

Wortmannin Inactivates Phosphoinositide 3-Kinase by Covalent Modification of Lys-802, a Residue Involved in the Phosphate Transfer Reaction

MATTHIAS P. WYMANN,^{1*} GINETTE BULGARELLI-LEVA,¹ MARKETA J. ZVELEBIL,²
LUCIANO PIROLA,¹ BART VANHAESEBROECK,² MICHAEL D. WATERFIELD,^{2,3}
AND GEORGE PANAYOTOU²

Institute of Biochemistry, University of Fribourg, CH-1700 Fribourg, Switzerland,¹ and Ludwig Institute for Cancer Research, London WIP 8BT,² and Department of Biochemistry and Molecular Biology, University College London, London WCE 6BT,³ United Kingdom

Received 13 November 1995/Returned for modification 11 December 1995/Accepted 21 December 1995

Wortmannin at nanomolar concentrations is a potent and specific inhibitor of phosphoinositide (PI) 3-kinase and has been used extensively to demonstrate the role of this enzyme in diverse signal transduction processes. At higher concentrations, wortmannin inhibits the ataxia telangiectasia gene (ATM)-related DNA-dependent protein kinase (DNA-PK_{cs}). We report here the identification of the site of interaction of wortmannin on the catalytic subunit of PI 3-kinase, p110 α . At physiological pH (6.5 to 8) wortmannin reacted specifically with p110 α . Phosphatidylinositol-4,5-diphosphate, ATP, and ATP analogs [adenine and 5'-(4-fluorosulfonylbenzoyl)adenine] competed effectively with wortmannin, while substances containing nucleophilic amino acid side chain functions had no effect at the same concentrations. This suggests that the wortmannin target site is localized in proximity to the substrate-binding site and that residues involved in wortmannin binding have an increased nucleophilicity because of their protein environment. Proteolytic fragments of wortmannin-treated, recombinant p110 α were mapped with anti-wortmannin and anti-p110 α peptide antibodies, thus limiting the target site within a 10-kDa fragment, colocalizing with the ATP-binding site. Site-directed mutagenesis of all candidate residues within this region showed that only the conservative Lys-802-to-Arg mutation abolished wortmannin binding. Inhibition of PI 3-kinase occurs, therefore, by the formation of an enamine following the attack of Lys-802 on the furan ring (at C-20) of wortmannin. The Lys-802-to-Arg mutant was also unable to bind FSBA and was catalytically inactive in lipid and protein kinase assays, indicating a crucial role for Lys-802 in the phosphotransfer reaction. In contrast, an Arg-916-to-Pro mutation abolished the catalytic activity whereas covalent wortmannin binding remained intact. Our results provide the basis for the design of novel and specific inhibitors of an enzyme family, including PI kinases and ATM-related genes, that play a central role in many physiological processes.

The expanding family of phosphatidylinositol (PtdIns) 3-kinases consists of enzymes composed of various catalytic subunits of the p110 α (26, 75), p110 β (27), p110 γ (65), and Vps34p (57, 74) types. [The term "PtdIns 3-kinase" was recently used for the human homolog of Vps34p utilizing solely PtdIns as a substrate. We therefore use phosphoinositide 3-kinase (PI 3-kinase) for enzymes phosphorylating PtdIns, PtdIns 4-P, and PtdIns(4,5)P₂ in vitro. PtdIns 3-kinase is also used when a statement applies to the family of these enzymes.] The p110 α and p110 β subtypes form tight heterodimers with a p85 regulatory subunit, which embodies an SH3 domain, two SH2 domains, and a BCR domain (17, 46, 60). Interaction between p110 α or p110 β and p85 is mediated through the N terminus of the catalytic subunit and the inter-SH2 region of p85 (14). This heterodimeric phosphoinositide 3-kinase (PI 3-kinase) is activated during the translocation to autophosphorylated growth factor receptors (30) or their substrates (e.g., the insulin receptor substrate 1), where the SH2 domains of p85 interact with phosphorylated YXXM motives (2, 19, 32, 48; see references 11 and 80 for reviews). Other PI 3-kinases seem to propagate signals from seven transmembrane helix receptors

as they are activated by G-protein $\beta\gamma$ -subunits (62, 65). The first of this kind has been cloned recently and named p110 γ (65). It has been shown that PI 3-kinases are the terminal enzymes in the synthesis of D-3-phosphorylated phosphoinositides (24, 63) and that these lipids are poor substrates for phosphatidylinositol-specific phospholipases C (58). PI 3-kinases therefore produce novel lipid second messengers that are believed to be a key step in receptor signalling by growth factors, cytokines, and hormones (30, 71).

The yeast Vps34 gene product represents yet another class of PtdIns 3-kinases: associated with and activated by a Vps15p serine/threonine kinase, Vps34p phosphorylates solely PtdIns to PtdIns 3-P and, in contrast to the p110 α - γ , does not accept PtdIns 4-P and PtdIns(4,5)P₂ as substrates (57). Moreover, it has been demonstrated that a functional lipid kinase is essential for cellular sorting processes (25, 61).

In higher eukaryotes, manipulation of PI 3-kinase activity was achieved by mutation of the docking sites of the p85 regulatory subunits on growth factor receptors (36, 78) or overexpression of dominant negative p85 (Δ p85) (77). These labor-intensive approaches, however, are limited to growth factor-mediated activation of the lipid kinase. The use of chemical, low-molecular-weight inhibitors of PI 3-kinase, on the other hand, provides a fast and easy way to explore the importance of this enzyme in any cell surface receptor-mediated signalling pathway. Wortmannin has recently been de-

* Corresponding author. Mailing address: Inst. Biochemistry, Rue du Musée 5, CH-1700 Fribourg, Switzerland. Phone: 41 37 29 86 55. Fax: 41 37 29 97 35. Electronic mail address: MatthiasPaul.Wymann@UniFR.ch.

scribed as a potent and specific inhibitor of PI 3-kinase (1, 44, 82, 83) and has since been widely used because of its advantageous properties. Wortmannin is cell permeable and commercially available and has, at concentrations at which it fully inhibits PI 3-kinase, very little effect on other signalling molecules. At submicromolar concentrations, wortmannin does not interfere with the activities of protein kinase C (PKC); calmodulin-dependent, cyclic AMP (cAMP)-dependent, and cGMP-dependent protein kinases (42); mitogen-activated protein kinase (43); p70^{S6k} (10); and the platelet-derived growth factor receptor tyrosine kinase (82). Under these conditions, wortmannin has no influence on the levels of PtdIns 4-P and PtdIns(4,5)P₂ in resting cells (1) and was reported not to inhibit PtdIns 4-kinase in vitro (44) but to inhibit a novel, weakly membrane-associated PtdIns 4-kinase at elevated concentrations (41). The release of calcium from intracellular stores in response to serpentine receptor ligand binding remains unaffected by wortmannin, illustrating that activation of phospholipase C, inositol(1,4,5)P₃, and diacylglycerol production remains intact in the presence of the inhibitor. With a 50% inhibitory concentration (IC₅₀) of about 200 nM, wortmannin has recently been shown to inhibit DNA-dependent protein kinase (DNA-PK_{cs}), which is involved in the control of DNA repair mechanisms (22).

Wortmannin at nanomolar concentrations, on the other hand, has been shown to inhibit the activation of neutrophil NADPH oxidase by *N*-formyl-Met-Leu-Phe, complement factor 5a, leukotriene B₄, platelet activation factor, and interleukin-8 but has no effect on the phorbol ester-induced response or the enzyme activity in vitro (1, 3, 44). Later, platelet-derived growth factor receptor-mediated fibroblast motility and membrane ruffling (77, 82), histamine release in mast cells (83), insulin-stimulated glucose uptake in various tissues (29, 43, 85), nerve growth factor-dependent survival and differentiation of PC12 cells (33, 84), platelet aggregation (35), and vesicular sorting of cathepsin D to lysosomes (7, 13) were added to the list of wortmannin-inhibited and possibly PI 3-kinase-controlled cellular processes.

Although it is not yet clear how inactivation of PtdIns 3-kinase by wortmannin affects these cell responses, a number of candidate downstream enzymatic activities are affected by the presence of the inhibitor or are modulated by PtdIns(3,4,5)P₃. Serum activation of the mitogen-activated protein kinase pathway, phosphorylation of glycogen synthase kinase 3 (12, 76) and p70^{S6k} (10), phospholipase D activity (5, 20, 49), and activation of the GTP-binding protein Rac (9, 23), the PKC δ , ϵ , τ (69), and ζ (40) isoforms, and, recently, PKB/c-Akt protein kinase (8, 18) have been proposed to depend on functional PI 3-kinase.

The family of PtdIns 3-kinases is constantly expanding, and related proteins include products of the ATM-related genes (for a review, see reference 87) and the targets of the immunosuppressant rapamycin (TOR, FRAP, and RAFT1 [6, 37, 51, 52]), with (so far) unknown activities. It has been suggested that targets of rapamycin (TORs) are downstream of PI 3-kinase because of the differential actions of rapamycin and wortmannin on p70^{S6k} phosphorylation (8, 10). An interaction of wortmannin with TORs was also considered (72).

The importance that wortmannin has gained as a tool to explore PtdIns 3-kinase signalling demands a better understanding of its inhibitory mechanism. In this work, we identified the reaction site of wortmannin on PI 3-kinase. Additionally, we showed that the lysine residue involved in wortmannin binding is likely to play a central role in the phosphotransfer reaction. Together, the results elucidate the inactivation mechanism of PtdIns 3-kinases by wortmannin and provide a fix

point for the alignment of the lipid kinase catalytic center with cAMP-dependent protein kinase (PKA).

MATERIALS AND METHODS

Anti-wortmannin, anti-p110 antisera, and immunoblots. Polyclonal antibodies were raised in rabbits. Wortmannin was dissolved in 50 μ l of dimethyl sulfoxide at 40 mg/ml and subsequently mixed with 950 μ l of 2-mg/ml bovine serum albumin (BSA) in phosphate-buffered saline (PBS; 8 mM Na₂HPO₄ · 2H₂O, 1.4 mM KH₂PO₄, 2.6 mM KCl, 136 mM NaCl [pH 7.4]) for 24 h at room temperature. Serum collected after five injections of wortmannin-BSA conjugate was passed over a BSA-Sepharose column to remove anti-BSA antibodies. Anti-5'-*p*-fluorosulfonylbenzoyladenine FSBA antibodies were produced in a similar way. The peptides FLVEQMRRPDMFDAL-Cys and Cys-GGWTTKMDWIF-HTIKQHALN, corresponding to amino acids 734 to 748 and 1049 to 1068 (C terminus) of p110 α , respectively, were coupled to keyhole limpet hemocyanin with the bifunctional linker sulfosuccinimidyl 4-(*N*-maleimidomethyl) cyclohexane-1-carboxylate (SMCC; Pierce). Immunoglobulin G fractions from antipeptide antisera were purified on γ -bind Sepharose (Pharmacia). Anti-p85 α rabbit antisera were obtained by immunization with a (His)₆ tag fusion protein containing amino acids 6 to 112 of bovine p85 α .

Wortmannin labelling of PI 3-kinase, proteolytic digests. Immobilized glutathione *S*-transferase (GST)-PI 3-kinase fusion protein or soluble p85/p110 α complex was taken up in PBS and usually incubated with 100 nM wortmannin on ice for 15 min. PI 3-kinase immobilized on glutathione or phosphotyrosine beads was washed with PBS-0.5% Triton X-100 to remove excess inhibitor before denaturation, while soluble protein was either denatured directly or precipitated by the method in reference 79. Modified conditions and wortmannin concentrations are described in the figure legends. Unless indicated otherwise, samples were denatured in the presence of β -mercaptoethanol, and subjected to standard sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (38), and transferred to polyvinylidene difluoride membranes (Millipore) by the method of Towbin et al. (70). Primary antibodies were detected by using goat anti-rabbit horseradish peroxidase conjugate (Sigma) and enhanced chemiluminescence (ECL; Amersham). Reactions of 200 μ M FSBA with PI 3-kinase complex, immobilized on glutathione or phosphotyrosine beads, were carried out in PBS-0.1% Triton X-100 for 15 min at 37°C. The beads were washed four times with PBS-0.5% Triton X-100 and denatured in sample buffer also containing 5 mM dithiothreitol (DTT).

