Lipid kinase and protein kinase activities of G-protein-coupled phosphoinositide 3-kinase γ : structure–activity analysis and interactions with wortmannin

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Signalling via seven transmembrane helix receptors can lead to a massive increase in cellular PtdIns(3,4,5) P_3 , which is critical for the induction of various cell responses and is likely to be produced by a trimeric G-protein-sensitive phosphoinositide 3-kinase (PI3K γ). We show here that PI3K γ is a bifunctional lipid kinase and protein kinase, and that both activities are inhibited by wortmannin at concentrations equal to those affecting the p85/p110 α heterodimeric PI3K (IC₅₀ approx. 2 nM). The binding of wortmannin to PI3K γ , as detected by anti-wortmannin antisera, closely followed the inhibition of the kinase activities. Truncation of more than the 98 N-terminal amino acid residues from PI3K γ produced proteins that were inactive in wortmannin binding and kinase assays. This suggests that regions apart from the core catalytic domain are important in catalysis and inhibitor

interaction. The covalent reaction of wortmannin with PI3K γ was prevented by preincubation with phosphoinositides, ATP and its analogues adenine and 5'-(4-fluorosulphonylbenzoyl)-adenine. Proteolytic analysis of wortmannin-prelabelled PI3K γ revealed candidate wortmannin-binding peptides around Lys-799. Replacement of Lys-799 by Arg through site-directed mutagenesis aborted the covalent reaction with wortmannin and the lipid kinase and protein kinase activities completely. The above illustrates that Lys-799 is crucial to the phosphate transfer reaction and wortmannin reactivity. Parallel inhibition of the PI3K γ -associated protein kinase and lipid kinase by wortmannin and by the Lys-799 $\rightarrow$ Arg mutation reveals that both activities are inherent in the PI3K γ polypeptide.

INTRODUCTION

The seven-transmembrane helix receptor-induced production of 3-phosphorylated phosphoinositides was first observed by Traynor-Kaplan et al. [1], who showed that the stimulation of neutrophils by the chemotactic peptide *N*-formyl-Met-Leu-Phe led to a massive increase in PtdIns(3,4,5) P_3 level. Since then it has been demonstrated that phosphoinositide 3-kinases (PI3Ks) catalyse the last step in the formation of D-3-phosphorylated phosphoinositides [2] and that the latter are poor substrates for PtdIns-specific phospholipases C [3]. Surface receptor-mediated PI3K activation is therefore thought to lead to the production of lipid second messengers that are mandatory for cell activation by chemotactic and growth factors, cytokines and hormones [4].

Cell activation through protein tyrosine kinase growth factor receptors involves the translocation of a p85/p110 heterodimeric PI3K complex to phosphorylated Tyr-Xaa-Xaa-Met motifs [5]. The coupling of PI3K activation to serpentine receptors, however, remained obscured until a novel G-protein $\beta\gamma$ subunit-activated enzyme was characterized [6,7]. Such a PI3K (named PI3K γ) was recently cloned and has indeed been shown to be responsive to G-protein α and $\beta\gamma$ subunits [7].

PI3K γ , as well as PI3Ks of the p110 α and β types, phosphorylates PtdIns, PtdIns4P and PtdIns(4,5) P_2 *in vitro*, and process PtdIns(4,5) P_2 as the major substrate *in vivo*. Vps34p (vacuolar protein sorting defective) [8,9] and *Drosophila* PI3K_68D homologues [10], in contrast, produce only PtdIns3P from PtdIns.

Serpentine receptor ligand-mediated cell responses, such as the

neutrophil respiratory burst induced by *N*-formyl-Met-Leu-Phe, complement factor 5a, leukotriene B₄ or interleukin 8, were shown to be inhibited by nanomolar concentrations of wortmannin [11,12]. Wortmannin has been recently identified as a specific inhibitor of PI3Ks [12–14] and has since been widely applied in investigations of the importance of the lipid kinase in platelet aggregation, growth factor-induced membrane ruffling in fibroblasts, epithelial cells and adipocytes, insulin-dependent glucose uptake and glucose transporter translocation, mitotic and apoptotic processes, and IgE-stimulated histamine release in mast cells, among others (for references see [15]).

