Design of Drug-Resistant Alleles of Type-III Phosphatidylinositol 4-Kinases Using Mutagenesis and Molecular Modeling†

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Received August 31, 2007; Revised Manuscript Received November 26, 2007

ABSTRACT: Molecular modeling and site directed mutagenesis were used to analyze the structural features determining the unique inhibitor sensitivities of type-III phosphatidylinositol 4-kinase enzymes (PI4Ks). Mutation of a highly conserved Tyr residue that provides the bottom of the hydrophobic pocket for ATP yielded a PI4KIIIβ enzyme that showed greatly reduced wortmannin sensitivity and was catalytically still active. Similar substitutions were not tolerated in the type-IIIα enzyme rendering it catalytically inactive. Two conserved Cys residues located in the active site of PI4KIIIα were found responsible for the high sensitivity of this enzyme to the oxidizing agent, phenylarsine oxide. Mutation of one of these Cys residues reduced the phenylarsine oxide sensitivity of the enzyme to the same level observed with the PI4KIIIβ protein. In search of inhibitors that would discriminate between the closely related PI4KIIIα and -IIIβ enzymes, the PI3Kγ inhibitor, PIK93, was found to inhibit PI4KIIIβ with significantly greater potency than PI4KIIIα. These studies should aid development of subtype-specific inhibitors of type-III PI4Ks and help to better understand the significance of localized PtdIns4*P* production by the various PI4Ks isoforms in specific cellular compartments.

Inositol phospholipids emerge as universal regulators of membrane-associated signaling events and membrane trafficking (*2*−*4*). These lipids are formed from phosphatidylinositol (PtdIns[1]) by sequential phosphorylations of the hydroxyl groups in their inositol ring by inositol lipid kinases. Phosphatidylinositol 4-kinases (PI4Ks) are the enzymes that produce PtdIns4*P*, a minor regulatory lipid that was long considered only as an intermediate in the production of other phosphoinositides, such as PtdIns(4,5)P_2 and PtdIns(3,4,5)P_3. More recently, PtdIns4*P* has been increasingly recognized as a regulatory lipid that controls the recruitment of adaptor proteins and lipid-transport proteins to ER and Golgi membranes (*5*). Therefore, interest in PI4Ks is increasing. PI4Ks are classified into two major classes: the type-II PI4Ks (-α and -β forms) are proteins of ∼56 kDa in size that are tightly associated with membranes due to their palmitoylation (*6*, *7*). These enzymes have only been recently cloned and, although they represent the majority of PI4K activities associated with membranes, their functional significance has just started to emerge. Type-III PI4Ks (-α and -β forms), on the other hand, are soluble peripheral membrane proteins that are greatly conserved from plants to humans and are homologous to PI3Ks (*8*−*10*).

The importance of the presence of multiple PI4K enzymes in the same cell is still not fully understood, due to the lack of specific inhibitors to study the functions of the individual enzymes. Based on yeast studies, both type-III PI4Ks are essential for viability, and they assume nonredundant functions. Pik1p (the yeast orthologue of PI4KIIIβ), which is mainly localized to the Golgi, plays a role in Golgi-to-plasma membrane secretion at the late Golgi secretory pathway (*11*−*13*), while the functions of Stt4p (the ortholog of PI4KIIIα) are more diverse, including roles in aminophospholipid synthesis (*14*), signaling to MAP kinases via the PKC pathway (*15*), and to PLD at the plasma membrane (*16*). Much less is known about these proteins in mammalian cells,

† This research was supported in part by the Intramural Research Program of the National Institute of Child Health and Human Development of the National Institutes of Health. A.B. is also a Bolyai Fellow of the Hungarian Academy of Science and is supported by the Hungarian Scientific Research fund (OTKA PF-63893).

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[1] Abbreviations: PAO, phenylarsine oxide; PI3K, phosphatidylinositol 3-kinase; PI4K, phosphatidylinositol 4-kinase; PtdIns, phosphatidylinositol; PtdIns4*P*, phosphatidylinositol 4-phosphate; PtdIns(4,5)P_2, phosphatidylinositol 4,5-bisphosphate; PtdIns(3,4,5)P_3, phosphatidylinositol 3,4,5-trisphosphate; Wm, wortmannin.

10.1021/bi7017927 CCC: $40.75
Published on Web 01/19/2008

but PI4KIIIβ, which is a Golgi-localized protein, is important for Golgi-to-plasma membrane secretion (*17*, *18*), while PI4KIIIα appears to regulate the production of the plasma membrane pool of PtdIns4*P*, although the enzyme is primarily localized to the ER/Golgi in mammalian cells (*8*, *19*). Type-II PI4Ks are mostly associated with endosomes and the TGN, and recently the type-IIα enzyme was implicated in EGF receptor trafficking and degradation (*20*).

The ideal way to study the functions of these proteins would be to use specific inhibitors for the distinct isoforms of these enzymes. Unfortunately, little effort has been exerted in this research direction. Wortmannin (*21*, *22*) and LY294002 (*23*) have been invaluable tools to study PI3Ks, and their use has also contributed to our knowledge of type-III PI4Ks (*24*). However, Wm has four major disadvantages when studying the functions of PI4Ks. First, Wm inhibits PI3Ks at much lower concentrations than is necessary to inhibit the type-III PI4Ks. Second, and especially problematic, Wm cannot discriminate between PI4KIIIα and -β (*25*). Third, the effects of Wm are irreversible.[2] Finally, its dose-dependent effects are greatly influenced by the duration of its administration (*26*, *27*). Phenylarsine oxide (PAO) has also been used often as a PI4K inhibitor (*28*), and while this drug has probably several cellular targets, among the PI4Ks it is most potent against the type-IIIα enzyme (*29*). The present study was undertaken to investigate the structural properties of the catalytic domains of type-III PI4Ks, in comparison with those of PI3Ks using molecular modeling. These studies combined with site-directed mutagenesis allowed the generation of PI4K enzymes that are significantly less sensitive to their commonly used inhibitors and that could be used in pharmacological rescue experiments. Moreover, these studies also identified an inhibitor that discriminates between the two closely related type-III PI4Ks. Our studies should aid efforts to search for, and possibly design, inhibitors that would specifically target individual PI4K enzymes.