PI 3-kinase samples for digestion with various proteases were labelled with 200 nM wortmannin as indicated above. Subsequently, 6.5 mM NaCNBH₃ was added for 1 h to reduce and stabilize the Schiff base formed between the kinase and wortmannin. Whenever soluble PI 3-kinase was used, labelling was performed in the respective digestion buffers. GST-PI 3-kinase was labelled and reduced in PBS, and the beads were washed twice with digestion buffer. Large fragments were obtained with factor Xa (Promega) (1.5 μ g of protease in 0.1 M NaCl-20 mM Tris · HCl [pH 8.0]) and Glu-C (*Staphylococcus aureus* V8 protease [Boehringer]; 0.4 μ g in 50 mM NH₄HCO₃ [pH 7.8]) and were separated by SDS-PAGE (10% polyacrylamide). Peptides smaller than 30 kDa were generated with Lys-C (Promega) (2 μ g in 25 mM Tris-1 mM EDTA [pH 7.7]), trypsin (Sigma) (0.04 μ g in 50 mM Tris-0.1 mM CaCl₂ [pH 7.8]), and Arg-C (clostripain [Promega], 0.2 μ g; the kinase was denatured in 1 mM DTT-1 M urea-20 mM Tris [pH 7.8] for 5 min at 90°C and subsequently digested). Digestions were carried out at 37°C for 3 h in a total volume of 80 μ l, and the products were denatured and separated on Tricine gels prepared as described previously (55).

Introduction of point mutations into p110 α . The transformer site-directed mutagenesis kit (Clontech) with *AflIII*-*BglII* *trans*-oligonucleotide primers and the respective mutagenesis primers was used to produce the desired Lys \rightarrow His, Lys \rightarrow Arg, and Cys \rightarrow Ser codon exchanges in the p110 α cDNA, cloned as a *Bam*HI-*Hind*III fragment (26) in the multicloning site of pUC19, and to introduce at the same time new restriction sites for plasmid selection (the mutants were named KR1 [K733H], KR2 [C769S/K776R], and KR3 [K802R]). The triple mutant KR4 (C862S/K863R/K867R) was produced by the ligation of a synthetic adapter into the *Hin*PI-*Pst*I region of p110 α DNA (corresponding to amino acids 860 to 871).

All nucleotide exchanges were confirmed by DNA sequencing (Sequenase 2.0; U.S. Biochemicals). The respective mutations were subsequently reintroduced as *Xba*I-*Pst*I or *Bbs*I-*Pst*I fragments into wild-type p110 α harbored in the pSCT1 and p36C expression vectors (see below) to generate the desired mutant proteins. General procedures were carried out as described previously (53).

Production of recombinant proteins. For transient expression of the p85 α /p110 α PtdIns complex, human embryo kidney 293 cells (obtained from the American Type Culture Collection) were transfected with 1 μ g of adenovirus late promoter expression vector pMT2 (31) containing the coding sequence of p85 α (26, 46) and 10 μ g of the cytomegalovirus promoter-based expression vector pSCT1 (50) carrying the wild-type and KR mutants of p110 α . Expression vectors and 5 μ g of calf thymus carrier DNA were preprecipitated for 10 min in a calcium phosphate solution, and this was subsequently added to 10-cm-diameter petri dishes containing 293 cells cultured in Dulbecco's modified Eagle's medium (Gibco/BRL) plus 5% fetal calf serum (the detailed procedure will be published elsewhere). The medium was exchanged 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% glycerol, 1 mM MgCl₂, 1 mM CaCl₂, 5 mM EDTA, 20 mM NaF, 1 mM sodium-*o*-vanadate, 20 μM leupeptin, 18 μM pepstatin, and 1% Nonidet P-40. Cleared-cell lysates (12,000 × *g* for 15 min at 4°C) were subjected to immunoprecipitation, wortmannin binding, and kinase assays. The protein subjected to SDS-PAGE or kinase assays corresponded typically to one-quarter of a 10-cm petri dish. Endogenous p85α could not be detected in 293 cells, whereas p85β was present (T4 anti-p85β antibodies were kindly given by I. Gout).

Recombinant p110α baculovirus was produced essentially as described previously (26). Briefly, Sf9 cells cultured in IPL41 (Gibco/BRL) with 10% fetal calf serum were cotransfected with 5 μg of each p36C transfer vector (47) containing mutated p110α cDNA and 0.25 μg of linear BaculoGold DNA (PharMingen) essentially as described by the manufacturer. Recombinant virus was plaque purified and amplified (45). For protein production, cells were harvested 60 h after transfection with recombinant p110α and p85α virus, centrifuged and lysed by mechanical disruption in a Dounce homogenizer in 20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES)–2 mM DTT–10 mM NaF–100 μM Na₃VO₄–5 mM EDTA (pH 7.5) supplemented with the protease inhibitors phenylmethylsulfonyl fluoride (1 mM), aprotinin (10 μg/ml), leupeptin (20 μM), and pepstatin (18 μM). The lysate was cleared by ultracentrifugation, and the supernatant was incubated with phosphotyrosine-Actigel beads (Sterogene). The beads with the associated p85α/p110α PI 3-kinase complex were washed (20 mM phosphate buffer [pH 7.5], 0.2 M NaCl) and used directly for experiments. The production of p85α/GST-p110α^{R916P} was described previously (15).

Immunoprecipitations. Recombinant PI 3-kinase subunits p85α, p110α, or complexes were immunoprecipitated from 293 cell lysates with anti-C-terminal antibodies and anti-p85 antisera by using protein A-Sepharose (Pharmacia) or with monoclonal anti-p85α antibodies (U13; a kind gift from I. Gout) by using anti-mouse immunoglobulin G agarose beads (Sigma) to immobilize the immunocomplexes. Precipitates were subsequently washed three times with 0.1 M Tris-HCl (pH 7.4)–0.5 M LiCl and three times with the respective reaction buffers.

PI 3-kinase and protein kinase assays. The assay for PI 3-kinase activity in immunoprecipitates and protein from insect cells was carried out essentially as described previously (30). PI 3-kinase samples were incubated with an [γ -³²P]ATP (3,000 Ci/mmol; Amersham)–PtdIns–phosphatidylserine (PS)–Mg²⁺ mix for 10 min at 30°C, lipids were extracted, and PtdIns 3-P was quantified after thin-layer chromatography (1, 30).

The protein serine kinase activity of PI 3-kinase was assayed as described previously (15) with minor modifications. Immunoprecipitated PI 3-kinase or PI 3-kinase immobilized on phosphotyrosine beads was washed twice with kinase buffer (50 mM HEPES [pH 7.4], 150 mM NaCl, 5 mM EDTA, 5 mM DTT, 10 mM MnCl₂, 0.01% Triton X-100), and an equal volume of kinase buffer with doubly concentrated ATP (to give a final concentration of 20 μM and 10 μCi of [γ -³²P]ATP per experiment) was added to start the reaction. After a 20-min incubation at 30°C, samples were denatured, separated by SDS-PAGE, and exposed to Kodak X-Omat films for autoradiography.

Molecular modelling. An initial model of the p110α catalytic domain was built on the basis of the crystallographic structure of PKA with bound PKA inhibitor-peptide and ATP (39). To improve the alignment of these two low-homology molecules, special attention was given to residues conserved within each family and to functional residues (for a review, see reference 64). Each family of structures was aligned by the MULTALIGN method of Barton and Sternberg (4). Predicted (89) and X-ray-determined secondary-structure information was used as further constraints for the alignment. The final alignment (45% homology and 11% identity) was then used to construct the model by using the suite of programs within Quanta. The target (p110α) protein and template (PKA) were aligned. Where the target sequence matched the template molecule, the residue coordinates from the template were transformed directly to the target. Where no equivalent atoms were found in the template molecule for the target protein, reference was made to a side chain rotamer library. This defines the most common conformation found for each side chain type (66). Gaps in the target sequence were subjected to local energy minimization to bring the core ends together and to alleviate local conformational strain. Insertions in the target sequence were modelled by searching a fragment database of high-resolution structures to find an appropriate template. The final structure was subjected to 100 steps of steepest gradient minimization by the CHARMM program to make minor shifts in the coordinate positions, thereby alleviating steric clashes between atoms and obtaining a reasonable peptide geometry. Wortmannin was fitted into the ATP-binding site with ATP as a template. The docking of wortmannin was refined manually, and the complex was energetically minimized. This cycle of adjustment was repeated several times to obtain the model presented here.

RESULTS

Specific interaction of wortmannin with p110α. While conditions for PtdIns 3-kinase assays are usually optimized to produce a maximal effect of wortmannin, the parameters for the present study were adapted to increase specificity rather

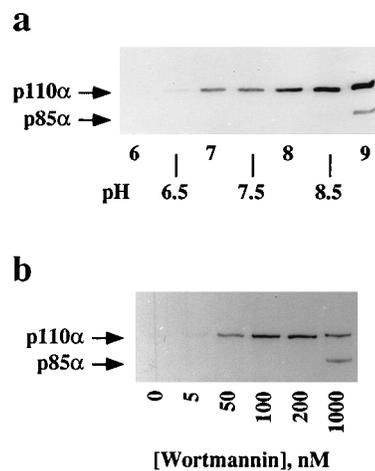


FIG. 1. Conditions for covalent modification of PI 3-kinase by wortmannin. Purified recombinant PI 3-kinase p110α/p85α complex was incubated with wortmannin as described in Materials and Methods. Wortmannin-labelled proteins were subsequently subjected to SDS-PAGE and probed with anti-wortmannin antisera on immunoblots. (a) Binding of 100 nM wortmannin was achieved at the indicated pHs in 0.2 M phosphate buffer. (b) PI 3-kinase complex was incubated with increasing concentrations of wortmannin.

than sensitivity so as to avoid the detection of nonspecific interactions of nucleophilic amino acid residues with wortmannin.

When wortmannin at nanomolar concentrations was incubated with recombinant bovine p85α/p110α complex, the inhibitor-protein conjugates could be detected after separation on reducing SDS-PAGE with anti-wortmannin antibodies. At physiological pH, wortmannin reacted covalently and specifically with p110α but not with p85α. At pHs below 6, no wortmannin binding could be detected. On the other hand, when the pH was increased beyond 8.5, wortmannin attached to p85α as well (Fig. 1a). Nonspecific wortmannin binding was also observed when the concentration of the inhibitor was increased to 1 μM or more (Fig. 1b), while p110α alone was stained at lower inhibitor concentrations. Nonspecific labelling of p85 could be somewhat reduced by the inclusion of detergent (0.1% Triton X-100 [compare Fig. 1 and 8]). Detectable labelling under the stringent conditions used (0°C for 15 min) could be achieved with as little as 5 nM wortmannin (Fig. 1b). Identical results were obtained with immobilized GST-p110α/p85α complex (data not shown). At high pH or excessive inhibitor concentrations, wortmannin reacts with virtually any protein containing lysine residues, a feature that was exploited when producing anti-wortmannin antibodies (see Materials and Methods).

