kα, are not inhibited by micromolar concentrations of the drug, suggesting that additional residues within the active site confer wortmannin sensitivity.

Members of this extended family have diverse cellular functions. For example, the yeast protein MEC1 and its *Drosophila* homologue MEI41 are checkpoint control genes which appear to monitor the state of the genome at the G₁/S and G₂/M transitions (28, 46, 47). Another family member, DNA-PK, was originally identified as a DNA-dependent protein kinase (48) but was subsequently shown to function in immunoglobulin gene rearrangement and DNA repair (29).

PI4Kβ has properties similar to the wortmannin-sensitive PtdIns 4-kinase described by Nakanishi *et al.* (1). The partially purified enzyme was inhibited by wortmannin with an IC₅₀ of ~50 nM, not dissimilar to the 120–140 nM IC₅₀ observed for PI4Kβ. The wortmannin-sensitive enzyme had an apparent molecular mass of 125 kDa, as judged by gel filtration, in agreement with the ~110-kDa molecular mass observed for PI4Kβ. The reported enzymatic properties of this protein are also very similar to those of PI4Kβ. It is likely that we have cloned the PtdIns 4-kinase that regulates the formation of agonist-sensitive inositol phospholipids that are required for intracellular signaling in some cells.

It should be noted that a PtdIns 4-kinase from the particu-long fraction of *Schizosaccharomyces pombe* has been observed to be sensitive to the wortmannin analogue demethoxyviridin (49). Curiously, this enzyme was not inhibited by wortmannin. Additionally, attempts to isolate a drug-sensitive PtdIns 4-kinase from rat brain particulate fractions were unsuccessful (49). Although we have detected PI4Kβ in rat brain, both our experiments and those of Nakanishi *et al.* (1) suggest that it is only loosely associated with the membrane. Furthermore, PI4Kβ is not the major PtdIns 4-kinase present in membrane fractions, and therefore lipid kinase assays on these crude fractions would not be expected to show wortmannin sensitivity.

Taken together, these data suggest that we need to reevaluate the interpretation of experiments employing wortmannin as an inhibitor in biological assays. For example, recent experiments have demonstrated that wortmannin inhibits the proper targeting of the lysosomal enzyme cathepsin D in a variety of cell types (50, 51). The concentration of wortmannin used was as high as 1 μM with an estimated IC₅₀ of ~100 nM. Clearly, these elevated levels of wortmannin could be inhibiting PI4Kβ thereby implicating it in protein trafficking. Furthermore, both wortmannin and demethoxyviridin have been reported to inhibit phospholipase D, PtdIns-phospholipase C, and phospholipase A₂ *in vivo* (41, 52). It is likely that this inhibition is a downstream effect of the inhibition of PtdIns 3-kinase and possibly PI4Kβ in these cells, as little direct inhibition of these enzymes was observed *in vitro* at μM concentrations of wortmannin. The assumption that PtdIns 3-kinase is a critical mediator of all the myriad pathways inhibited by wortmannin is likely to be an oversimplification.

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