EXPERIMENTAL PROCEDURES

Materials. Wortmannin and LY294002 were purchased from Calbiochem, and PIK93 was synthesized as described previously (*30*). Phosphatidylinositol and PAO were obtained from Sigma. [γ-^{32}P]ATP (6000 Ci/mmol) was from NEN (General Electric), and [^{3}H]wortmannin-17-ol (19.7 Ci/mmol) was from DuPont NEN. Unfortunately, the latter product has now been discontinued for a long time. [^{3}H]L-Serine (20-40 Ci/mmol) was from General Electric (Piscataway, NJ). All other materials were of the highest analytical grade.

Molecular Biology and Mutagenesis. The bacterial expression plasmid for GST-fused PI4KIIIβ has been described previously (*31*), and so were the HA-tagged forms of the type-II enzymes for eukaryotic expression (*29*). Bovine PI4KIIIβ was HA-tagged at the N-terminus using the pcDNA3.1-PI4KIIIβ as the template (*10*), while bovine PI4KIIIα was epitope tagged at its C-terminus in the pcDNA3.1(+) vector. For bacterial expression of a PI4KIIIα enzyme, a GST fusion construct was generated from the C-terminal fragment (residues 874−2044) of the bovine PI4KIIIα using the pET23b plasmid (Novagen) as a backbone. This plasmid was fashioned to generate a GST fusion protein construct using the GST fragment with its original proteolytic and restriction sites from the pGEX6P (Amersham) plasmid.

Mutagenesis was performed with the QuikChange mutagensis kit of Promega following the manufacturer's instructions. After verifying the mutations with dideoxy sequencing, the mutated fragments were exchanged between the wild-type and mutated enzymes with suitable restriction sites to avoid the generation of unwanted mutations outside the sequenced regions.

Production of Proteins in Bacteria. All four PI4Ks were expressed in the BL21 strain of *E. coli*, induced, purified essentially as described earlier (*31*) and cleaved either with PrerScission or TEV protease or eluted with gluthathione.

PI Kinase Assays. PI kinase assays were performed either with the bacterially expressed and GST purified enzymes on the beads or with the enzymes expressed in COS-7 cells and immunoprecipitated from cell lysates essentially as described previously (*24*, *31*). Briefly, the assay mixture contained ~1 mM PtdIns in the form of Triton micelles and 0.1 mM (1−2 μCi) [γ-^{32}P]ATP in 20 mM Tris buffer (pH 7.4) and in the presence of 20 mM $MgCl_2$ and the enzyme. When inhibitors were added, they were preincubated with the enzymes for 10 min prior to the addition of ATP. Reactions were run at room temperature for 10−20 min and were terminated by the addition of 3 mL of $CHCl_3$:CH_3OH:*cc*HCl (200:100:0.75 (v/v). The organic solvent phase was separated from [γ-^{32}P]ATP by adding 0.6 mL of 0.6 N HCl, mixing vigorously, and letting it stand for phase separation. The upper phase (aqueous) was discarded, and 1.5 mL of $CHCl_3$:CH_3OH:0.6 N HCl (3:48:47, v/v) was added to the lower phase, followed by mixing and phase separation. The lower phase was then transferred to scintillation vials and, after evaporation, the radioactivity counted with Econofluor as a scintillant.

For K_m determinations, the concentration of unlabeled ATP was changed, and the incubation time was chosen to be on the linear part of the reaction kinetics. For calculations, the simple Lineweaver−Burk analysis was used.

Wortmannin Binding. Aliquots of the purified and cleaved wild-type or mutant PI4KIIIβ enzymes containing equal amounts of protein were incubated for 20 min at room temperature in a total volume of 100 μL of PBS containing 0.4 μCi of [^{3}H]WT, which corresponded to 200 nM concentration of WT. Proteins were precipitated with TCA (5% final) and subjected to SDS PAGE and Western blotting. PVDF membranes were then sprayed with ENHANCE (DuPont), and after drying they were exposed at −70 °C for 1 week.

Analysis of Protein Kinase Activity. Aliquots of the purified and cleaved wild-type or mutant PI4KIIIβ enzymes were incubated in 100 μL of 50 mM Tris-HCl pH 7.4, 100 mM NaCl, 3−10 mM MnCl2, 40 μM ATP, and 5 μCi of [γ-^{32}P]ATP for 30 min at 37 °C. Wm or DMSO was added 10 min prior to ATP. After phosphorylation, the protein was subjected to SDS PAGE, and phosphorylation was quantitated using a PhosphoImager (Molecular Dynamics).

Studies in COS-7 Cells. COS-7 cells were transfected with the indicated constructs cloned in pcDNA3.1 plasmids and

[2] The effects of wortmannin can be reversed by strong illumination especially with wavelengths below 450 nm (*1*).

selected in 800 μg/mL G418. Clones were tested with Western analysis to determine the expression of the protein. Cells were grown on 25 mm glass coverslips and transfected with a construct composed of the CERT-PH domain fused to mRFP for 24 h for confocal analysis. Live cells were examined at 35 °C in a Zeiss LSM510 laser confocal microscope as described in detail elsewhere (*32*). The ceramide and sphingomyelin labeling with [^{3}H]serine has been previously described (*33*). Briefly, COS-7 cells were seeded on 12 well plates at a density of 3×10^5 cells/well. After 1 day in culture, cells were incubated in a serine-free medium for 4 h before labeling with 100 μCi/mL [^{3}H]serine for 60 min. When inhibitors were used, they were added 10 min prior to labeling and they were present throughout the labeling period. Incubations were terminated by removal of the labeling medium and the addition of ice-cold 5% PCA. Lipids were extracted, deacylated and separated as described in (*33*).