Competition of PI 3-kinase substrates with wortmannin. Wortmannin could act as either a competitive or noncompetitive inhibitor of PI 3-kinase. To test the two possibilities, PI 3-kinase was incubated with sonicated mixtures of phosphoinositides and PS, before wortmannin was added. While lipid suspensions containing PtdIns(4,5)P₂ protected PI 3-kinase from modification by wortmannin, PtdIns, PS, or Triton-X100 alone had no effect up to 1 mg/ml (Fig. 2a). These results suggest that wortmannin might interact with the head group-binding site for PtdIns(4,5)P₂. It has been previously reported that high concentrations of ATP reduce the inhibitory action of wortmannin on PI 3-kinase (82). The same was true for the covalent reaction of wortmannin with PI 3-kinase; ATP and the ATP analogs FSBA and adenine at 1 mM all interfered with the alkylation of PI 3-kinase when added before the in-

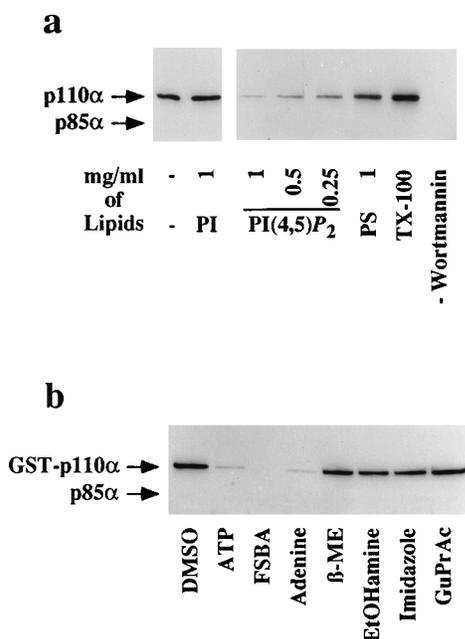


FIG. 2. Substrates competing with wortmannin binding. PI 3-kinase was subjected to wortmannin labelling (100 nM) in the presence of the enzyme substrates PtdIns (PI), PtdIns(4,5)P₂, ATP, ATP analogs, and nucleophilic substances. The reaction of PI 3-kinase with wortmannin was verified as in Fig. 1. (a) Recombinant p110α/p85α complex was added to lipid suspensions made up from the indicated lipids (phosphoinositide mixtures also contained one-quarter of PS; Triton-X 100 [TX-100] was present at 0.1%). Wortmannin or dimethyl sulfoxide (DMSO) only (-Wortmannin) were subsequently added. (b) Immobilized GST-p110α/p85α complex was preincubated with 1 mM ATP, FSBA, adenine, β-mercaptoethanol (β-ME), ethanolamine (EtOHamine), or β-guanidinopropionic acid (GuPrAc) for 30 min at 37°C. The samples were cooled to 0°C before incubation with wortmannin.

hibitor (Fig. 2b). FSBA reacts covalently with reactive nucleophilic amino acid residues and was previously used to map the nucleotide-binding sites of various ATP- and GTP-utilizing enzymes (reference 34 and references therein). Although FSBA reacts nonspecifically to a certain extent (34) (see below), the present results suggest that wortmannin alkylates a nucleophilic residue within the ATP-binding site of PI 3-kinase.

To mimic the nucleophilic attack of amino acid side chains on wortmannin, substances similar to the respective amino acids but lacking the α-amino group were used. An excess of β-mercaptoethanol, ethanolamine, imidazole, and guanidinopropionic acid did not compete with PI 3-kinase and did not destroy the reactivity of wortmannin under the conditions used (Fig. 2b). This demonstrates that the nucleophilicity of the wortmannin-reactive amino acid residue is determined by its environment within the catalytic site of the enzyme rather than by its characteristics in aqueous solution. The lack of any effect of ethanolamine also shows that the primary amino group in ATP and its analogs does not have the potential to inactivate wortmannin under the given conditions.

Proteolytic mapping of the wortmannin-binding site. When wortmannin-labelled GST-p110α/p85α complex was partially digested, factor Xa produced relatively small amounts of the expected p110α full-length protein cleaved adjacent to the Ile-Glu-Gly-Arg intersection of GST-p110. The major wortmannin-labelled product of factor Xa digestion was of 52 kDa, while Glu-C (*S. aureus* V8 protease) generated a wortmannin-containing 55-kDa peptide (Fig. 3). After the removal of anti-

wortmannin antibodies, immunoblots were reprobed with anti-p110 C-terminal antibodies. Superposition of the signals obtained from anti-wortmannin and anti-p110 C-terminal immunoblots showed that GST-p110α, p110α, and the 52- and 55-kDa peptides contained both the wortmannin-binding site and the C terminus of p110α (Fig. 3). The 52- and 55-kDa peptides remained firmly attached to the glutathione beads, since none of the 52-kDa and very little of the 55-kDa peptide could be detected in the supernatant of the digest. Separate experiments with wortmannin absent showed that PI 3-kinase activity of the immobilized GST-p110α/p85α complex was increased 2.3-fold by factor Xa and 3.8-fold by Glu-C cleavage, indicating that the enzyme remained fully functional after the above protease treatment.

Once the N-terminal half of p110 could be excluded from binding wortmannin covalently, the interaction site was further mapped with proteases generating smaller peptides. In the course of these studies, we realized that binding of wortmannin to PI 3-kinase was covalent but reversible. Attempts to isolate wortmannin-labelled peptides failed because of the acid lability of the wortmannin-enzyme interaction and because labelled peptides could not be recovered from various high-pressure liquid chromatography columns at neutral pH. Assuming that the acid-labile function might be a Schiff base, model reactions with lysine and morpholino adducts of wortmannin (kindly provided by T. G. Payne, Sandoz, Basel, Switzerland) were carried out to find ways to stabilize the putative amine-wortmannin interaction (for reactions of wortmannin with amines, see reference 21). Thus, NaCNBH₃ was finally chosen to reduce wortmannin-p110. Reduction with NaCNBH₃, as described in Materials and Methods, considerably decreased the release of wortmannin from p110α when the mixture was exposed to low pH (pH 3 to 4 [data not shown]). Because of its longer half-life, reduced, wortmannin-labelled p110α was used subsequently for peptide analysis by Tricine gel electrophoresis and immunodetection. Proteases were always used at various concentrations to monitor the formation of peptides, which were then analyzed by superimposing anti-wortmannin and anti-peptide immunoblots. Because stripping and reprobing of membranes increased background chemiluminescence, parallel (not serial) immunoblots are shown in Fig. 4.

Digestion with Lys-C protease produced two groups of wortmannin-labelled peptides with apparent molecular masses of approximately 15 and 7 kDa, which were not cross-detected with anti-C terminal antibodies (Fig. 4a). Trypsin and Arg-C, on the other hand, produced various C-terminal peptides that

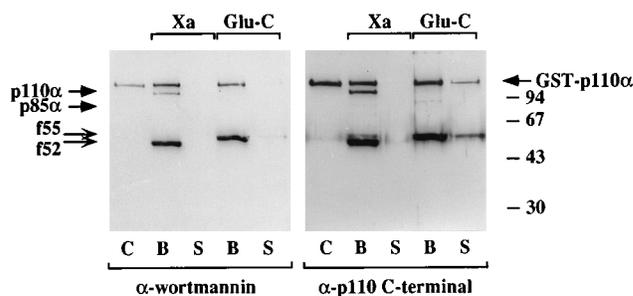


FIG. 3. Partial digests of GST-p110α/p85α complex by factor Xa and Glu-C. Wortmannin-labelled PI 3-kinase immobilized on glutathione-Sepharose beads was digested as described in Materials and Methods. Beads (B) and supernatants (S) following protease treatment and undigested PI 3-kinase (C) were denatured separately and examined on immunoblots for the presence of wortmannin. The membranes were then stripped and reprobed with anti-p110 C-terminal antibodies.

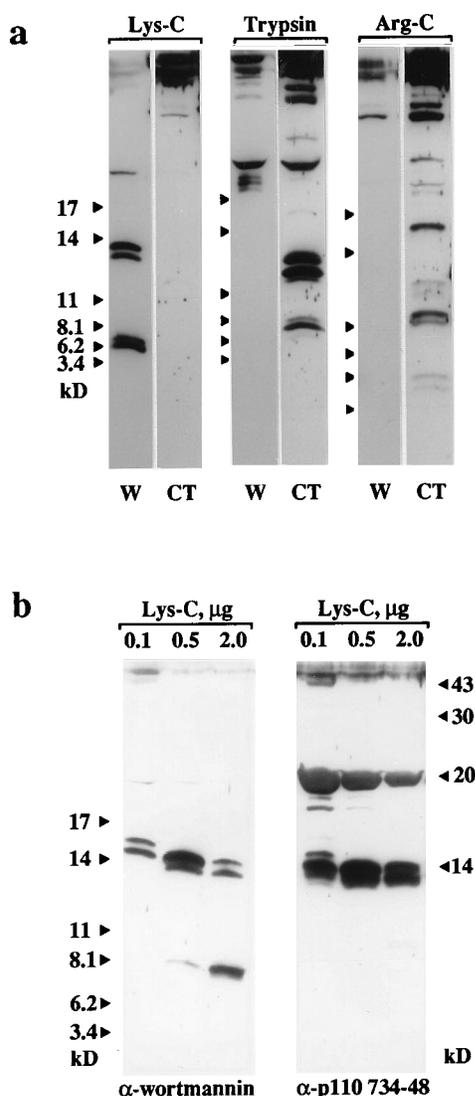


FIG. 4. Localization of wortmannin-labelled peptides. Immobilized GST-p110 α /p85 α complex was incubated with 200 nM wortmannin, digested with the indicated proteases (Lys-C at 2 μ g when not otherwise indicated, trypsin at 0.04 μ g, Arg-C at 2 μ g), and subjected to Tricine gel electrophoresis and immunoblotting. (a) Detection of wortmannin-labelled peptides with anti-wortmannin antiserum (W) or peptides including the C terminus with anti-p110 α C-terminal antiserum (CT). (b) Lys-C concentration-dependent formation of wortmannin-labelled peptides (α -wortmannin) or peptides containing the internal sequence 734 to 748 of p110 α (α -p110 734-48). Triangles mark the positions of peptide molecular mass standards (to the left, Sigma [2.5 to 17 kDa]; to the right, Pharmacia [14 to 94 kDa]).

remained undetected with anti-wortmannin antibodies (in the range of 7 to 15 kDa). Peptides staining for wortmannin and the C terminus were more than 40 kDa in Arg-C partial digests.

As C-terminal peptides of more than 40 kDa were found to be labelled by wortmannin and those of less than 15 kDa were not (as illustrated in Fig. 5), a region of approximately 25 kDa (amino acids 720 to 935) was estimated for the wortmannin-binding site. This localization corresponds to the ATP-binding site.

A complete digestion of p110 α with Lys-C theoretically generates 72 peptides, of which 12 are larger than 3 kDa and are therefore putative candidates for the wortmannin-labelled

peptide family with an apparent molecular mass of 7 kD (Fig. 4). Only four of these peptides, however, fall into the region of amino acids 720 to 935. If the ϵ -amino group of a lysine reacted with wortmannin, one would expect that the lysine-specific protease would not cleave C-terminal to the alkylated residue. A Lys-C digest would then result in 32 peptides which are larger than 3 kDa, with 6 candidates located within the 25-kDa wortmannin-binding region (Table 1). Of these peptides, however, only two (F734-K802 and R777-K863) would give rise to a 15-kDa wortmannin-labelled peptide with one additional un-cleaved amide bound distal to one of the neighboring lysines. If the 15-kDa wortmannin signal was the F734-K863 peptide, this would also explain the appearance of multiple bands, since it can be further extended to lysines 729, 724, or 867.

To check this possibility, wortmannin-labelled p110 α was digested with Lys-C and immunoblots were probed with anti-wortmannin and anti-peptide antibodies (p110 α 734 to 748). These experiments showed clearly that the 15-kDa peptides contain the wortmannin attachment site as well as the peptide sequence 734 to 748. However, the wortmannin-labelled 7-kDa peptides that were formed as the 15-kDa group was further digested remained undetected by anti-p110 α 734 to 748 antisera (Fig. 4b). Because of its proximity to the gel front, the 734 to 776 fragment not labelled with wortmannin produced only a weak signal with an apparent molecular mass of about 3.2 kDa in anti-peptide immunoblots (calculated mass, 4.9 kDa [data not shown]). These results clearly exclude Lys-776 from being the covalent binding site of wortmannin and point to an interaction at Lys-802.

Wortmannin and FSBA binding to Lys-to-Arg mutants of p110 α . Lysines within the putative wortmannin-binding site K733-K867 were replaced by arginines (histidine for Lys-733) by site-directed mutagenesis. Selected cysteines within the same region were mutated to serine at the same time. Mutated p110 α was subsequently coexpressed with p85 α in 293 cells and immunoprecipitated with monoclonal anti-p85 α antibodies or anti-p110 α C-terminal antibodies from cell lysates. Expression levels of wild-type p110 α /p85 α complex and mutant PI 3-kinase protein were all equal, as demonstrated by Coomassie blue and colloidal gold staining (Fig. 6a and b). The amount of endogenous PI 3-kinase from 293 cells was too small to be detected. When anti-PI 3-kinase immunoprecipitates were subjected to wortmannin labelling, wild-type p110 α (wt), p110 α ^{K733H} (KR1), p110 α ^{C769S/K776R} (KR2), and p110 α ^{C862S/K863/K867R} (KR4) all bound wortmannin covalently whereas the p110 α ^{K802R} (KR3) mutant was undetected by anti-wortmannin antisera (Fig. 6b and d). Identical results were obtained with recombinant PI 3-kinase complexes (wt and KR2-4) isolated from the baculovirus/Sf9 cell expression system (data not shown). A covalent interaction of wortmannin with Lys-802, as confirmed by the KR3 mutation, is in agreement with the results obtained with substrate competition and proteolytic digests.