How wortmannin-mediated inactivation of PI3K interferes with these cell responses has yet to be elucidated. The number of signalling molecules that have been suggested to be downstream targets of 3-phosphorylated phosphoinositides, however, is increasing: activation of the mitogen-activated protein kinase pathway [16,17], phosphorylations of glycogen synthase kinase 3 [18,19] and p70^{S6} kinase [20–22], phospholipase D activity [23], the activation of the GTP-binding protein Rac [24,25] and the protein kinase C δ , ϵ , τ [26] and ζ [27] isoforms have all been proposed to depend on functional PI3K. Protein kinase B/c-Akt protein kinase was recently added to this list [28,29] and was shown to mediate the phosphorylation of glycogen synthase kinase 3 [30]. Wortmannin at submicromolar concentrations does not directly interfere with the enzyme activities above but was shown to inhibit a weakly membrane-associated phosphoinositide 4-kinase, DNA-dependent protein kinase and myosin light-chain kinase at elevated concentrations [15].

It has been demonstrated that wortmannin reacts with Lys-802

Abbreviations used: FSBA, 5'-*p*-fluorosulphonylbenzoyl-adenine; GST, glutathione S-transferase; HR, homology region; PI, phosphoinositide; PI3K, phosphoinositide 3-kinase.

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of the catalytic subunit of the p85/p110 α heterodimeric PI3K to form a Schiff base. This lysine residue is situated within the conserved core catalytic domain and is crucial to the phosphate transfer reaction [15]. Moreover, the residue is shared by all PI3Ks and related molecules such as the ataxia telangiectasia-related genes, DNA-dependent protein kinase, the targets of the immunosuppressant rapamycin (TOR, FRAP and RAFT1) and phosphoinositide 4-kinases (for sequence alignments and references see [15]).

PI3K γ displays many lysine residues within the predicted wortmannin-interacting region and thus provides a challenge for the specificity of the proposed covalent reaction site. The interaction of wortmannin with PI3K γ suggests the involvement of multiple domains of the enzyme, which seem also to be necessary for kinase activity. Moreover, we demonstrate that PI3K γ can act as a bifunctional kinase phosphorylating phosphoinositides and protein.

MATERIALS AND METHODS

Materials

PtdIns(4,5) P_2 was from Boehringer Mannheim; other phosphoinositides were from Sigma; precoated TLC plates (Silica-gel 60, 0.2 mm) were from Merck; glutathione-Sepharose was from Pharmacia; DMSO, 5'-*p*-fluorosulphonylbenzoyladenine (FSBA) and Triton X-100 were from Fluka, Buchs, Switzerland. Sf9 cells were from Innotech; cell culture media, antibiotics and additions were from Gibco/BRL. Peptide standards for gel electrophoresis were purchased from Sigma and Pharmacia. Wortmannin was kindly provided by Dr. T. G. Payne (Sandoz, Basle, Switzerland).

Anti-PI3K γ antisera

Anti-PI3K γ antisera were raised in rabbits against a peptide corresponding to amino acid residues 742–756 (NSQLPSSFR-VPYDPG) coupled to keyhole limpet haemocyanin for immunization. Antibodies were affinity-purified on peptide-Affigel (Pierce) columns. Anti-wortmannin antisera were produced as described [15].

Cloning and mutagenesis

A pAcG2T baculovirus transfer vector (obtained from I. Jones [31]) encoding glutathione S-transferase (GST) was used to generate a GST–PI3K γ full-length construct (codons 4–1068, with the assumed start codon at cctccATGGAG) [7] and to prepare various GST-tagged fragments of the lipid kinase: the internal *Sma*I site was used to generate a GST–PI3K γ fragment from amino acid residues 98–1068 (GST–PI3K γ /98–1068); the two internal *Bgl*II sites allowed the in-frame deletion of residues 75–398 (GST–PI3K γ /Δ75–398); the conserved catalytic domain could be joined to GST at the *Eco*RI site (GST–PI3K γ /740–1068); and the same restriction site was used to terminate an N-terminal *Bam*HI fragment to generate GST–PI3K γ /4–740.