Molecular Modeling of PI4Ks. Homology models of the bovine type-III PI4Ks (accession numbers: AAC48729 for β form and AAC48730 for α form) were built with either ATP, wortmannin or PIK93 bound, using the crystal structure of PI3Kγ with the particular ligand bound (pdb codes: 1e8x, 1e7u, 2chz, respectively) as template. The SegMod algorithm (*34*) implemented in the program GeneMine was used to build each model.

RESULTS

Comparison of PI3K and Type-III PI4Ks Using Sequence Alignment and Molecular Modeling. Multiple-sequence alignments of the catalytic domains of the various classes of PI 3- and 4-kinases demonstrate a large degree of conservation among these proteins (Figure 1). Such alignments are of great value to pinpoint differences that are conserved within the individual classes of the enzymes. The published X-ray structure of PI3Kγ (*35*, *36*) has made it possible to analyze these differences structurally and to predict their importance for the catalytic process. We used homology-based molecular modeling to compare the structures of the catalytic domains of the two type-III PI4Ks, PI4KIIIα and PI4KIIIβ, with that of PI3Kγ. These models are built assuming that the catalytic domains of PI4Ks fold similarly to that of PI3Kγ. Figure 2A shows the model of PI4KIIIβ with residues that are identical to PI3Kγ colored red. Although the overall homology within this whole segment is only 21%, it increases closer to the ATP binding site (46% within 7 Å of ATP and 65% within 5 Å). The catalytic domains of both PI4K models are formed by two half-lobes, a smaller N-terminal and a larger C-terminal lobe (Figure 2A), with the ATP molecule sandwiched in between. The two sides are linked together by a short intervening sequence, and a highly conserved Tyr residue (Y867 in PI3Kγ) located on a long loop forms the bottom of the nucleotide binding pocket (Figure 2A). Importantly, the major contacts with the adenosine ring and the phosphate groups are highly conserved between the three molecules.

Mutagenesis of PI4KIIIβ. These sequence comparisons and the structural models were used to design a PI4K with diminished Wm sensitivity. The advantage of such an enzyme is that it could be used in expression studies where the endogenous enzymes are inhibited by Wm treatment. Wm binds to the ATP binding site of PI3Ks and covalently attaches to Lys 833 of PI3Kγ (*37*). This Lys residue corresponds to K549 and K1791 in PI4KIIIβ and -α, respectively, shown in the model either with the ATP (Figure 2B) or Wm (Figure 3) molecules. Since this residue is essential to the binding of ATP, its mutation renders the enzyme catalytically inactive. Instead, we sought a site of Wm interaction that, when mutated, would weaken Wm binding without a major effect on ATP binding. Since the yeast orthologue of the PI4KIIIβ enzyme, Pik1p, is resistant to Wm, we reasoned that creation of such a mutant was feasible, and we looked for regions within the Wm binding pocket that differ significantly between the yeast Pik1p enzyme and other PI 3- or 4-kinases.

Two aromatic residues were found in the right position. One was the Tyr within the P866−Y867 sequence (PI3Kγ numbering) that is highly conserved between the PI 3- and type-III PI4-kinases. This Tyr residue, which corresponds to Y583 and Y1825 in PI4KIIIβ and -α, respectively, forms the bottom of the binding pocket both for ATP (Figure 2B) and for Wm (Figure 3). Remarkably, in the Pik1 homologues of lower eukaryotes these two residues are replaced by Arg-Met (Figure 1, upper panel, highlighted in deep blue). The other residue, which corresponds to F961 of PI3Kγ, faces the adenine ring and corresponds to a Ser in Pik1p and an Ile in all type-III PI 4-kinases. Mutations were generated in the bacterially expressed recombinant bovine PI4KIIIβ (*31*), and the enzymatic activities of equal amounts of enzymes were determined in the presence of 0.1 or 1 mM ATP. Substitution of the P582−Y583 sequence with Arg-Met or Leu-Met greatly reduced Wm sensitivity (Figure 4A and Table 1). However, the catalytic activities of these mutants were also sharply reduced (Table 1). In contrast, substitution of only the Tyr with Met (Pro-Met) resulted in a similarly reduced Wm sensitivity, but the activity of this mutant was much better preserved (Figure 4A and Table 1). Mutation of I670−I671 to Val-Ser (as in Pik1p) reduced Wm sensitivity but also greatly reduced the catalytic activity compared to the wild-type PI4KIIIβ (Figure 4A and Table 1).

The binding of [^{3}H]Wm and the Wm-sensitivity of the autophosphorylation (*31*) of selected PI4KIIIβ enzymes were also tested. As shown in Figure 4B, Wm binding of the Y583M mutant was negligible, and its autophosphorylation was not inhibited by 3 μM Wm, a concentration that completely eliminated the autophosphorylation of the wild-type enzyme. We also tested whether mutations of conserved Ser and Thr residues within the tail of PI4KIIIβ affect autophosphorylation or the enzyme's catalytic activity. Neither of these substitutions (S781A, T787A and T797A) affected autophosphorylation or changed significantly the catalytic property of the enzyme (data not shown).

Mutagenesis of the PI4KIIIα Enzyme. To generate a bacterially expressed enzyme, the C-terminal 1171 residues of bovine PI4KIIIα were fused to GST based on an earlier report describing this construct as the minimally active catalytic module (*38*). Expression of this protein, indeed, yielded a catalytically active enzyme that was extremely sensitive to oxidation as has been observed for the mammalian PI4KIIIα enzyme. This result, and the high sensitivity

[3] Although the modeling was done on the bovine PI4K sequences, in the regions shown the bovine and human protein sequence is identical.