Wild-type and mutant PI 3-kinases were also incubated with FSBA and subsequently examined with anti-FSBA antisera for the presence of sulfonated protein. Although FSBA is considered a specific probe for ATP-binding sites (34), we observed nonspecific modification of p85 (Fig. 6e) and immunoglobulin G heavy and light chains in immunoprecipitates (data not shown). Further experiments were therefore carried out with recombinant p110/p85 complex isolated from insect cells. With this purified, immobilized protein, FSBA staining of wt, KR2, and KR4 was prominent while the labelling of KR3 was virtually absent (Fig. 6e). Although samples were extensively washed and denatured in the presence of β -mercaptoethanol and DTT, FSBA binding to p85 persisted.

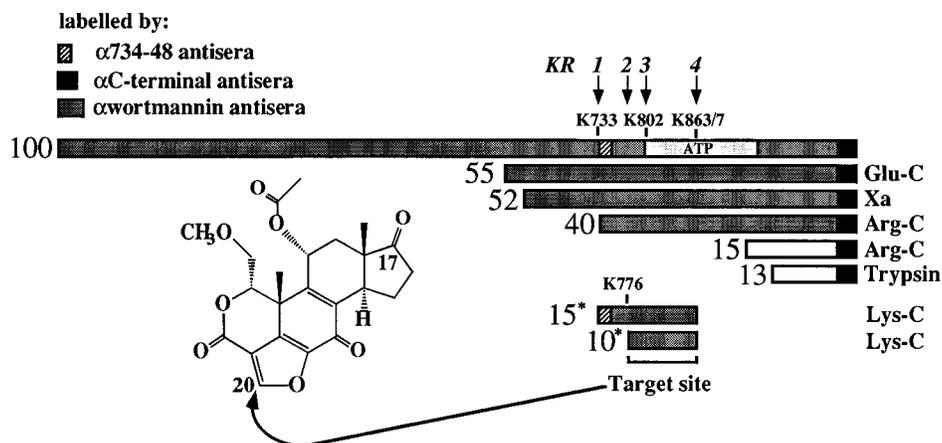


FIG. 5. The wortmannin target site deduced from digest patterns as displayed in Fig. 3 and 4. Selected peptides were aligned with full-length p110 α by using the C terminus or the internal sequence 734 to 748 as markers. Peptides detected by anti-wortmannin antibodies are displayed in gray. Numbers indicate the apparent molecular masses in kilodaltons (asterisks indicate expected molecular masses) as obtained by the indicated proteolytic treatments.

Lipid and protein kinase activities of Lys-to-Arg mutants of p110 α . The transiently overexpressed and immunoprecipitated proteins were assayed for lipid and protein kinase activities. While 293 cells transfected with p85 α and one of the wt, KR1, KR2, or KR4 p110 α vectors produced 35 to 40 times the amount of PtdIns 3-P than did cells that received p85 only, the PI 3-kinase activity from p85/KR3-transfected cells achieved only levels characteristic of mock-transfected cells (Fig. 7a). That the remaining activity in KR3-transfected cells is due to coprecipitation of endogenous PI 3-kinase is illustrated by the fact that the p85 α /KR3-p110 α complex purified from Sf9 insect cells yielded less than 0.1% of the activity of the wt, KR2, and KR4 protein from the same source (data not shown).

As in the lipid kinase assay, the KR3 mutant p110 α was impaired in its ability to phosphorylate coexpressed p85 α whereas the wt and the rest of the KR mutants all heavily phosphorylated the p85 α band (Fig. 7b). The same results were obtained with protein from Sf9 cells, when the KR3 mutant showed no phosphorylation of p85 α at all (data not shown).

It has been shown previously that mutations within the conserved DXHXXN kinase motif completely destroy the lipid kinase activity of Vps34p (57) as well as the lipid and protein

kinase activity of p110 α (DRHNSN in p110 α , where R = Arg-916 [15]). When a p85 α /GST-p110 α R916P mutant (RP) PI 3-kinase was used to assay wortmannin binding, the inactive p110 subunit still bound wortmannin covalently. The reaction with the inhibitor was observed at similar concentrations to those with the wild-type lipid kinase and occurred in a specific manner (Fig. 8). The labelling efficiency of the RP mutant, however, was reduced, and more protein was needed to yield comparable signals in the anti-wortmannin immunoblots. This indicates that the interaction of wortmannin with the catalytic subunit of PI 3-kinase is optimal with an intact ATP binding site but is not strictly coupled to kinase activity.

Structure prediction of the wortmannin-p110 α complex.

The availability of the crystal structure of PKA prompted us to construct a model of the catalytic domain of p110 α so that, with our assignment of the wortmannin-binding residue, a possible structure of the p110 α -wortmannin interaction site could be put forward. Modelling was performed as described in Materials and Methods. The overall root mean square deviation of the modelled structure from the original template structure was 2.5 Å (0.25 nm) after minimization. Two significant insertions had to be added to the p110 α model with respect to PKA. The first is 8 residues long and partially covers a groove in p110 α which is equivalent to the binding site of the PKA inhibitor peptide. However, a smaller substrate such as PI would still fit into the active site of p110 α together with ATP. The second insertion (10 residues) is located in such a way as to replace the N-terminal helix which is found in PKA but seems to be absent in the PI-kinase catalytic domains. The environment of the ATP-binding site contains the conserved Lys-802 (equivalent to Lys-72 in PKA) and the stabilizing Glu-821 (Glu-91 in PKA). Lys-802 could also interact with the α - and β -phosphates of ATP (data not shown) as proposed for Lys-72 in PKA (39). When wortmannin was docked into this site with the ATP molecule as the template, the C-20 became positioned so that it was near Lys-802. Residues that may stabilize wortmannin in this site are Pro-786, Asp-787, Ile-788, Ser-919, Asp-933, His-936, and Lys-802. Wortmannin fits well into this pocket (Fig. 9). Lys-802 could attack C-20 of wortmannin while still being stabilized by Glu-821. Although the model of the p110 α catalytic center as shown in Fig. 9 is preliminary and further studies are in progress to improve it, it

TABLE 1. Predicted Lys-C peptide pattern for wortmannin-labelled p110 α ^a

Starting residue	C-terminal lysine residue	Putative wortmannin-binding site	Expected mol mass (Da)	Within expected region
673	700	678	3,283	No
628	655	640	3,472	No
679	711	700	3,893	No
885	924	886	4,396	Yes
987	1030	1024	5,035	No
730	776	733	5,465	Yes
974	1024	986	6,143	No
887	941	924	6,176	Yes
803	867	863	7,390	Yes
734	802	776	8,221	Yes
777	863	802	10,277	Yes

^a Peptides larger than 3 kDa starting at amino acids above 600 are listed by increasing molecular masses. The molecular mass was calculated under the assumption that a wortmannin-lysine adduct at the putative position masks the cleavage site for Lys-C.

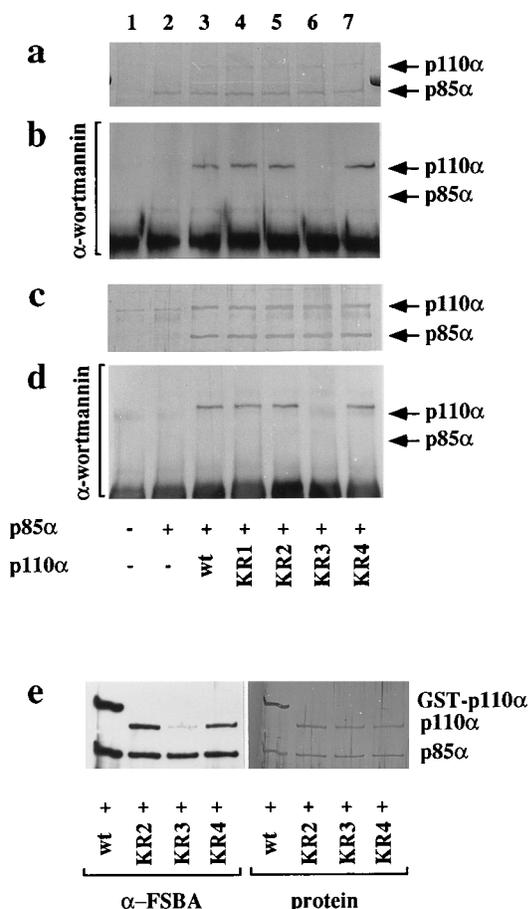


FIG. 6. Wortmannin and FSBA binding on overexpressed p110 α Lys-to-Arg mutants. (a to d) Human embryonic kidney 293 cells were cotransfected with p85 α and p110 α DNA as indicated at the bottom of panel d. PI 3-kinase was immunoprecipitated from cell lysates with monoclonal anti-p85 α antibodies (a and b) or anti-p110 α rabbit antisera (c and d) (see Materials and Methods for details). Expression of p85 α or p110 α was verified by staining for total protein (a and c). (a) Coomassie blue-stained SDS-PAGE of immunoprecipitates from 293 cells. (b) Anti-p85 α immunoprecipitates were incubated with 100 nM wortmannin and subsequently probed on immunoblots for covalent binding of wortmannin by using anti-wortmannin antisera. (c) Colloidal gold total protein staining of the polyvinylidene difluoride membrane after wortmannin detection in panel d. (d) Anti-p110 α immunoprecipitates treated as described for the samples in panel b. (e) Recombinant wild-type and mutant p110 α /85 α complexes were isolated from insect cells on glutathione (wt) or phosphotyrosine (KR) beads. Samples were labeled with 200 μ M FSBA and subsequent immunoblots were developed with anti-FSBA antibodies (left) before the membranes were stained for total protein (right) as in panel c.

is in good agreement with the biochemical data described above.

DISCUSSION

Wortmannin first received widespread attention as an inhibitor of the agonist-induced superoxide anion production in neutrophils. Although the cellular target of wortmannin was unknown at that time, Baggolini et al. (3) demonstrated that the furan ring structure of the substance was important for its action, because derivatives with an opened or protected furan ring were ineffective in biological assays. After the identification of wortmannin as a specific inhibitor of PI 3-kinase (1, 44, 82, 83), it was found that 17-[³H],17-hydroxy wortmannin labelled a 110-kDa PI 3-kinase from neutrophil cytosol. The

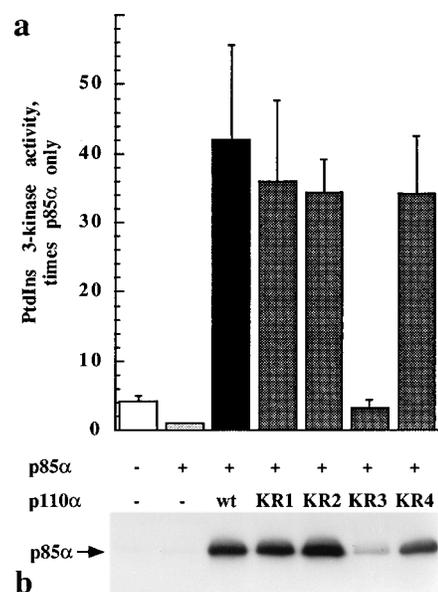


FIG. 7. Lipid and protein kinase activities of p110 α lysine mutants. PI 3-kinase was immunoprecipitated with anti-p85 α antiserum from 293 cells transfected with p85 α and p110 α DNA as described in the legend to Fig. 6. (a) Immunoprecipitates were subjected to PI 3-kinase activity assays. The amount of radioactivity incorporated in [³²P]PtdIns-3P is displayed in relation to the activity obtained from cells transfected with p85 α DNA only ($n = 3$; bars indicate standard error of the mean). (b) Immunoprecipitates were assayed for p85 α phosphorylation.

resistance of this association to denaturation under reducing conditions suggested a covalent interaction of wortmannin and the catalytic p110 subunit of PI 3-kinase (68).