Point mutations were introduced into PI3K γ by the unique site elimination method [32], using *Afl*III/*Bgl*III *trans* oligonucleotide primers. The simultaneously added mutagenesis primer was used to produce the desired Lys→Arg codon exchange in the PI3K γ cDNA (cloned as an *Eco*RI–*Eco*RI fragment into the multicloning site of pUC19), and to generate at the same time a new *Nco*I restriction site for plasmid selection

(PI3K γ K799R). Mutations were confirmed by DNA sequencing (Sequenase 2.0, US Biochemicals). The respective mutated DNA sequences were subsequently reintroduced as *Eco*47III–*Xba*I fragments into wild-type PI3K γ harboured in derivatives of the CMV-promoter expression vector pSCT1 [33] and pAcG2T to generate the desired mutant proteins.

Recombinant proteins

The expression of GST–PI3K γ fusion protein has been described previously [7]. Sf9 cells, cultured in IPL41 (Gibco/BRL) with 10% (v/v) fetal calf serum, 1% lipid concentrate and 50 μ g/ml gentamycin, were co-transfected with 5 μ g of each GST–PI3K γ construct in the pAcG2T transfer vector and 0.25 μ g of linear BacculoGold DNA (Pharmingen) essentially as described by the manufacturer. Recombinant virus was plaque purified and amplified. Cells were harvested 48–60 h after transfection with recombinant PI3K γ baculovirus and lysed in 50 mM Tris (pH 7.5)/150 mM NaCl/1% (w/v) Triton X-100/5 mM EDTA/50 mM NaF/20 μ M leupeptin/2 mM PMSF/10 mM benzamidine/1 mM sodium orthovanadate. The GST fusion proteins were purified on glutathione beads (Pharmacia) essentially as described [7]. Recombinant p110 α /p85 complex was produced essentially as described [15].

For transient expression of GST–PI3K γ fusion proteins, human embryo kidney 293 cells (ATCC) were transfected with 10 μ g of the CMV promoter-based expression vectors carrying the wild-type and KR mutant of GST–PI3K γ . Expression vectors and 5 μ g of calf thymus carrier DNA were pre-precipitated in calcium phosphate, and subsequently added to 10 cm Petri dishes of 293 cells cultured in Dulbecco's modified Eagle's medium (Gibco/BRL) with 5% (v/v) fetal calf serum. The medium was replaced after 12 h and cells were lysed 48 h later in 20 mM Tris (pH 8.0)/138 mM NaCl/2.7 mM KCl, supplemented with 5% (w/v) glycerol, 1 mM MgCl₂, 1 mM CaCl₂, 5 mM EDTA, 20 mM NaF, 1 mM sodium orthovanadate, 20 μ M leupeptin, 18 μ M pepstatin and 1% (w/v) NP40. Cleared cell lysates (12000 g, 15 min, 4 °C) were applied to glutathione beads (see above). Wortmannin binding and kinase assays were performed on the immobilized protein. The amount of protein applied to SDS/PAGE or kinase assays corresponded typically to one-eighth of a 10 cm Petri dish.

PI3K and protein kinase assays

PI3K activity was measured essentially as described [34]. PI3K samples were exposed to [γ -³²P]ATP (Amersham, 3000 Ci/mmol), PtdIns, phosphatidylserine and Mg²⁺ for 10 min at 30 °C, before lipids were extracted and PtdIns3-*P* was quantified after TLC on silica 60 plates.

To assay protein phosphorylation, immobilized GST–PI3K γ was washed twice with kinase buffer without ATP [50 mM Hepes (pH 7.4)/150 mM NaCl/5 mM EDTA/5 mM dithiothreitol/10 mM MgCl₂/0.01% Triton X-100] and resuspended in the same buffer (MgCl₂ concentrations were varied where indicated). An equal volume of kinase buffer supplemented with ATP (to give a final concentration of 20 μ M and 10 μ Ci of [γ -³²P]ATP per experiment) was added to initialize the phosphorylation reaction. Incubation for 20 min at 30 °C was followed by denaturation, SDS/PAGE and autoradiography (with Kodak X-Omat films).