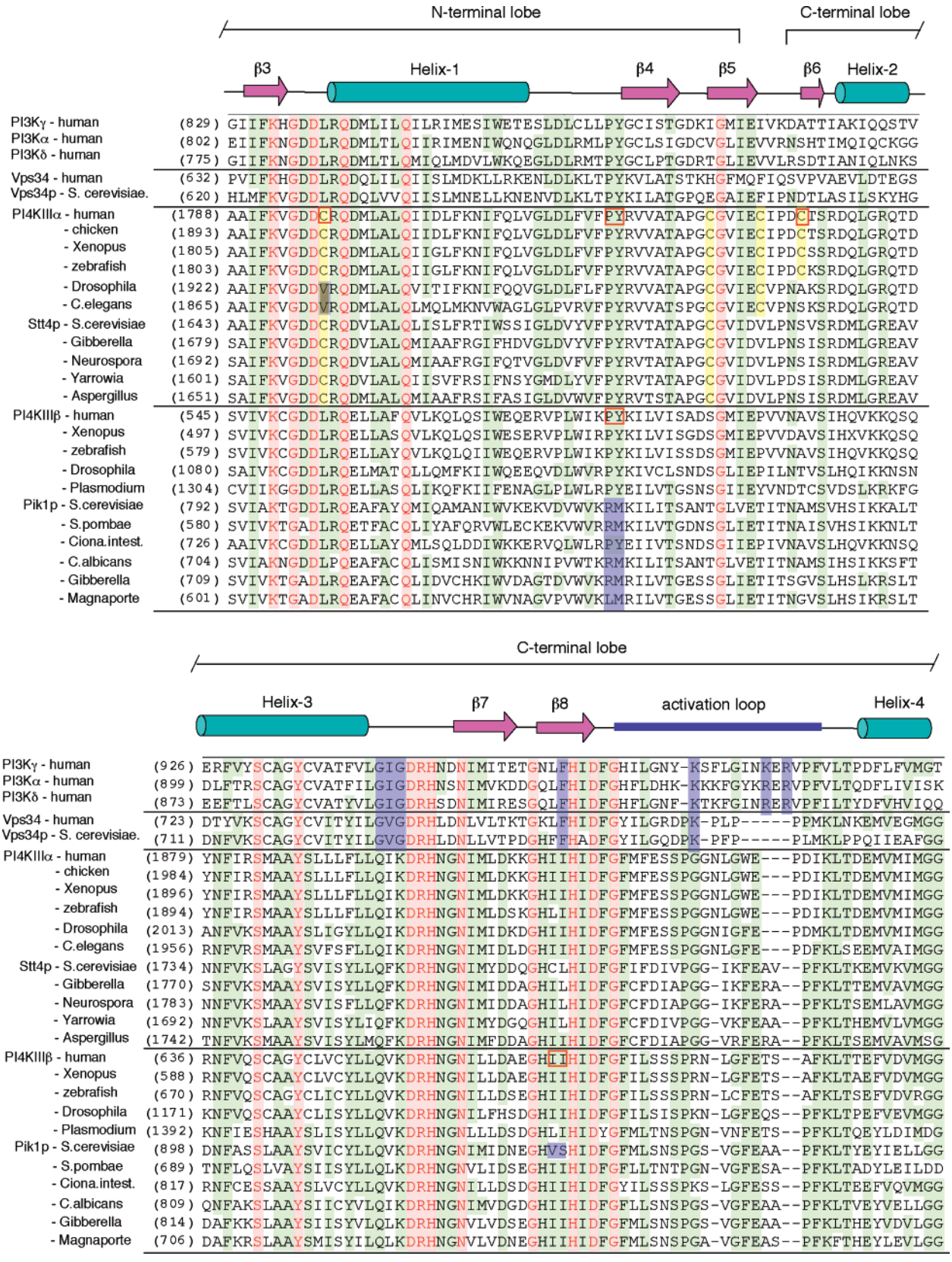

FIGURE 1: Multiple-sequence alignment of the catalytic domains of type-III PI4Ks and the human representatives of PI3Ks.[3] Residues conserved across all species and the different classes of enzymes are labeled red, while those showing limited conservation are labeled light green. Secondary structure elements based on the PI3Kγ structure are shown above the alignment. Regions of interest are highlighted with purple background and are discussed in the text. The Cys residues within PI4KIIIα are highlighted with a yellow background. Mutated residues are labeled with the red boxes. The multiple alignment was made with the Vector NTI software.

of the PI4KIIIα enzyme to the oxidizing agent PAO (*29*), prompted us to evaluate the roles of Cys residues in the high PAO sensitivity. Comparison of the sequences and models of the two PI4Ks revealed the presence of two cysteines (C1839 and C1843) facing the adenine ring from each side in the short linker region connecting the N- and C-terminal