To determine the exact site of this covalent interaction, we optimized the reaction conditions for wortmannin with PI 3-kinase, aiming at a most specific reaction of the inhibitor with p110 α . When added in excess, the physiological substrates of

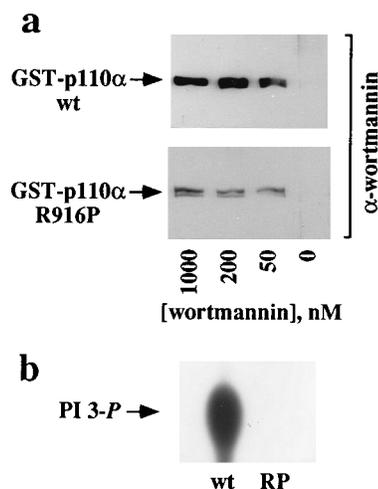


FIG. 8. Covalent reaction of wortmannin with an inactive PI 3-kinase complex. (a) Purified recombinant GST-p110 α /p85 α and Arg-to-Pro mutant GST-p110 α ^{R916P}/p85 α complex (twice the amount of protein) were exposed to the indicated concentrations of wortmannin in the presence of 0.1% Triton X-100 and then subjected to SDS-PAGE and immunoblotting with anti-wortmannin antibodies. (b) [³²P]PtdIns-3P formed by GST-p110 α /p85 α (wt) or GST-p110 α ^{R916P}/p85 α (RP) complex in a PI 3-kinase assay.

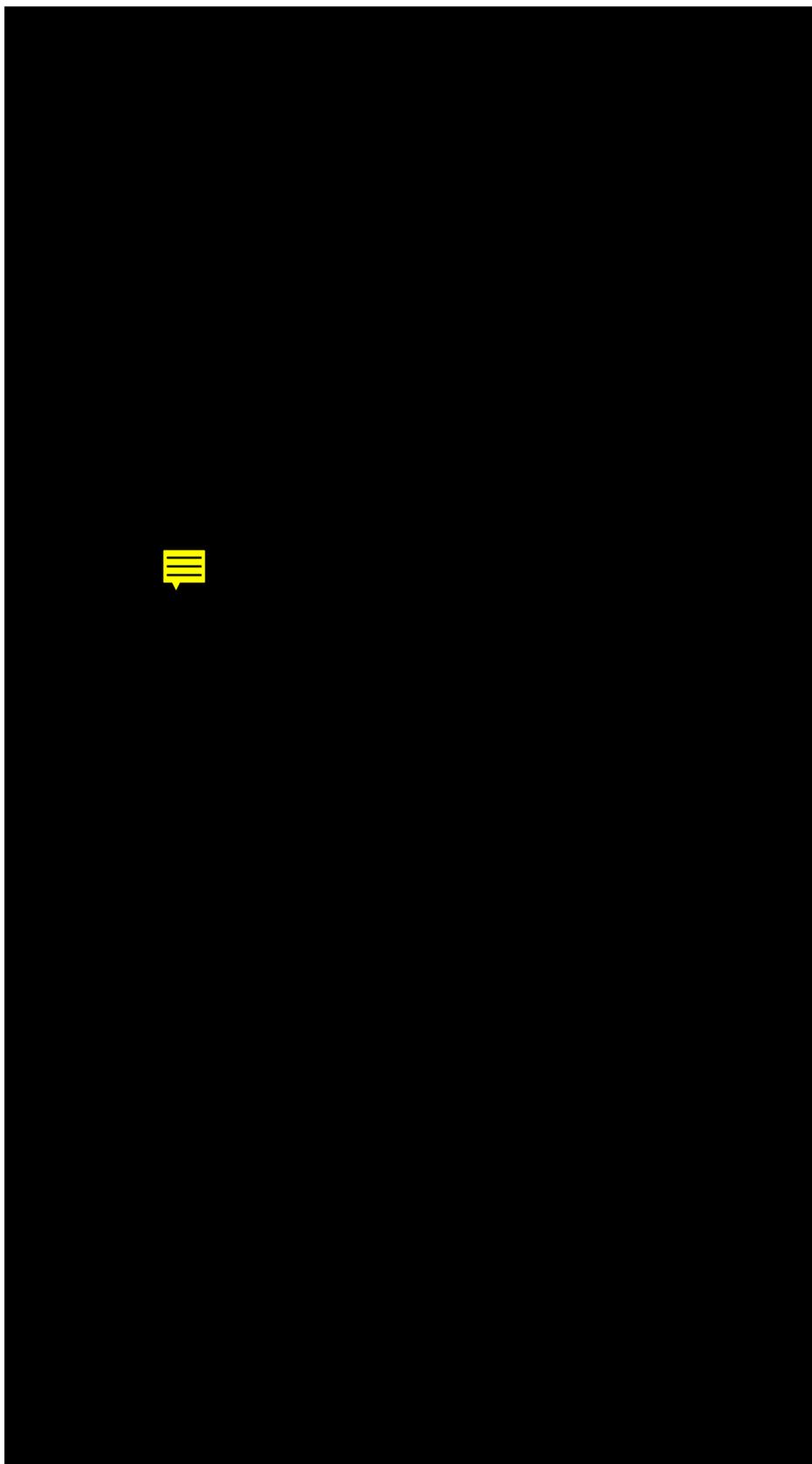


FIG. 9. Modelling of the wortmannin-p110 α complex. (a) Three-dimensional representation of the backbone of p110 α with wortmannin docked into the putative catalytic site. Lys-802 is represented by balls and sticks and is colored blue, while the stabilizing Glu-821 is shown in red. Wortmannin is represented as van der Waals spheres in white. (b) Schematic representation of the wortmannin molecule and protein amino acids that surround it within the binding site. Dashed lines indicate possible hydrogen bonds and/or electrostatic interactions between residues and wortmannin. Half circles indicate hydrophobic interactions.

TABLE 2. Alignment of wortmannin-reactive lysine in p110 α with related proteins^a

Protein ^b	Sequence	End residue	Concn for FSBA/WT ^c
BT p110 α	SAKRPLWLNWENPDIMSELLFQNN II FKNGDDLRLQDMLTLQIIRIME	821	FSBA-/WT= <u>K</u> 802, nM
HS p110 α	SAKRPLWLNWENPDIMSELLFQNN II FKNGDDLRLQDMLTLQIIRIME	821	nM
HS p110 β	KYMDSKMKPLWL V YNNKVF G EDSVGVIFKNGDDLRLQDMLTLQMLRLMD	824	nM
HS p110 γ	SKKKPLWLEFKCADPTALSN-ETIGIIFKNGDDLRLQDMLLILQILRIME	851	nM/B (unpublished data)
HS Vps34	TATLFK S ALMPAQLFFKTEDGGKYPVIFKNGDDLRLQDQLILQIISLMD	655	nM/B (unpublished data)
SC Vps34	TSKVFKSSLSPLKITFKTTLNQP Y HLMFKVGGDDLRLQDQLVVQIISLMD	643	> μ M
AT Vps34	SSLFKSALHPLRLTFRTPEEGRSCKLIFKNGDDLRLQDQLVVQMVWLMMD	583	?
SC TOR2	VFSVSISSKQRPRKFCIKGSDGKDYKYVLKGHEDIRQDSLVMQFLGLVN	2147	?
HS FRAP	SLQVITSKQRPRKLTLMG S NGHEFVFLKGHEDLRQDERVMQFLGLVN	2206	?
RAFT1	SLQVITSKQRPRKLTLMG S NGHEFVFLKGHEDLRQDERVMQFLGLVN	2206	?
HS DNA-PK	RVTVMASLRPRKRIIRGHDEREHPFLVKG G EDLRLQDQRVEQLFQVMN	3771	nM
HS P14K	LRCRSDSEDECESTQEADGQKISWQAAIFKVGDDCRQDMLALQIIDL F K	621	> μ M
SC P14K	DWATKKERIRKTS E YGHFNWDLCSVIAKTGGDLRLQEAFA Y QMIQAMA	815	μ M
MLCK	RLGSGKFGQVFR L VEKK----TGK V WAGKFFKAYS A KEKENIRDEIS I	K = 548	FSBA-K548/ μ M/B
PKA	TLGTGSFGRV M LK H KE----S N HYAMKILDKQ V VK L KQIEHTL N E	K = 72	FSBA-K72
BT p110 α	pyqclsigdcvglievvrnshtimqi q cgkglkgalqf	<u>k</u> = 863	Misalignment

^a Lipid and protein kinase sequences were aligned around Lys-802 (K) of the p110 α catalytic subunit of PI 3-kinase.

^b AT, *Arabidopsis thaliana*; BT, bovine; HS, human; SC, *Saccharomyces cerevisiae*.

^c The concentration range at which wortmannin inhibits the respective activities is indicated. B, covalent wortmannin binding was observed (for references, see the text. FSBA-X and WT=X denote identified reaction sites of kinases with either FSBA or wortmannin.

PI 3-kinase, PtdIns(4,5)P₂ and ATP, both prevented the reaction of wortmannin with p110 α . The kinase motifs DXHXXN and DFG are conserved in p110 α (26). It has been shown that point mutations within these motifs destroy the lipid kinase activities of p110 α (15) and Vps34p (57). Because of the preceding glycine-rich region (Gly-837, Gly-842, and Gly-846 in p110 α [Table 2]), Lys-863 was originally aligned by Hiles et al. (26) with Lys-72 of PKA (67). This roughly localized the ATP-binding site within the 842G-DFG935 region of p110 α .

The charged 941KKKKFGYKRER951 stretch of p110 α resembles the K(X)_nKXXX (n = 3 to 7) motif that was found to bind PtdIns(4,5)P₂ in gelsolin (86) and might therefore constitute the binding site for the 4,5-phosphates of the lipid. The fact that PtdIns(4,5)P₂ but not PtdIns, PS, or detergent diminished the wortmannin-p110 interaction may indicate that the concentrated positive charges somehow participate in the non-covalent binding of wortmannin. The competitors in this experiment, however, were present in mixed micelles and vesicles. Since it was previously observed that the physical properties of lipids can influence their effects on lipid-binding proteins (e.g., on gelsolin [28]), these results must be viewed with caution. Together, these results define a minimal putative 13-kDa region for noncovalent wortmannin-binding overlapping with the ATP- and PtdIns(4,5)P₂-binding sites from approximately G-824 to R-951.

To evaluate the putative reaction sites within the target region defined by proteolytic digests (Lys-802 within the wortmannin-labelled R-777 to K-863 peptide [see Results and Fig. 5]), we used site-directed mutagenesis to exchange putative wortmannin-reactive lysines. The aim of these manipulations was to leave the positive charges in place while reducing the nucleophilicity of the respective side chains. Lysines were therefore replaced by arginines (except for the K733H mutation). Because arginines are always protonated under physiologic conditions and histidines are secondary amines, both are weak nucleophiles. The observation that the exchange of Lys-733, Lys-776, Lys-863, and Lys-867 had no consequences and only the KR3 mutant (K802R) was no longer able to bind wortmannin covalently confirmed the interpretation of the peptide and substrate competition data. This is also in agreement with the fact that Lys-C digestion of the wortmannin-preincubated KR2 PI 3-kinase led to accumulation of the 15-

kDa wortmannin-labelled peptides while the 7-kDa peptides were no longer present (data not shown).

By analogy to chemical reactions performed to produce wortmannin derivatives (21), the ϵ -amino group of Lys-802 attacks C-20 of wortmannin, leading to the opening of the furan ring and the formation of an enamine. The enamine is in equilibrium with a Schiff base, which is relatively stable at physiological pH but is easily hydrolyzed under acidic conditions and can be reduced by NaCNBH₃ in its imine form. A similar reaction mechanism is likely to be found for demethoxyviridin, another inhibitor of PI 3-kinase effective at nanomolar concentrations (5, 81), because the substance contains an identical furan ring system to that in wortmannin.