Analytical digests of wortmannin-labelled PI3K γ

Immobilized GST–PI3K γ was washed with PBS/0.05% Triton X-100 and incubated with 200 nM wortmannin (stock solutions

were made up 1000-fold concentrated in DMSO) on ice for 15 min; NaCNBH₃ (6.5 mM) was added in some cases for 1 h to stabilize the wortmannin-PI3K γ interaction. After the removal of excess inhibitor with PBS/0.05% Triton X-100, immobilized PI3K was washed twice with the respective digestion buffers. Lys-C (1 μ g; Promega) in 25 mM Tris/1 mM EDTA (pH 7.8) and trypsin (0.05 μ g; Boehringer Mannheim) in 50 mM Tris/0.1 mM CaCl₂ (pH 7.8) were used to generate small fragments. Other proteases (Arg-C and Glu-C; Promega) were used as indicated by the supplier. Prelabelled PI3K was digested for 3 h at 37 °C in a total volume of 80 μ l. After denaturation in the presence of 2-mercaptoethanol, peptides were separated by Tricine/gel electrophoresis [35] and then transferred to PVDF membranes (Millipore). Wortmannin-labelled peptides were detected by anti-wortmannin antibodies, secondary goat anti-(rabbit IgG)-horseradish peroxidase conjugates (Sigma) and enhanced chemiluminescence (ECL; Amersham).

RESULTS AND DISCUSSION

Wortmannin-binding and inhibition of PI3K γ

In view of the potent inhibition of serpentine receptor-mediated PtdIns(3,4,5)P₃ production and cell responses by wortmannin, we investigated the inactivation mechanism of PI3K γ by this substance. When GST-p110 α /p85 α or GST-PI3K γ was incubated with increasing concentrations of wortmannin under identical conditions, the inhibitor displayed similar IC₅₀ values

Figure 1 Interaction of wortmannin with PI3K γ

Immobilized, recombinant GST-PI3K γ and GST-p110 α /p85 PI3K complexes were exposed to the indicated concentrations of wortmannin as indicated in the Materials and methods section. (a) PI3K activity was assayed by the formation of [³²P]PtdIns3-P after wortmannin incubation. Symbols: ○, concentration-dependent inhibition of GST-p110 α /p85 α ; ●, results for GST-PI3K γ (means $\pm$ S.D., $n = 4$). (b) Concentration-dependent covalent binding of wortmannin to GST-PI3K γ . PI3K γ samples were incubated with wortmannin as above, denatured under reducing conditions and subjected to SDS/PAGE. Protein-bound wortmannin was then detected with anti-wortmannin antisera on immunoblots (α -Wt).

Figure 2 Competition of substrates with wortmannin for PI3K γ binding

GST-PI3K γ was exposed to 200 nM wortmannin in the presence of the kinase substrates PtdIns (PI), PtdIns(4,5)P₂ (PIP₂), and ATP and ATP analogues. Wortmannin-binding was detected as in Figure 1(b). (a) GST-PI3K γ was incubated with pre-prepared PtdIns and PtdIns(4,5)P₂ suspensions at the given concentrations, before wortmannin was added. (b) Immobilized PI3K γ was preincubated with DMSO (—), 1 mM ATP, 1 mM FSBA or 1 mM adenine for 30 min at 37 °C, before the samples were cooled to 0 °C for the incubation with wortmannin.

(approx. 2 nM) for both lipid kinases, as measured by the formation of [³²P]PtdIns3P from PtdIns and [γ -³²P]ATP. Covalent binding of wortmannin to GST-PI3K γ was detected by anti-wortmannin antisera, occurred in parallel to inhibition and was saturated at 20 nM (Figure 1). The inhibitory concentrations presented here are lower than the values published earlier for a partly purified G-protein $\beta\gamma$ subunit-stimulated PI3K from U937 cells (43 nM, and 17 nM for the IC₅₀ for p85/p110 inhibition measured in parallel [6]). As the inhibition of PI3Ks by wortmannin is mediated by a covalent modification of the catalytic subunit [15,36], reaction time, pH, buffer composition and temperature all influence the inhibitor's potency and might explain the observed differences.