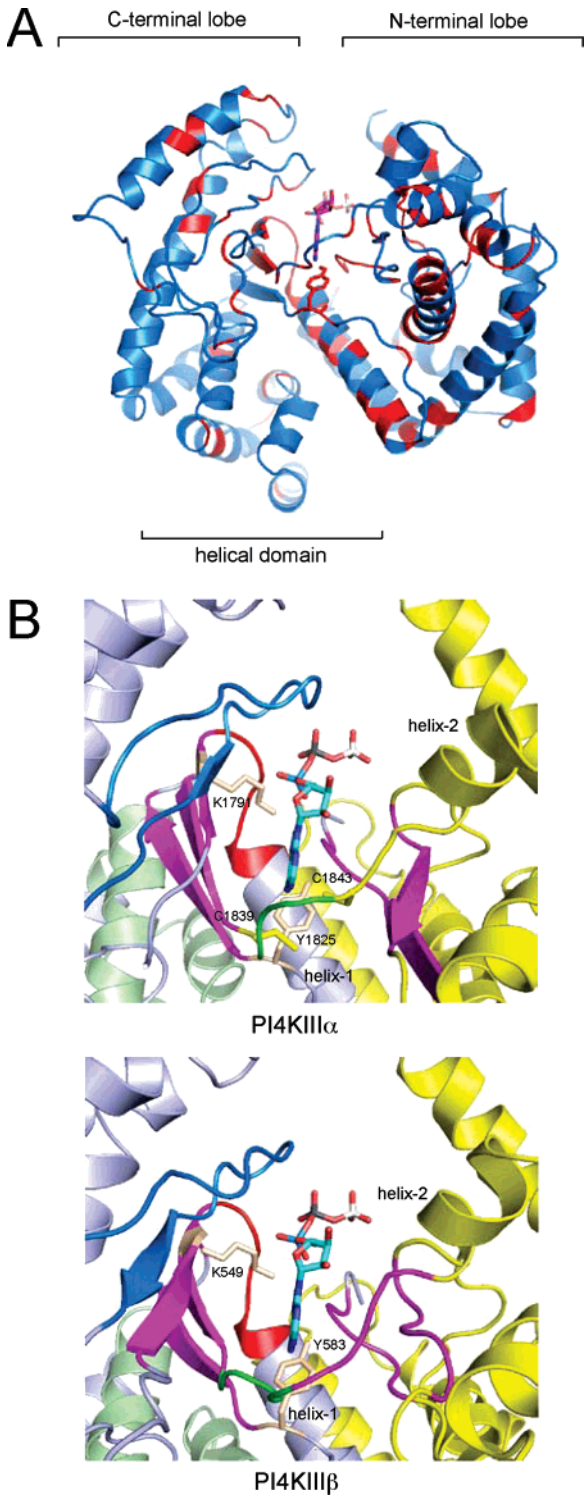

FIGURE 2: Molecular models of the catalytic domains of type-III PI4Ks based on the PI3Kγ structure bound with ATP. (A) Residues of PI4KIIIβ identical to those of PI3Kγ are colored red. The ATP binding site is formed by C- and N-terminal lobes (light blue and yellow, respectively, in panel B). In both lobes several antiparallel β-strands (purple in panel B) contribute to the sides of the nucleotide binding pocket. The all-helical domain (also called the lipid kinase unique domain) is packed against the bottom part of the C-terminal lobe (shown in light green in panel B), but it shows little sequence identity and the model has significant uncertainty in those regions. (B) Structural models of PI4KIIIα (upper) and PI4KIIIβ (lower) with ATP. The segments corresponding to the upper wall of the binding site in the C-terminal lobe are the least conserved (marine blue). Important residues that participate in ATP binding and PAO sensitivity are highlighted. The pictures were made using PyMol.

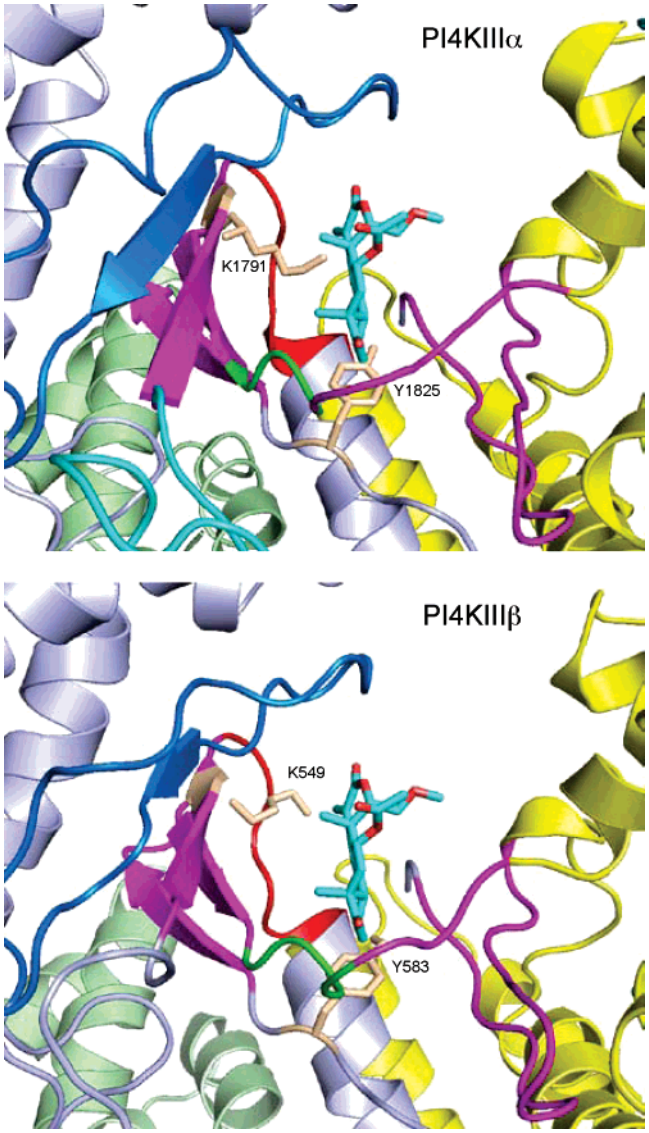

FIGURE 3: Structural models of PI4KIIIα (upper) and PI4KIIIβ (lower) with wortmannin in the binding pocket. The segments corresponding to the upper wall of the binding site in the C-terminal lobe are the least conserved (marine blue). The Lys residue that covalently binds Wm and the conserved Tyr are highlighted. The pictures were made using PyMol.

lobes of the catalytic pocket. (Figures 1 and 2B). Also tested was an additional Cys (C1796) that is also highly conserved within the Gly-Asp-Asp-Cys-Lys segment of PI4KIIIα and that corresponds to a Leu in all PI3Ks and PI4KIIIβ enzymes (Figure 1A). Substitution of C1796 with either Ser or Leu yielded an enzyme with no catalytic activity. However, substitution of Cys1843 with Ser did not diminish the catalytic activity of the enzyme, but reduced its PAO sensitivity to the level that was observed with the PI4KIIIβ enzyme (Figure 5A). Interestingly, the mutant enzyme showed a slight enhancement of activity by low concentrations of PAO for which we do not have an explanation. However, this substitution failed to protect the enzyme from oxidation as judged by the rapid loss of activity without DTT. It is notable that the PI4KIIIα homologues of yeast and fungi lack both of these cysteines and the *Drosophila* and *Caenorhabditis elegans* homologues lack one of them, probably making these enzymes less sensitive to PAO, but this was not tested experimentally.