The covalent character of the interaction also explains the low inhibitory concentrations of wortmannin when compared with quercetin derivatives. Quercetin and the more specific LY294002 derivative (59, 73) have no site for nucleophilic attack and were reported to inhibit PtdIns kinases at micromolar concentrations. This is comparable to the effect of wortmannin derivatives with hydrolyzed furane ring structures (3, 83). LY294002 was shown to inhibit PI 3-kinase competitively with respect to ATP (73), while wortmannin was classified as a noncompetitive inhibitor (44). However, as we show here, wortmannin does react covalently within the ATP-binding site and thus produces the characteristics of an apparent noncovalent inhibitor by reducing the amount of functional enzyme and the apparent V_{max} while the K_m of unreacted PI 3-kinase remains unchanged.

FSBA was previously used to map the ATP-binding sites of various proteins, including PKA and smooth muscle myosin light-chain kinase (MLCK). The isolation of sulfonated peptides led to the identification of the FSBA-reactive residues, which are Lys-72 in PKA (88) and Lys-548 in MLCK (34). Of the three KR mutants produced in insect cells, only KR3 did not react with FSBA, whereas the K776R, K763R and K767R replacements had no effect. Although we obtained nonspecific FSBA staining of p85, the differential staining of the p110 subunits indicates that K-802 is the residue modified by FSBA.

The sulfonated residues in PKA and MLCK were shown to play crucial roles in the phosphotransfer reaction (39, 67), and both enzymes were irreversibly inhibited by FSBA. When wt and KR mutant PI 3-kinase were tested for lipid and kinase

activity, wt, KR1, KR2, and KR4 phosphorylated PtdIns, as well as the coexpressed p85 regulatory subunit. KR3, on the other hand, was inactive in both assays. Taken together, our results demonstrate that K-802 has a similar function to K-72 in PKA, which was proposed to interact with the α - and β -phosphate groups of ATP (39).

An alignment of PtdIns 3-kinases, TORs, DNA-PK_{cs}, PtdIns 4-kinases, and two protein kinases around the Lys-802-p110 α conserved region illustrates that the identified lipid kinases are devoid of a typical P-loop (Table 2) (for a review of protein kinase motifs, see reference 54). The TOR genes—without an attributed activity so far—contain as the only members of this family two conserved glycine residues, which might constitute a degenerated P-loop.

On the basis of the alignments in Table 2, it is likely that K-548 of MLCK will turn out to be the wortmannin-binding residue, because it has been verified that MLCK is irreversibly inhibited by micromolar concentrations of wortmannin (42). PtdIns 4-kinase is inactivated by FSBA, and it has been proposed that the unknown sulfonylated residue is a lysine (56). Lys-602 seems to be the most likely candidate at present.

Reflections about the reaction sequence of wortmannin and FSBA with the catalytic center of kinases may yield some general predictions: both substances associate noncovalently with the substrate-binding site of the respective kinase in a first step. Once the inhibitors are held in place by noncovalent interactions, the high local concentrations of the reaction partners (e.g., Lys-802 and wortmannin) drive the reaction immediately towards the covalent conjugate. While the first step depends on the dissociation constant of the inhibitor, the on-rate of the covalent reaction is dictated by the nucleophilicity of the attacking lysine residue. Because this residue is present in all lipid kinases and most protein kinases, differences and specificities in wortmannin reactivity must be sought at the level of noncovalent interactions. This is supported by the differential reactivity of yeast and human Vps34p with wortmannin, because both enzymes catalyze identical reactions and contain the K-802-equivalent residue.

PI 3-kinase, rendered nonfunctional by the R916P mutation within the DXHXXN motif, still could bind wortmannin covalently, although less efficiently than wild-type kinase did. Wortmannin also reacted with PI 3-kinase in the presence of inactivating concentrations of detergent or with PI 3-kinase inactivated by the prephosphorylation of p85 by the intrinsic protein kinase activity of p110. Together, these results indicate that kinase activity and intact substrate binding are not obligatory for a covalent reaction of wortmannin, although the latter is optimal if the ATP-binding site is intact. As wortmannin and FSBA both bind within the ATP-binding site of the prephosphorylated p85/p110 complex, one might assume that the phosphorylation of Ser-608 on p85 α (15)—deactivating the PI 3-kinase complex—blocks lipid binding rather than access of ATP to the catalytic site. A contribution of p85 in the binding of PtdIns(4,5)P₂ was suggested previously (16).

The identified covalent interaction site and the proposed reaction mechanism presented here explain the highly efficient inhibition of PtdIns 3-kinases by wortmannin, because the inhibitor not only blocks substrate binding but also alkylates a Lys residue, whose nucleophilicity is crucial for the catalytic process. Wortmannin can be predicted to react at high concentrations with known or not yet identified protein and lipid kinases if they show homologies within the PI 3-kinase Lys-802 region and contain classical kinase domains (like DXHXXN and DFG [e.g., MLCK]). The presence of lipid-binding sites and an acid residue corresponding to Glu-821 could increase the sensitivity to wortmannin, because the former expose hy-

drophobic surfaces for wortmannin interaction and the latter might increase the nucleophilicity of the Lys-802 homolog residue. The present data contribute to the correct interpretation of data obtained with the inhibitor with respect to PtdIns 3-kinase involvement in various cell responses. When wortmannin is used, the covalent nature of the reaction has to be recalled and the reaction temperature, buffer composition, and incubation time must be considered. As shown above, nonspecific reactions are to be expected at pHs above 8, when the nucleophilicity of lysines is dramatically increased by deprotonation. As for all covalent reactions, IC₅₀s are misleading if they are not strictly used comparatively and were not obtained under identical conditions. Although wortmannin inhibits PI 3-kinases at very low concentrations, careful controls are essential to rule out cross-interferences with other signalling pathways.

The model of the p110 α -wortmannin complex that we have derived is certainly speculative and would have to be confirmed by crystallography. However, it is in very good agreement with the experimental data presented here and serves as a template for further site-directed mutagenesis studies to test and improve its validity. The localization and modelling of the wortmannin-binding site provide the basis for ongoing experimental approaches exploring the noncovalent wortmannin interaction within the catalytic cleft of PI 3-kinase. This information might lead to the design of subunit-specific inhibitors for PI 3-kinases and related enzymes. Unlike wortmannin, such inhibitors might be able to inhibit one specific member of the PI 3-kinase family while others function normally, be it for research or therapeutic uses.

ACKNOWLEDGMENTS

We thank R. Dhand for helpful discussions and technical advice with the baculovirus expression system, S. Rusconi for the donation of expression vectors, I. Gout for anti-p85 monoclonal antibodies, T. G. Payne for wortmannin and derivatives, P. Burn for peptide synthesis, and Akunna Akpan for excellent technical assistance.

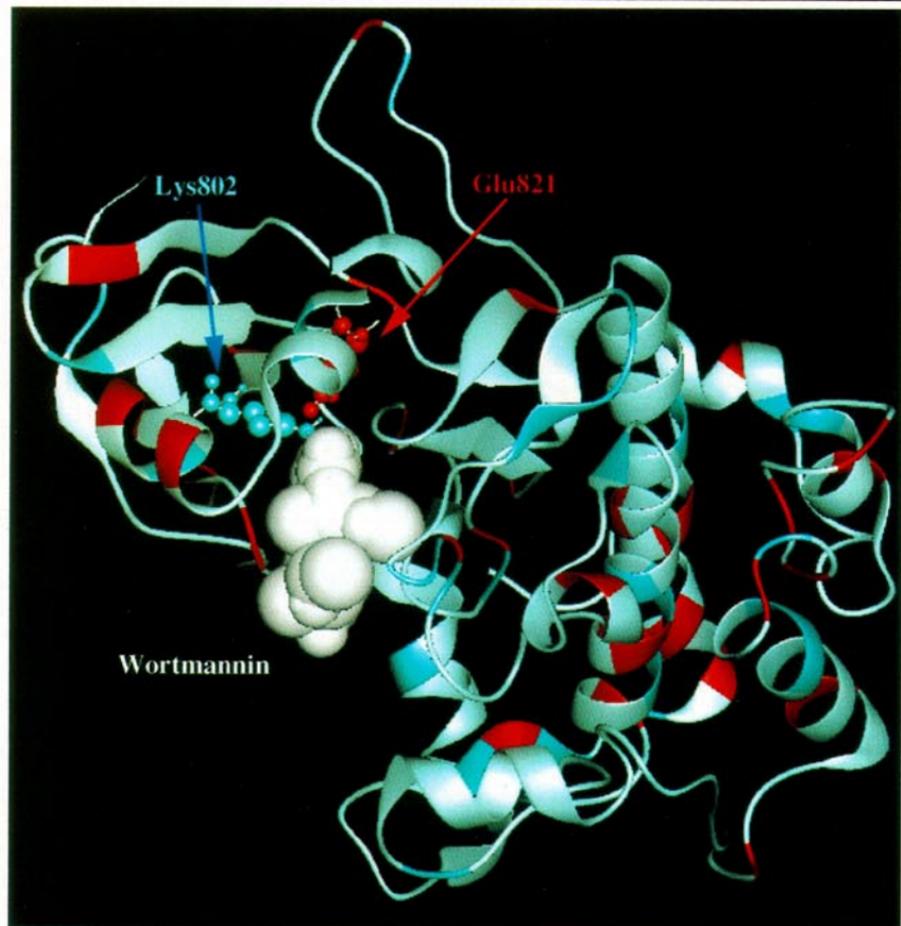
This project was supported by the Swiss National Science Foundation grant 31-40745.94. M.P.W. was a recipient of a British/Swiss Joint Research Programme travel stipend.

REFERENCES

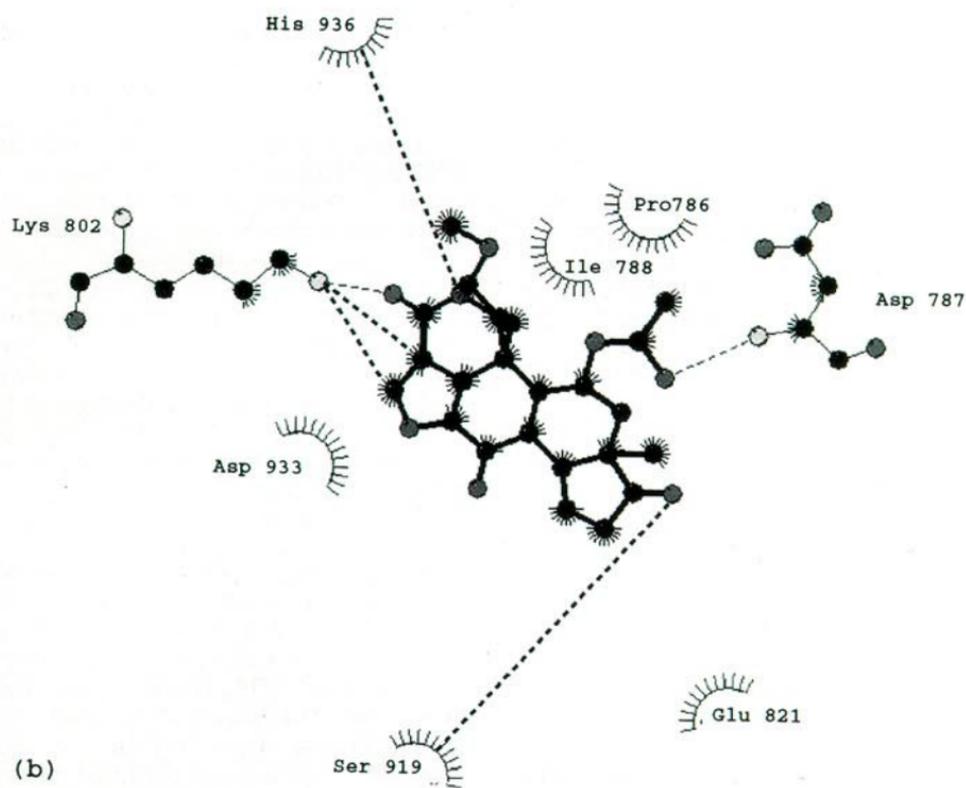
1. Arcaro, A., and M. P. Wymann. 1993. Wortmannin is a potent phosphatidylinositol 3-kinase inhibitor: the role of phosphatidylinositol 3,4,5-trisphosphate in neutrophil responses. *Biochem. J.* **296**:297–301.
2. Backer, J. M., M. G. Myers, Jr., S. E. Shoelson, D. J. Chin, X. J. Sun, M. Miralpeix, P. Hu, B. Margolis, E. Y. Skolnik, and J. Schlessinger. 1992. Phosphatidylinositol 3'-kinase is activated by association with IRS-1 during insulin stimulation. *EMBO J.* **11**:3469–3479.
3. Baggiolini, M., B. Dewald, J. Schnyder, W. Ruch, P. H. Cooper, and T. G. Payne. 1987. Inhibition of the phagocytosis-induced respiratory burst by the fungal metabolite wortmannin and some analogues. *Exp. Cell Res.* **169**:408–418.
4. Barton, G. J., and M. J. Sternberg. 1987. A strategy for the rapid multiple alignment of protein sequences: confidence levels from tertiary structure comparisons. *J. Mol. Biol.* **198**:327–337.
5. Bonser, R. W., N. T. Thompson, R. W. Randall, J. E. Tateson, G. D. Spacey, H. F. Hodson, and L. G. Garland. 1991. Demethoxyviridin and wortmannin block phospholipase C and D activation in the human neutrophil. *Br. J. Pharmacol.* **103**:1237–1241.
6. Brown, E. J., M. W. Albers, T. B. Shin, K. Ichikawa, C. T. Keith, W. S. Lane, and S. L. Schreiber. 1994. A mammalian protein targeted by G1-arresting rapamycin-receptor complex. *Nature (London)* **369**:756–758.
7. Brown, W. J., D. B. DeWald, S. D. Emr, H. Plutner, and W. E. Balch. 1995. Role for phosphatidylinositol 3-kinase in the sorting and transport of newly synthesized lysosomal enzymes in mammalian cells. *J. Cell Biol.* **130**:781–796.
8. Burgering, B. M. T., and P. J. Coffer. 1995. Protein kinase B (c-Akt) in phosphatidylinositol-3-OH kinase signal transduction. *Nature (London)* **376**:599–603.
9. Chuang, T.-H., B. P. Bohl, and G. M. Bokoch. 1993. Biologically active lipids are regulators of Rac-GDI complexation. *J. Biol. Chem.* **268**:26206–26211.