Stephens et al. [37] have recently shown that a pig homologue of PI3K γ co-purifies with a p101 protein of yet unknown function. It will have to be verified whether a p101/PI3K γ complex displays a decreased sensitivity to wortmannin compared with the recombinant PI3K γ used in this study. An effect of p101 on wortmannin interaction with PI3K γ , however, seems unlikely because the IC₅₀ of 2 nM obtained *in vitro* is close to the concentration of wortmannin required to inhibit various cellular responses: interference with G-protein-coupled surface receptor-induced PtdIns(3,4,5)P₃ production has been demonstrated at concentrations as low as 5 nM [12] and leads to the abortion of the *N*-formyl-Met-Leu-Phe-induced respiratory burst in neutrophils [11,12] or the thrombin-induced phosphorylation of pleckstrin in platelets [38,39]. Stimulation of the same cell responses by

the protein kinase C activator PMA were not affected by wortmannin.

PI3K γ is the only PI3K subtype that has been shown to be coupled to trimeric G-protein activation so far and is present in cells of the haematopoietic lineage [7,40,41]. Altogether this makes PI3K γ the most likely target to rationalize wortmannin-mediated inhibition of G-protein-coupled receptor-induced responses. Overlapping inhibitory concentrations for the α and γ PI3K subtypes, in contrast, illustrate that wortmannin concentration dependences should not be used to discriminate between the contribution of these PI3K subtypes to distinct cell responses.

Competition of phosphoinositides and ATP with wortmannin

To localize the inhibitor reaction site in relation to the substrate interaction domains, lipids and ATP analogues were assayed for their capacity to prevent PI3K γ labelling by wortmannin. Pre-incubation of GST-PI3K γ with increasing amounts of phosphoinositides protected the PI3K from the reaction with wortmannin, as shown in Figure 2(a). Whereas PtdIns prevented wortmannin binding only above 1 mg/ml, PtdIns(4,5) P_2 displayed a complete protection at one-tenth of this concentration. A similar pattern was observed for the p85/p110 α PI3K complex, but considerably higher phosphoinositide concentrations had to be used to abolish wortmannin binding in those assays [15]. A difference in lipid interaction of the two enzymes might also be reflected by the different detergent sensitivities of the α and the γ type PI3Ks: whereas the activity of the first is augmented at 0.0125–0.025% NP40 and decreases thereafter, these detergent concentrations completely abolish PtdIns3- P production by PI3K γ (results not shown).

Distal to the highly conserved kinase domain, p110 α contains the positively charged K⁹⁴¹KKKFGYKRER⁹⁵¹ sequence, which resembles the PtdIns(4,5) P_2 -binding K(X)_nKXXX ($n = 3-7$) consensus motif in gelsolin [42]. In PI3K γ , in contrast, four of the lysine residues within this polybasic stretch are replaced (Y⁹³⁸KSEFLGINKER⁹⁴⁸). In spite of this difference it has been shown that p85/p110 α and PI3K γ have the same substrate specificities [7,43]. Further studies will be needed to prove whether this motif actually determines the preference for various phosphoinositides or whether other polybasic regions N-terminal to the core catalytic domain in PI3K γ contribute to the interaction with the PtdIns(4,5) P_2 headgroup.

ATP and its analogues FSBA and adenine all interfered with wortmannin binding to GST-PI3K γ when added before the inhibitor (Figure 2b). FSBA has previously been used to map nucleotide-binding sites of various protein kinases [44] and lipid kinases [15]. The highly conserved DXHXXN and DFG kinase motifs present in all PI3Ks localize the ATP-binding site of PI3K γ approximately to the region of P⁷⁷⁶–DFG⁹³². Because the contact of wortmannin and PI3K γ results in a covalent modification of the enzyme (with a decrease in $V_{\max}$ but not in K_m), the characteristics of the inhibition seem to be non-competitive when assayed by conventional methods. The above results, however, demonstrate clearly that the substrate-binding sites overlap with domains involved in inhibitor interaction.