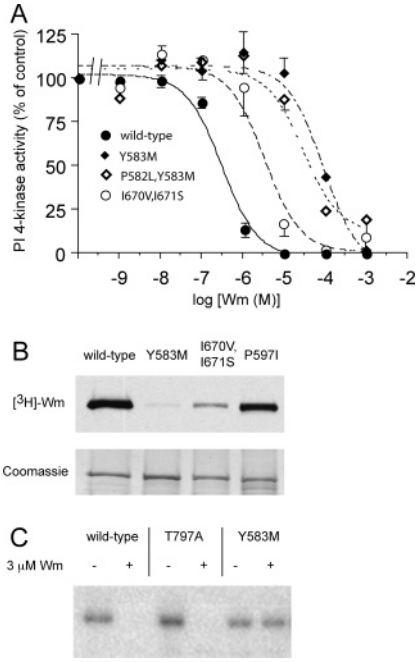

FIGURE 4: Inhibitory dose−response curves for wortmannin (Wm) in wild-type and mutant forms of recombinant PI4KIIIβ expressed in bacteria as GST fusion proteins. The enzymes were preincubated with the indicated concentrations of Wm for 10 min before the addition of [γ-^{32}P]ATP (0.1 mM final) to the kinase buffer that contained 1 mM PtdIns as substrate. See Experimental Procedures for details. (A) Activity values are expressed as percent of the activity found without Wm (DMSO). Means ± SEM of 3−6 determinations are shown. For the K_m and relative V_{max} values see Table 1. Wortmannin binding (B) and autophosphorylation (C) of recombinant PI4KIIIβ mutants. Note the reduced Wm binding to mutants (Y583M; I670V, I671S; P597I) that show decreased Wm sensitivity and the complete inhibition of the autophosphorylation by 3 μM Wm in the wild-type or tail mutant enzyme but not in the Y583M mutant.

Table 1: Summary of Kinetic Parameters of the Mutant PI4KIIIβ Enzymes[a]

	IC_{50}			
	Wm (μM)	LY294002 (mM)	ATP K_m (mM)	rel V_{max} (% of wt)
PI4KIIIβ (wt)	0.32	0.30	0.5	100
I670V, I671S	3.53	1.00	1.0	2.2
I670L, I671F	0.27	0.21	0.9	~10[a]
P597I	0.12	1.00	0.8	1.0
P582L, Y583M	31.0	nd[b]	nd	~1.0[a]
P582R, Y583M	48.2	nd	nd	~1.0[a]
Y583M	93.8	1.00	2.3	90

[a] Estimated values based on relative initial activity measured using same amounts of enzymes. [b] Not determined.

Identification of Inhibitors That Discriminate between PI4KIIIα and -β. Wortmannin has been a valuable tool to discriminate between the functions of type-II and type-III PI 4-kinases. Although Wm also inhibits PI3Ks, comparison of the doses of Wm that affect a particular cellular function can help determine whether the process is mediated by PI 3- or 4-kinases. Unfortunately, Wm is almost equipotent on PI4KIIIα and -β, and therefore, the functions of these proteins cannot be discriminated by Wm. It would be very useful to identify inhibitors that discriminate between the two proteins. During a recent screen of a chemically diverse panel of PI 3-kinase inhibitors, we identified the phenylthiazole compound, PIK93, as a potent inhibitor of PI4KIIIβ

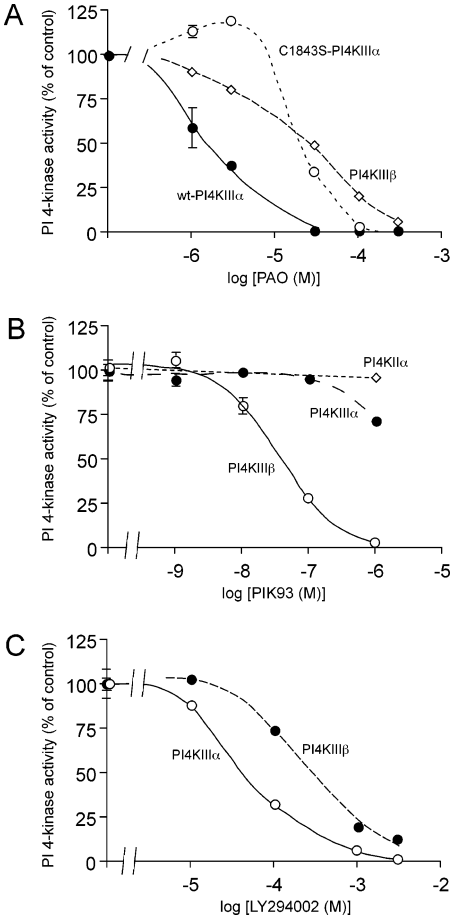

FIGURE 5: Differential inhibitor sensitivity of type-III PI4Ks. (A) PI4KIIIα shows high sensitivity to the alkylating agent, phenylarsine oxide (PAO), due to the presence of two exposed cysteins in the ATP binding pocket. Mutation of one of these residues (C1843S) significantly reduces the PAO sensitivity of the enzyme. Representative of two similar observations performed in duplicate. (B) PI4KIIIβ (but not PI4KIIIIα) is highly sensitive to the PI3K inhibitor, PIK93, while the type-II PI4Kα shows complete resistance. Representative results from two similar observations performed in duplicate. (C) The sensitivities of both PI4Ks to LY294002 are low, but PI4KIIIα is more sensitive to the compound than PI4KIIIβ. Representative results are shown from two similar observations.