10. Chung, J., T. C. Grammer, K. P. Lemon, A. Kazlauskas, and J. Blenis. 1994. PDGF- and insulin-dependent pp70^{S6k} activation mediated by phosphatidylinositol-3-OH kinase. *Nature (London)* **370**:71–75.
11. Claesson-Welsh, L. 1994. Platelet-derived growth factor receptor signals. *J. Biol. Chem.* **269**:32023–32026.
12. Cross, D. A., D. R. Alessi, J. R. Vandenhede, H. E. McDowell, H. S. Hundal, and P. Cohen. 1994. The inhibition of glycogen synthase kinase-3 by insulin or insulin-like growth factor 1 in the rat skeletal muscle cell line L6 is blocked by wortmannin, but not by rapamycin: evidence that wortmannin blocks activation of the mitogen-activated protein kinase pathway in L6 cells between Ras and Raf. *Biochem. J.* **303**:21–26.
13. Davidson, H. W. 1995. Wortmannin causes mistargeting of procathepsin D. Evidence for the involvement of phosphatidylinositol 3-kinase in vesicular transport to lysosomes. *J. Cell Biol.* **130**:797–805.
14. Dhand, R., K. Hara, I. Hiles, B. Bax, I. Gout, G. Panayotou, M. J. Fry, K. Yonezawa, M. Kasuga, and M. D. Waterfield. 1994. PI 3-kinase: structural and functional analysis of intersubunit interactions. *EMBO J.* **13**:511–521.
15. Dhand, R., I. Hiles, G. Panayotou, S. Roche, M. J. Fry, I. Gout, N. F. Totty, O. Truong, P. Vicendo, K. Yonezawa, M. Kasuga, S. A. Courtneidge, and M. D. Waterfield. 1994. PI 3-kinase is a dual specific enzyme: autoregulation by an intrinsic protein serine-kinase activity. *EMBO J.* **13**:522–533.
16. End, P., I. Gout, M. J. Fry, G. Panayotou, R. Dhand, K. Yonezawa, M. Kasuga, and M. Waterfield. 1993. A biosensor approach to probe the structure and function of the p85 α subunit of the phosphatidylinositol 3-kinase complex. *J. Biol. Chem.* **268**:10066–10075.
17. Escobedo, J. A., S. Navankasattusas, W. M. Kavanaugh, D. Milfay, V. A. Fried, and L. T. Williams. 1991. cDNA cloning of a novel 85 kD protein that has SH2 domains and regulates binding of PI3-kinase to the PDGF β -receptor. *Cell* **65**:75–82.
18. Franke, T. F., S.-I. Yang, T. O. Chan, K. Datta, A. Kazlauskas, D. K. Morrison, D. R. Kaplan, and P. N. Tsichlis. 1995. The protein kinase encoded by the *Akt* proto-oncogene is a target of the PDGF-activated phosphatidylinositol 3-kinase. *Cell* **81**:727–736.
19. Fry, M. J., G. Panayotou, R. Dhand, F. Ruiz Larrea, I. Gout, O. Nguyen, S. A. Courtneidge, and M. D. Waterfield. 1992. Purification and characterization of a phosphatidylinositol 3-kinase complex from bovine brain by using phosphopeptide affinity columns. *Biochem. J.* **288**:383–393.
20. Gelas, P., V. von Tscharnner, M. Record, M. Baggiolini, and H. Chap. 1992. Human neutrophil phospholipase D activation by N-formylmethionyl-leucylphenylalanine reveals a two-step process for the control of phosphatidylcholine breakdown and oxidative burst. *Biochem. J.* **287**:67–72.
21. Haefliger, W., and D. Hauser. 1975. Einführung der Corticoid-Seitenkette bei Wortmannin. *Helv. Chim. Acta* **58**:1629–1633.
22. Hartley, K. O., D. Gell, G. C. M. Smith, H. Zhang, N. Divecha, M. A. Connelly, A. Admon, S. P. Lees-Miller, C. W. Anderson, and S. P. Jackson. 1995. DNA-dependent protein kinase catalytic subunit: a relative of phosphatidylinositol 3-kinase and the ataxia telangiectasia gene product. *Cell* **82**:849–856.
23. Hawkins, P. T., A. Eguinoa, R.-G. Qiu, D. Stokoe, F. T. Cooke, R. Walters, S. Wennström, L. Claesson-Welsh, T. Evans, M. Symons, and L. R. Stephens. 1995. PDGF stimulates an increase in GTP-Rac via activation of phosphoinositide 3-kinase. *Curr. Biol.* **5**:393–403.
24. Hawkins, P. T., T. R. Jackson, and L. R. Stephens. 1992. Platelet-derived growth factor stimulates synthesis of PtdIns(3,4,5)P₃ by activating a PtdIns(4,5)P₂ 3-OH kinase. *Nature (London)* **358**:157–159.
25. Herman, P. K., and S. D. Emr. 1990. Characterization of VPS34, a gene required for vacuolar protein sorting and vacuole segregation in *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* **10**:6742–6754.
26. Hiles, I. D., M. Otsu, S. Volinia, M. J. Fry, I. Gout, R. Dhand, G. Panayotou, F. Ruiz Larrea, A. Thompson, and N. F. Totty. 1992. Phosphatidylinositol 3-kinase: structure and expression of the 110 kd catalytic subunit. *Cell* **70**:419–429.
27. Hu, P., A. Mondino, E. Y. Skolnik, and J. Schlessinger. 1993. Cloning of a novel, ubiquitously expressed human phosphatidylinositol 3-kinase and identification of its binding site on p85. *Mol. Cell. Biol.* **13**:7677–7688.
28. Janmey, P. A., K. Iida, H. L. Yin, and T. P. Stossel. 1987. Polyphosphoinositide micelles and polyphosphoinositide-containing vesicles dissociate endogenous gelsolin-actin complexes and promote actin assembly from the fast-growing end of actin filaments blocked by gelsolin. *J. Biol. Chem.* **262**:12228–12236.
29. Kanai, F., K. Ito, M. Todaka, H. Hayashi, S. Kamohara, K. Ishii, T. Okada, O. Hazeki, M. Ui, and Y. Ebina. 1993. Insulin-stimulated GLUT4 translocation is relevant to the phosphorylation of IRS-1 and the activity of PI3-kinase. *Biochem. Biophys. Res. Commun.* **195**:762–768.
30. Kaplan, D. R., M. Whitman, B. Schaffhausen, D. C. Pallas, M. White, L. C. Cantley, and T. M. Roberts. 1987. Common elements in growth factor stimulation and oncogenic transformation: 85 kd phosphoprotein and phosphatidylinositol kinase activity. *Cell* **50**:1021–1029.
31. Kaufmann, R. J., P. Murtha, D. E. Ingolia, C.-Y. Yeung, and R. E. Kellems. 1989. The phosphorylation state of eucaryotic initiation factor 2 alters translational efficiency of specific mRNAs. *Mol. Cell. Biol.* **9**:946–958.
32. Kazlauskas, A., and J. A. Cooper. 1990. Phosphorylation of the PDGF receptor β subunit creates a tight binding site for phosphatidylinositol 3-kinase. *EMBO J.* **9**:3279–3286.
33. Kimura, K., S. Hattori, Y. Kabuyama, Y. Shizawa, J. Takayanagi, S. Nakamura, S. Toki, Y. Matsuda, K. Onodera, and Y. Fukui. 1994. Neurite outgrowth of PC12 cells is suppressed by wortmannin, a specific inhibitor of phosphatidylinositol 3-kinase. *J. Biol. Chem.* **269**:18961–18967.
34. Komatsu, H., and M. Ikebe. 1993. Affinity labelling of smooth-muscle myosin light-chain kinase with 5'-(p-(fluorosulphonyl)benzoyl)adenosine. *Biochem. J.* **296**:53–58.
35. Kovacsics, T. J., C. Bachelot, A. Toker, C. J. Vlahos, B. Duckworth, L. C. Cantley, and J. H. Hartwig. 1995. Phosphoinositide 3-kinase inhibition spares actin assembly in activating platelets but reverses platelet aggregation. *J. Biol. Chem.* **270**:11358–11366.
36. Kundra, V., J. A. Escobedo, A. Kaslauskas, H. K. Kim, S. G. Rhee, L. T. Williams, and B. R. Zetter. 1994. Regulation of chemotaxis by the platelet-derived growth factor receptor- β . *Nature (London)* **367**:474–476.
37. Kunz, J., R. Henriquez, U. Schneider, M. Deuter Reinhard, N. R. Movva, and M. N. Hall. 1993. Target of rapamycin in yeast, TOR2, is an essential phosphatidylinositol kinase homolog required for G1 progression. *Cell* **73**:585–596.
38. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (London)* **227**:680–685.
39. Madhusudan, E. A. Trafny, N. H. Xuong, J. A. Adams, L. F. Ten Eyck, S. S. Taylor, and J. M. Sowadski. 1994. cAMP-dependent protein kinase: crystallographic insights into substrate recognition and phosphotransfer. *Protein Sci.* **3**:176–187.
40. Nakanishi, H., K. A. Brewer, and J. H. Exton. 1993. Activation of the ζ isozyme of protein kinase C by phosphatidylinositol 3,4,5-trisphosphate. *J. Biol. Chem.* **268**:13–16.
41. Nakanishi, S., K. J. Catt, and T. Balla. 1995. A wortmannin-sensitive phosphatidylinositol 4-kinase that regulates hormone-sensitive pools of inositol-phospholipids. *Proc. Natl. Acad. Sci. USA* **92**:5317–5321.
42. Nakanishi, S., S. Kakita, I. Takahashi, K. Kawahara, E. Tsukuda, T. Sano, K. Yamada, M. Yoshida, H. Kase, Y. Matsuda, Y. Hashimoto, and Y. Nonomura. 1992. Wortmannin, a microbial product inhibitor of myosin light chain kinase. *J. Biol. Chem.* **267**:2157–2163.
43. Okada, T., Y. Kawano, T. Sakakibara, O. Hazeki, and M. Ui. 1994. Essential role of phosphatidylinositol 3-kinase in insulin-induced glucose transport and antilipolysis in rat adipocytes—studies with a selective inhibitor wortmannin. *J. Biol. Chem.* **269**:3568–3573.
44. Okada, T., L. Sakuma, Y. Fukui, O. Hazeki, and M. Ui. 1994. Blockage of chemotactic peptide-induced stimulation of neutrophils by wortmannin as a result of selective inhibition of phosphatidylinositol 3-kinase. *J. Biol. Chem.* **269**:3563–3567.
45. O'Reilly, D. R., L. K. Miller, and V. A. Luckow. 1992. Baculovirus expression vectors—a laboratory manual. W. H. Freeman & Co., New York.
46. Otsu, M., I. D. Hiles, I. Gout, M. J. Fry, F. Ruiz-Larrea, G. Panayotou, A. Thompson, R. Dhand, J. Hsuan, N. Totty, A. D. Smith, S. J. Morgan, S. A. Courtneidge, P. J. Parker, and M. D. Waterfield. 1991. Characterisation of two 85 kd proteins that associate with receptor tyrosine kinases, middle-T/pp60^{src} complexes, and PI3-kinase. *Cell* **65**:91–104.
47. Page, M. J. 1989. p36C: an improved baculovirus expression vector for producing high levels of mature recombinant proteins. *Nucleic Acids Res.* **17**:454.
48. Panayotou, G., B. Bax, I. Gout, M. Federwisch, B. Wroblewski, R. Dhand, M. J. Fry, T. L. Blundell, A. Wollmer, and M. D. Waterfield. 1992. Interaction of the p85-subunit of PI 3-kinase and its N-terminal SH2-domain with a PDGF receptor phosphorylation site—structural features and analysis of conformational changes. *EMBO J.* **11**:4261–4272.
49. Reinhold, S. L., S. M. Prescott, G. A. Zimmerman, and T. M. McIntyre. 1990. Activation of human neutrophil phospholipase D by three separable mechanisms. *FASEB J.* **4**:208–214.
50. Rusconi, S., Y. Severne, O. Georgiev, I. Galli, and S. A. Wieland. 1990. A novel expression assay to study transcriptional activators. *Gene* **89**:211–221.
51. Sabatini, D. M., H. Erdjument-Bromage, M. Lui, P. Tempst, and S. H. Snyder. 1994. RAFT: A mammalian protein that binds to FKBP12 in a rapamycin-dependent fashion and is homologous to yeast TORs. *Cell* **78**:35–43.
52. Sabers, C. J., M. M. Martin, G. J. Brunn, J. M. Williams, F. J. Dumont, G. Wiederrecht, and R. T. Abraham. 1995. Isolation of a protein target of the FKBP12-rapamycin complex in mammalian cells. *J. Biol. Chem.* **270**:815–822.
53. Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. *Molecular cloning: a laboratory manual*, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
54. Saraste, M., P. R. Sibbald, and A. Wittinghofer. 1990. The P-loop—a common motif in ATP- and GTP-binding proteins. *Trends Biochem. Sci.* **15**:430–434.
55. Schägger, H., and G. Von Jagow. 1987. Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range of 1 to 100 kDa. *Anal. Biochem.* **166**:368–379.
56. Scholz, G., G. J. Barritt, and F. Kwok. 1992. Affinity labelling of the active