Protein kinase activity of PI3K γ

It was reported earlier that the catalytic subunit p110 α could phosphorylate Ser-608 of the associated p85 α regulatory subunit and thus down-regulate the lipid kinase activity of the complex [45,46]. The incubation of GST-PI3K γ with [γ -³²P]ATP, in the presence of either Mn²⁺ (results not shown) or Mg²⁺, led to a

Figure 3 Protein kinase activity of PI3K γ

Immobilized GST-PI3K γ was incubated in kinase buffer with [γ -³²P]ATP. Autoradiographs of proteins separated by SDS/PAGE are shown. The arrows point to the GST-PI3K γ protein band. (a) Phosphorylation of GST-PI3K γ in the presence of the indicated amounts of MgCl₂. (b) Concentration-dependent inhibition of GST-PI3K γ phosphorylation by wortmannin. After the incubation of PI3K γ with wortmannin, the inhibitor was removed and the samples were subjected to the protein kinase assay in the presence of 10 mM MgCl₂.

prominent labelling of the immobilized enzyme. Protein phosphorylation was found to be inhibited by wortmannin at concentrations equally effective in lipid kinase assays (Figure 3).

In contrast with the Ser-608 phosphorylation of p85 α , which occurs exclusively in the presence of high concentrations of Mn²⁺, serine phosphorylation of PI3K γ did take place under physiological conditions but did not markedly affect its lipid kinase activity *in vitro* (M. P. Wymann, unpublished work), and pre-phosphorylation of PI3K γ did not interfere with subsequent binding of wortmannin (results not shown).

The abortion of the protein kinase activity by wortmannin and by a Lys-799 → Arg mutation (see below) exclude the possibility that PI3K γ was phosphorylated by an unidentified, associated kinase. The results illustrate that PI3K γ is indeed a bifunctional lipid kinase and protein kinase operating under physiological conditions, and indicate that PI3K γ -mediated protein phosphorylation might play a role in G-protein-coupled signalling. Currently, transphosphorylation of PI3K γ by the formation of homodimers cannot be excluded. Experiments to elucidate this process and to map the exact site of phosphorylation are in progress.

Domains necessary for PI3K function

Primary sequence alignments and modelling of PI3Ks have revealed three conserved homology regions (denoted HR) [47]. Whereas HR1 (residues 768–1068 in p110 α) contains the core catalytic domain with the well-conserved kinase and ATP-binding motifs, HR2 (residues 529–675 in p110 α) and HR3 (residues 324–410 in p110 α) have not yet been assigned a specific function in the catalytic process. In this study we have tested various truncated versions of PI3K γ at three levels of complexity: (1) the lipid kinase assay, demanding the presence of both ATP and lipid-binding sites; (2) the protein kinase assay, involving an ATP-binding site and a putative, not yet localized, substrate region; (3) wortmannin binding, which would be expected to depend only on a nucleophilic lysine residue in the HR1 domain [15].

Figure 4 Kinase activity and wortmannin binding of PI3K γ fragments

GST fusion proteins of full-length (wt, amino acid residues 4–1068) PI3K γ and segments of PI3K γ were purified from baculovirus-infected Sf9 cells. Numbers indicate the range of residues of PI3K γ fused to GST; Δ 75–398 marks an in-frame deletion from the full-length protein. Upper panel, schematic representation of PI3K γ and constructs used for functional studies. Ras-binding is depicted by analogy with p110 α ; binding sites for G-protein $\beta\gamma$ subunits and p101 are hypothetical and have yet to be confirmed. Interactions of wortmannin (Wt) and ATP occur within HR1 (see text). Lower panel, purified fusion proteins were separated by SDS/PAGE and revealed by Coomassie Blue staining (Coomassie). In parallel, GST–PI3K γ fragments were subjected to wortmannin labelling as in Figure 1(b) (α -wortmannin), phosphorylation as in Figure 3(a) (phosphorylation) or the PI3K lipid kinase assay (PI 3-P) described in the Materials and methods section. No formation of PtdIns3-P was detected for proteins applied to lanes 3–5 (means $\pm$ S.D., $n = 4$).