(*30*). As shown in Figure 5B, this compound was about 100-fold more potent against PI4KIIIβ than against PI4KIIIα (tested at 0.1 mM ATP concentration), and it was ineffective against the type-II PI4Ks. The PIK93 sensitivity of the PI4KIIIβ Y583M mutant was also tested and showed greatly reduced sensitivity to the inhibitor (not shown). We also examined the other widely used PI3K inhibitor, LY 294002, with the two PI 4-kinases. As shown in Figure 5C, LY 294002 showed low potency against either PI4K, but it was still more potent against the PI4KIIIα enzyme.

Expression of the Mutant Enzymes in Mammalian Cells. The Y583M mutation was then created in the bovine PI4KIIIβ enzyme in the mammalian expression plasmid so that the activity of the enzyme expressed in COS-7 cells could be tested. The Y583M mutant mammalian enzyme was catalytically still active and was not inhibited by Wm at concentrations that completely inhibited the endogenous or the expressed wild-type PI4KIIIβ (not shown). Interestingly, the Y583G substituted enzyme that showed an even better activity than the Y583M mutant in the bacterially expressed

recombinant protein (not shown) had only negligible activity when expressed in COS-7 cells. Our explanation for this discrepancy was that the folding of the mutant enzyme must have been affected at the higher temperature and/or in the different redox environment of the COS-7 cells, but these possibilities have not been pursued further in the present study. When the corresponding mutation (Y1825M) was created in the full-length bovine PI4KIIIα, it had negligible catalytic activity, in spite of the protein being expressed at levels equivalent to wild-type (not shown). The C1843S mutation in the full-length PI4KIIIα construct showed similarly reduced PAO sensitivity and an activity comparable to the wild-type enzyme (not shown).

The value of a Wm-insensitive PI4K enzyme was next evaluated in COS-7 cells stably expressing either the wild-type or the Y583M PI4KIIIβ enzyme. We chose to examine the Wm-sensitivity of the conversion of ceramide (Cer) to sphingomyelin (SM) in [^{3}H]serine-labeled cells that we recently demonstrated as dependent on PI4KIIIβ (*33*). This is due to the requirement of the ceramide transfer protein (CERT) pleckstrin homology (PH) domain to bind PtdIns4*P* for Golgi interaction (*39*). First we tested the localization of the CERT-PH-mRFP construct in COS-7 cells in which we knocked down the PI4KIIα enzyme so that PtdIns4*P* formation in the Golgi is primarily dependent on PI4KIIIβ. As shown in Figure 6A, under these conditions, 10 μM Wm rapidly reduced the Golgi localization of CERT-PH-mRFP either in untransfected control COS-7 cells or in cells expressing the wild-type enzyme (the latter not shown). In contrast, in cells expressing the Y583M mutant enzyme, a significant localization of the PH domain was still observed after treatment with Wm. Similarly, the conversion of Cer to SM showed a significantly reduced Wm sensitivity in cells expressing the Y583M mutant enzyme, compared either to control COS-7 cells or to those expressing the wild-type protein (Figure 6B). These experiments have confirmed that the Wm-sensitivity of Cer to SM conversion is due to the inhibition of the PI4KIIIβ enzyme and showed that this approach can be applied to other questions related to PI4KIIIβ function.

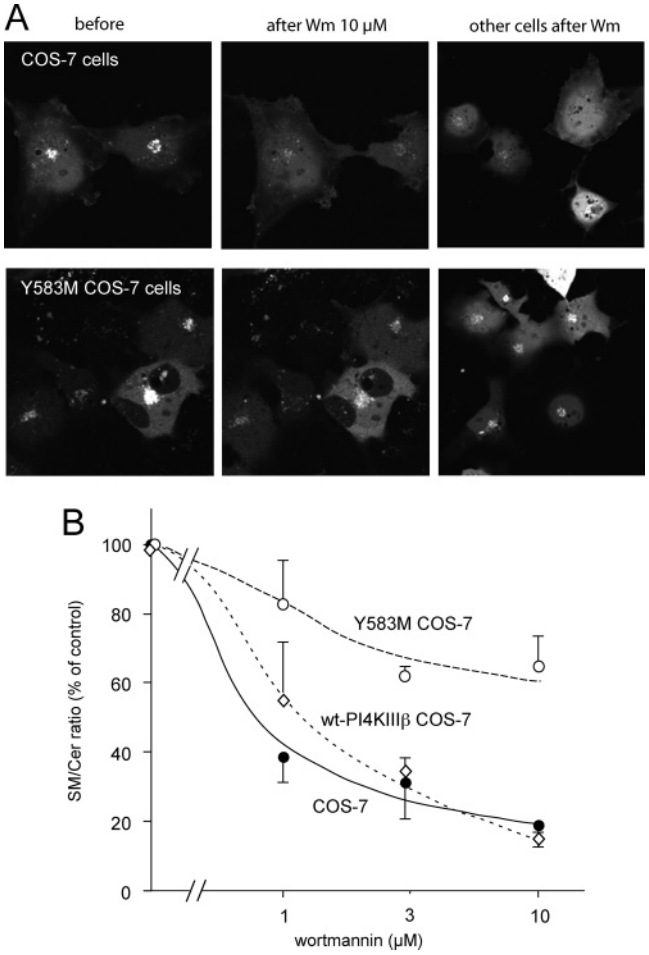

FIGURE 6: Wortmannin-resistance of ceramide transport to the Golgi in COS-7 cells stably expressing Y583M mutant PI4KIIIβ. COS-7 cells were stably transfected with either the wild-type or the Y583M mutant PI4KIIIβ. (A) Cells were treated with siRNA to downregulate the type-II PI4Ka enzyme (*19*) so that the recruitment of the CERT-PH domain to the Golgi is mostly dependent on PI4KIIIβ. In such cells, the localization of the CERT-PH-mRFP protein to the Golgi is largely eliminated after 10 min treatment with 10 μM Wm in control cells (upper panels), but not in cells expressing the Y583M mutant PI4KIIIβ (lower panels). (B) The conversion of ceramide (Cer) to sphingomyelin (SM) is inhibited by Wm in control cells but not in cells expressing the Y583M mutant enzyme. Cells were labeled with [^{3}H]serine, and the labeled sphingolipids were separated after glycerophospholipids were eliminated by alkaline methanolysis. Note the slight shift in the Wm-sensitivity in cells overexpressing the wild-type enzyme but a resistance in cells expressing the mutant. Means ± SEM of three experiments each performed in duplicate.