- site of brain phosphatidylinositol 4-kinase with 5'-fluorosulphonylbenzoyl-adenosine. *Eur. J. Biochem.* **210**:461-466.
57. **Schu, P. V., K. Takegawa, M. J. Fry, J. H. Stack, M. D. Waterfield, and S. D. Emr.** 1993. Phosphatidylinositol 3-kinase encoded by yeast *VPS34* gene essential for protein sorting. *Science* **260**:88-91.
 58. **Serunian, L. A., M. T. Haber, T. Fukui, J. W. Kim, S. G. Rhee, J. M. Lowenstein, and L. C. Cantley.** 1989. Polyphosphoinositides produced by phosphatidylinositol 3-kinase are poor substrates for phospholipases C from rat liver and bovine brain. *J. Biol. Chem.* **269**:31552-31562.
 59. **Shibasaki, F., Y. Fukui, and T. Takenawa.** 1993. Different properties of monomer and heterodimer forms of phosphatidylinositol 3-kinases. *Biochem. J.* **289**:227-231.
 60. **Skolnik, E. Y., B. Margolis, M. Mohammadi, E. Lowenstein, R. Fischer, A. Drepps, A. Ullrich, and J. Schlessinger.** 1991. Cloning of PI3 kinase-associated p85 utilizing a novel method for expression/cloning of target proteins for receptor tyrosine kinases. *Cell* **65**:83-90.
 61. **Stack, J. H., D. B. DeWald, K. Takegawa, and S. D. Emr.** 1995. Vesicle-mediated protein transport: regulation of interactions between the Vps15 protein kinase and the Vps34 PtdIns 3-kinase essential for protein sorting to the vacuole in yeast. *J. Cell Biol.* **129**:321-334.
 62. **Stephens, L., A. Smrcka, F. T. Cooke, T. R. Jackson, P. C. Sternweis, and P. T. Hawkins.** 1994. A novel phosphoinositide 3-kinase activity in myeloid-derived cells is activated by G protein $\beta\gamma$ subunits. *Cell* **77**:83-93.
 63. **Stephens, L. R., K. T. Hughes, and R. F. Irvine.** 1991. Pathway of phosphatidylinositol(3,4,5)-trisphosphate synthesis in activated neutrophils. *Nature (London)* **351**:33-39.
 64. **Sternberg, M. J., and M. J. Zvelebil.** 1990. Prediction of protein structure from sequences. *Eur. J. Cancer* **26**:1163-1166.
 65. **Stoyanov, B., S. Volinia, T. Hanck, I. Rubio, M. Loubtchenkov, D. Malek, S. Stoyanova, B. Vanhaesebroeck, R. Dhand, B. Nürnberg, P. Gierschik, K. Seedorf, J. J. Hsuan, M. D. Waterfield, and R. Wetzker.** 1995. Cloning and characterization of a G protein-activated human phosphatidylinositol-3 kinase. *Science* **269**:690-693.
 66. **Summers, N. L., and M. Karplus.** 1989. Construction of side chains in homology modelling. Application to the C-terminal lobe of rhizopuspepsin. *J. Mol. Biol.* **210**:785-811.
 67. **Taylor, S. S., D. R. Knighton, J. Zheng, L. F. Ten Eyck, and J. M. Sowadski.** 1992. Structural framework for the protein kinase family. *Annu. Rev. Cell Biol.* **8**:429-462.
 68. **Thelen, M., M. P. Wymann, and H. Langen.** 1994. Wortmannin binds specifically to phosphatidylinositol 3-kinase while inhibiting G protein coupled receptor signalling in neutrophil leukocytes. *Proc. Natl. Acad. Sci. USA* **91**:4960-4964.
 69. **Toker, A., M. Meyer, K. K. Reddy, J. R. Falck, R. Aneja, S. Aneja, A. Parra, D. J. Burns, L. M. Ballas, and L. C. Cantley.** 1994. Activation of protein kinase C family members by the novel polyphosphoinositides PtdIns-3,4-P2 and PtdIns-3,4,5-P3. *J. Biol. Chem.* **269**:32358-32367.
 70. **Towbin, H., T. Staehelin, and J. Gordon.** 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. USA* **76**:4350-4354.
 71. **Traynor-Kaplan, A. E., A. L. Harris, B. Thompson, P. Taylor, and L. A. Sklar.** 1988. An inositol tetrakisphosphate-containing phospholipid in activated neutrophils. *Nature (London)* **334**:353-356.
 72. **Ui, M., T. Okada, K. Hazeki, and O. Hazeki.** 1995. Wortmannin as a unique probe for an intracellular signalling protein, phosphoinositide 3-kinase. *Trends Biochem. Sci.* **20**:303-307.
 73. **Vlahos, C. J., W. F. Matter, K. Y. Hui, and R. F. Brown.** 1994. A specific inhibitor of phosphatidylinositol 3-kinase, 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one (LY294002). *J. Biol. Chem.* **269**:5241-5248.
 74. **Volina, S., R. Dhand, B. Vanhaesebroeck, L. K. MacDougall, R. Stein, M. J. Zvelebil, J. Domin, C. Panaretou, and M. D. Waterfield.** 1995. A human phosphatidylinositol 3-kinase complex related to the yeast Vps34p-Vps15p protein sorting system. *EMBO J.* **14**:3339-3348.
 75. **Volina, S., I. D. Hiles, E. Ormondroyd, D. Nizetic, R. Antonacci, M. Rocchi, and M. D. Waterfield.** 1994. Molecular cloning, cDNA sequence, and chromosomal localization of the human phosphatidylinositol 3-kinase p110 α (PIK3CA) gene. *Genomics* **24**:472-477.
 76. **Welsh, G. L., E. J. Foulstone, S. W. Young, J. M. Tavaré, and C. G. Proud.** 1994. Wortmannin inhibits the effects of insulin and serum on the activities of glycogen synthase kinase-3 and mitogen-activated protein kinase. *Biochem. J.* **303**:15-20.
 77. **Wennström, S., P. T. Hawkins, F. Cooke, K. Hara, K. Yonezawa, M. Kasuga, T. Jackson, L. Claesson-Welsh, and L. Stephens.** 1994. Activation of phosphoinositide 3-kinase is required for PDGF-stimulated membrane ruffling. *Curr. Biol.* **4**:385-393.
 78. **Wennström, S., A. Siegbahn, K. Yokote, A.-K. Arvidsson, C.-H. Heldin, S. Mori, and L. Claesson-Welsh.** 1994. Membrane ruffling and chemotaxis transduced by the PDGF β -receptor require the binding site for phosphatidylinositol 3' kinase. *Oncogene* **9**:651-660.
 79. **Wessel, D., and U. I. Flügge.** 1984. A method for the quantitative recovery of protein in dilute solution in the presence of detergents and lipids. *Anal. Biochem.* **138**:141-143.
 80. **White, M. F., and C. R. Kahn.** 1994. The insulin signaling system. *J. Biol. Chem.* **269**:1-4.
 81. **Woscholski, R., T. Kodaki, M. McKinnon, M. D. Waterfield, and P. J. Parker.** 1994. A comparison of demethoxyviridin and wortmannin as inhibitors of phosphatidylinositol 3-kinase. *FEBS Lett.* **342**:109-114.
 82. **Wymann, M. P., and A. Arcaro.** 1994. PDGF-induced phosphatidylinositol 3-kinase activation mediates actin rearrangements in fibroblasts. *Biochem. J.* **298**:517-520.
 83. **Yano, H., S. Nakanishi, K. Kimura, N. Hanai, Y. Saitoh, Y. Fukui, Y. Nonomura, and Y. Matsuda.** 1993. Inhibition of histamine secretion by wortmannin through the blockade of phosphatidylinositol 3-kinase in RBL-2H3 cells. *J. Biol. Chem.* **268**:25846-25856.
 84. **Yao, R., and G. M. Cooper.** 1995. Requirement for phosphatidylinositol-3 kinase in the prevention of apoptosis by nerve growth factor. *Science* **267**:2003-2006.
 85. **Yeh, J. I., E. A. Gulve, L. Rameh, and M. J. Birnbaum.** 1995. The effects of wortmannin on rat skeletal muscle. Dissociation of signaling pathways for insulin- and contraction-activated hexose transport. *J. Biol. Chem.* **270**:2107-2111.
 86. **Yu, F.-X., H.-Q. Sun, P. A. Janmey, and H. L. Yin.** 1992. Identification of a phosphoinositide-binding sequence in an actin monomer-binding domain of gelsolin. *J. Biol. Chem.* **267**:14616-14621.
 87. **Zakian, V. A.** 1995. *ATM*-related genes: what do they tell us about functions of the human Gene? *Cell* **82**:685-687.
 88. **Zoller, M. J., N. C. Nelson, and S. S. Taylor.** 1989. Affinity labeling of cAMP-dependent protein kinase with p-fluorosulfonylbenzoyl adenosine. *J. Biol. Chem.* **256**:10837-10842.
 89. **Zvelebil, M. J., G. J. Barton, W. R. Taylor, and M. J. Sternberg.** 1987. Prediction of protein secondary structure and active sites using the alignment of homologous sequences. *J. Mol. Biol.* **195**:957-961.



(a)



(b)