As shown in Figure 4, truncation of the first 98 N-terminal amino acid residues from PI3K γ increased the production of PtdIns3-P, phosphorylation and wortmannin-binding compared with the wild-type GST fusion protein. Whereas the N-terminal 123 residues were reported to mediate the binding of p110 α [48] and p110 β [49] to the inter-SH2 region of the p85 subunit, the function of the N-terminus of PI3K γ is unknown. The increase in activity in the GST–PI3K γ /98–1068 construct points to a possible regulatory role of the N-terminal region, but excludes its participation in the phosphate transfer reaction.

The in-frame deletion of residues 75–398 from PI3K γ remove putative binding sites for Ras and G-protein $\beta\gamma$ subunits and the HR3 domain. The Ras-binding site on p110 α has recently been localized to around Lys-227 on p110 α [50] (corresponding to Lys-217 in PI3K γ). The GST–PI3K γ /475–398 protein was well expressed in Sf9 cells, but did not retain any of the assayed functions. It was therefore not surprising that the GST–PI3K γ /4–740 (devoid of the kinase domain) and the GST–PI3K γ /740–1068 fusion protein (the latter diminished basically to the core catalytic domain HR1) did not display kinase activities. Although the HR1 domain contains all sites that were predicted to interact with wortmannin covalently bound to p110 α [15], the GST–PI3K γ /740–1068 protein did not bind wortmannin.

These results illustrate that most of PI3K γ is needed to yield a functional kinase, which is in agreement with results obtained for truncated p110 α constructs (B. Vanhaesebroeck, personal communication). Whether the conserved HRs are necessary for catalysis and substrate interaction or whether they are implicated in the correct folding of the PI3K γ is not entirely clear. Recombinant N-terminal fragments of PI3K γ used here, in contrast, maintained the effector-binding capacities of the full-length protein (T. Hanck, unpublished work). Assuming that the recombinant proteins used folded correctly, this would indicate that wortmannin binds to PI3K γ in a two-step mechanism: (1) reversible, non-covalent binding to sites not contained in HR1 might dictate the initial rate of the inhibitor–protein interaction, (2) once bound in this way, wortmannin reacts covalently with a nucleophilic residue within the ATP-binding site. Such a mechanism would open favourable perspectives for the development of phosphoinositide kinase subtype-specific inhibitors, as similarities between members of this family decrease outside HR1. That non-covalent wortmannin binding might be directed by other PI3K domains besides HR1 is supported by the retained wortmannin binding of a kinase-inactive p110 α Arg-916 $\rightarrow$ Pro mutant with a distorted ATP-binding site [15] and the existence of PI3Ks with decreased sensitivities to wortmannin (e.g. *Saccharomyces cerevisiae* Vps34 [9] and the mouse p170 with a C-terminal C2 domain [10]).

The wortmannin reaction site

In contrast with p110 α , PI3K γ displays numerous lysines (e.g. six in the K⁷⁶⁶CK–EFK⁷⁸⁰ sequence) close to the proposed covalent docking site of wortmannin. PI3K γ therefore provides a means of re-evaluating the possibility that Lys-802 of p110 α reacts with the non-covalently bound wortmannin owing to the absence of other nucleophilic residues in the catalytic cleft, or owing to an enhanced reactivity of the suspected ATP-binding lysine. The absence of wortmannin binding to fragments of PI3K γ , and an eventual involvement of several (on the primary sequence separated) protein domains, underlines the importance of using a native full-length protein to study wortmannin–PI3K γ interactions.

To localize the site of covalent wortmannin binding on PI3K γ ,

Figure 5 Partial proteolysis of wortmannin-labelled PI3K γ

GST-PI3K γ was incubated with 200 nM wortmannin and digested with either Lys-C (L) or trypsin (T) as described in the Materials and methods section. Peptide separation was achieved by Tricine/gel electrophoresis, and immunoblots were probed with anti-PI3K antibodies directed against the peptide 742–756 (α -742–756) or anti-wortmannin antisera (α -Wt). The locations of peptide molecular mass standards (Sigma, 2.5–17 kDa) are indicated (in kDa) at the left.