DISCUSSION

Mutagenesis and modeling of the type-III PI4Ks based on the crystal structure of PI3Kγ have provided several important clues concerning the catalytic properties and inhibitor sensitivities of these enzymes. Even before their cloning, these proteins had been predicted to be relatives of PI3Ks based on their sensitivities to PI3K inhibitors, most notably, wortmannin (*24*, *25*). Following their molecular cloning, sequence comparisons have proven the high homology between the catalytic domains of these enzymes, and the solved PI3Kγ structure provided a good template for modeling.

Inhibitor Sensitivities. Analysis of the Wm sensitivity of the various groups of the PI3- and 4-kinases revealed significant differences, even though the Lys residue that reacts covalently with Wm in the ATP binding pocket is highly conserved in all of these proteins. In this regard it was significant that Pik1p, the yeast orthologue of PI4KIIIβ shows very low sensitivity to Wm. This suggested that it might be possible to generate mutations that could decrease Wm sensitivity without losing the catalytic activity. Comparison of the sequences of numerous PI3- and -4-kinases in regions that form the ATP binding pocket, together with the molecular models, pointed to a unique variation conserved within the family of yeast and fungal Pik1p proteins. A ProTyr residue pair that is highly conserved in all PI3- and 4-kinases was replaced by an ArgMet sequence. This Tyr has a special position as it forms the bottom of the binding pocket encompassing the adenine ring. While the ArgMet substitution in the bovine PI4KIIIβ yielded a catalytically compromised enzyme, the ProMet mutant retained reasonable activity, and it was also transferable to enzymes expressed in mammalian cells. Remarkably, the same mutation was not tolerated in the PI4KIIIα enzyme

for reasons that are not obvious based on comparisons of the three models or sequences. However, the mutations of the same residue in PI3Kα also resulted in a catalytically inactive enzyme (*40*). Our finding is also consistent with the lack of any published report on Wm-resistant PI4KIIIα and the high sensitivity of the yeast orthologue, Stt4p, to this inhibitor (*41*). Also, no substitution corresponding to this Y residue is found in any of the homologue sequences retrieved from various genomes.

The extreme sensitivity of the PI4KIIIα enzyme to PAO (*29*) as well as to oxidation suggested that this enzyme has reactive pairs of SH groups in close proximity at a solvent-exposed surface. Comparison of the PI4K structural models and analysis of the multiple-sequence alignment pointed to two Cys residues (C1839 and C1843) in the short loop connecting the N- and C-terminal lobes of the ATP binding pocket in PI4KIIIα but not in that of PI4KIII*β*. Interestingly, these Cys residues are only present in the vertebrate enzymes. Indeed, the C1843S mutation significantly decreased the PAO sensitivity of the enzyme to a level comparable to that of PI4KIII*β*, without changing the sensitivity of the enzyme to oxidation (judged by rapid loss of activity without DTT). We assume that the high sensitivity of the enzymes to oxidation is related to two other Cys residues found in close proximity (C1796 and C1834) that are also present in almost all of the PI4KIIIα enzymes from yeast to humans (C1796 is not found in *Drosophila* and *C. elegans*). Since the C1796S mutation made the enzyme inactive, the validity of this hypothesis cannot be tested.

Substrate Preference. Although substrate preference of the PI4Ks was not directly tested experimentally, it is very likely that it is determined by the activation loop and the adjacent sequences. PI4Ks, like the PtdIns specific class III PI3Ks, only phosphorylate PtdIns. Both the PI4K enzymes and class III PI3Ks lack the positive residues within the activation loop characteristic of class I PI3Ks (Figure 1). Instead, they contain several Pro residues and a Lys at the C-terminally adjacent sequence. It is certainly the activation loop that also determines the position of the inositol ring in PtdIns to expose the OH to be phosphorylated, as observed with both the PI3K*γ* (*42*) and PIP kinases (*43*).

Inibitor Selectivities and Possibilities for New Inhibitors. These studies further analyzed the sensitivities of PI4Ks to PI3K inhibitors, especially to PIK93, the inhibitor recently described as significantly more potent on PI4KIII*β* than PI4KIIIα (*30*). The higher sensitivity of the *β* enzyme can be attributed to sequence differences in a segment corresponding to the "selectivity pocket" of PI3K*γ* (*30*). While this part of the molecule shows relatively low sequence identity and therefore the model has significant ambiguity there, it is almost certainly the region relevant for subtype-specific inhibition. These and other differences, such as the presence of the two cysteines within the ATP binding site in PI4KIIIα, suggest that there is a good possibility that specific PI4K inhibitors can be developed. Such inhibitors would greatly facilitate studies on the role of the various PI4Ks in the cell since the contribution of these enzymes to the production of PtdIns4*P* in specific cellular compartments is not known. Most studies on this topic have relied on the use of PI3K inhibitors exploiting the difference in sensitivity between PI4Ks and PI3Ks to these drugs. Unfortunately, knock down of the individual enzymes, the only approach that would selectively eliminate the function of the proteins, has many drawbacks due to the exposure of cells to the depletion of the protein for several days as opposed to minutes when using inhibitors.

Until more selective inhibitors become available, the experiments performed in COS-7 cells stably transfected with the Wm-resistant Pi4KIII*β* mutant enzymes showed the value of this chemical-genetic approach. The insensitivity of the mutant enzyme to Wm allows a selective assessment of PI4KIII*β* functions when the endogenous type-III PI4Ks are acutely inhibited. Although this approach will not alleviate the need for specific PI4K inhibitors, it may still facilitate progress in the field of biochemistry and cell biology of PI4Ks.

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BI7017927