recombinant GST-PI3K γ wild-type protein was prelabelled with wortmannin and digested with various site-specific proteases. The resulting peptides were subsequently separated by Tricine/gel electrophoresis and probed for the presence of the amino acid residues 742–756 epitope (using the anti-peptide antiserum H518; see the Materials and methods section) or the presence of wortmannin. When digested with Lys-C protease, a sharp H518-positive band was obtained at 13 kDa co-migrating with a wortmannin-labelled peptide (Figure 5). Trypsin digestion, in contrast, generated a 13 kDa peptide that was detected only with anti-peptide antisera. This indicates that the wortmannin-binding site is exclusively located less than 13 kDa upstream or downstream of the H518 epitope. Although 8, 11.5, 13 and 20 kDa wortmannin-reactive bands appear transiently in the Lys-C digests, a huge wortmannin-positive signal accumulates at approx. 7 kDa. As predicted from the primary sequence of PI3K γ , it is very unlikely that peptides of this size could be generated by Lys-C from the 13 kDa H518 upstream region, whereas the 13 kDa region downstream of the H518 epitope could give rise to a 7 kDa peptide family around the 783–839 core sequence when wortmannin blocks the Lys-C-mediated cleavage distal to Lys-799. This would locate the wortmannin-reactive site close to the ATP-binding site, which is in agreement with the observed competition of ATP and wortmannin, as well as with results obtained with p110 α [15].

To confirm that a covalent reaction of wortmannin with PI3K γ was indeed dependent on the presence of Lys-799, this lysine residue was mutated to arginine to reduce the nucleophilicity while maintaining the size and charge at this position. As shown in Figure 6, the Lys-799 $\rightarrow$ Arg mutation completely aborted wortmannin binding as well as lipid kinase and protein kinase activities in GST-PI3K γ fusion proteins overexpressed in 293 cells (identical results were obtained with protein purified

Figure 6 Wortmannin-binding, protein kinase and lipid kinase activities of a PI3K γ Lys-799 $\rightarrow$ Arg mutant

GST, GST-PI3K γ (PI3K) wild-type (wt) and Lys-799 $\rightarrow$ Arg mutant (KR) fusion proteins were transiently overexpressed in 293 cells and immobilized on glutathione-Sepharose beads as described in the Materials and methods section. (a) Top panel: Coomassie Blue staining of immobilized proteins after SDS/PAGE. Bottom panel: identical amounts of protein were used for labelling with 200 nM wortmannin and subsequent immunodetection with anti-wortmannin antibodies (α -Wt) or protein kinase assays ([γ - 32 P]ATP). The DNA species used for transfection are indicated at the bottom. (b) Production of PtdIns3-P (PI 3-P) by immobilized proteins from cells transfected with either GST or GST-PI3K γ fusion protein expression vectors (expressed as a percentage of wild type, $n = 4$).

from the baculovirus/Sf9 expression system; results not shown). It can therefore be concluded that Lys-799 is the residue attacking the carbon atom near position 20 of wortmannin to form a Schiff base. The fact that lysine residues close to the N-terminal part of HR1 (the Pro⁷⁸⁶-Asp-Ile⁷⁸⁸ loop in p110 α proposed to interact with wortmannin) were not sufficient to maintain the covalent linkage with the inhibitor could indicate that these residues are exposed to water, protonated and thus much less nucleophilic. A likely explanation for the increased nucleophilicity of Lys-802 in p110 α (Lys-799 in p110 γ) might be the formation of an ionic bridge with Glu-821 (Glu-818 in p110 γ) in the absence of a ligand binding within the catalytic pocket [15], as modelled on the basis of the solved crystal structure of cAMP-dependent protein kinase [51]. That this ionic bridge is modulating the nucleophilicity of Lys-799 and is indeed important for wortmannin binding is supported by preliminary experiments manipulating Glu-818 in PI3K γ (M. P. Wymann, unpublished work).

As with Lys-802 in the p85/p110 α complex, Lys-799 is needed for the phosphate transfer reaction to lipids and protein substrates. Moreover, the abortion of protein phosphorylation proves the capacity of PI3K γ to operate as a protein kinase, for which a physiological significance has yet to be determined.

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