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Form and flexibility in phosphoinositide 3-kinases

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Abstract

PI3Ks (phosphoinositide 3-kinases) have important roles in a variety of cellular activities, including survival, proliferation, growth, shape, migration and intracellular sorting. Consistent with their function in cell survival and growth, the gene for the class Iα PI3K catalytic subunit is a common site of gain-of-function mutations in cancers. Ongoing structural studies of these enzymes and the complexes they make with their regulatory subunits have helped to clarify the mechanistic basis of this role in tumour development. The broad spectrum of biological activities associated with various isotypes of class I PI3Ks has led to an intense search for isotype-specific inhibitors as tools in mammalian cell biology and for therapeutic application. Structural studies of the class I PI3Ks suggest that flexibility may be a component of the catalytic cycle of the enzymes.

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Roger Williams

Introduction

A variety of enzymes can modify the landscape of lipids present in membranes. Prominent members include the PI3Ks (phosphoinositide 3-kinases), a family of lipid kinases that generate inositol lipids phosphorylated at the D3 hydroxy group. These 3-phosphoinositides typically carry out their signalling role by membrane recruitment of effectors having phosphoinositide-recognition modules such as the FYVE, (Fab1p, YOTB, Vac1p, EEA1)/PH (pleckstrin homology)/GRAM (glucosyltransferases, Rab-like GTPase activators and myotubularins)/GLUE (GRAM-like ubiquitin-binding in EAP45) and PX (Phox homology) domains. Because of their roles in generating second messengers in many cell signalling processes, including the RTK (receptor tyrosine kinase) and GPCR (G-protein-coupled receptor) pathways, and their roles in intracellular traffic, these enzymes have been the focus of intense study. Several PI3K inhibitors are now in pre-clinical and clinical trials as possible therapeutic agents in a variety of diseases, such as cancer, rheumatoid arthritis and asthma. PI3Ks can be divided into three classes on the basis of their domain organizations. The class I enzymes have five domains, an N-terminal ABD (adaptor-binding domain), an RBD (Ras-binding domain), a C2 domain, a helical domain and a C-terminal catalytic domain [1]. The C2, helical and catalytic domains are shared by all three classes of PI3K. In the present review, we examine some recent developments in the field of PI3Ks in the light of structural work on these enzymes that may help to decipher mechanisms of both activation and inhibition of these potentially important pharmacological targets.

Class I PI3Ks

Class I PI3K activity is associated with tumorigenesis, cell proliferation, growth and survival. These enzymes are major players in intracellular signalling networks downstream of both RTKs and GPCRs [2,3]. *In vitro*, class I enzymes can use PtdIns, PtdIns4*P*, and PtdIns(4,5)P_2 as substrates, but *in vivo*, the substrate is PtdIns(4,5)P_2 [4]. PI3Ks of this class form heterodimers containing one of four related p110 catalytic subunits, p110α, p110β, p110δ or p110γ, and a regulatory subunit. The p110α isotype is primarily responsible for insulin-dependent signalling [5]. The p110β isotype has a role in platelet aggregation and thrombosis [6] and sustained insulin signalling [7]. Both the p110δ and

Key words: cancer, p85, p110, phosphoinositide 3-kinase (PI3K), phosphoinositide 3,4,5-trisphosphate.

Abbreviations used: ABD, adaptor-binding domain; AML, acute myeloblastic leukaemia; ATM, ataxia telangiectasia mutated; βARK1, β-adrenergic receptor kinase 1; cSH2, C-terminal Src homology 2 domain; GLUT4, glucose transporter 4; GPCR, G-protein-coupled receptor; GRAM, glucosyltransferases, Rab-like GTPase activators and myotubularins; iSH2, intervening coiled-coil domain between two Src homology 2 domains; LPA, lysophosphatidic acid; mTOR, mammalian target of rapamycin; nSH2, N-terminal Src homology domain; PI3K, phosphoinositide 3-kinase; PTEN, phosphatase and tensin homologue deleted on chromosome 10; PX, Phox homology; RBD, Ras-binding domain; RTK, receptor tyrosine kinase; SH, Src homology; TAC, transverse aortic constriction; UVRAG, UV irradiation resistance-associated gene; Vps, vacuolar protein sorting.

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p110γ isotypes are mainly expressed in leucocytes and have roles in lymphocyte activation [8,9], mast cell degranulation [10] and leucocyte chemotaxis [11–16].

Regulatory subunits

The class IA catalytic subunits, p110α, p110β and p110δ, associate with one of five p85-related regulatory subunits, p85α and its splice variants p55α and p50α, p85β and p55γ. Each of these regulatory subunits is thought to bind to any of the type IA catalytic subunits. The triple effect of p85-related polypeptides on the catalytic subunit is to stabilize it, to inhibit its basal activity and to recruit it to phosphorylated tyrosine residues of RTKs or adaptor proteins such as IRS (insulin receptor substrate) [17]. The full-length p85 subunit has an N-terminal SH (Src homology) 3 domain followed by a BCR (breakpoint cluster region) domain; however, common to all the p85-related variants is that they possess two SH2 domains, nSH2 (N-terminal SH2 domain) and cSH2 (C-terminal SH2 domain), separated by an intervening coiled-coil domain (iSH2). Class IA PI3Ks act downstream of RTKs. Binding of p85 SH2 domains to activated RTKs or their associated adaptors via phosphorylated Tyr-Xaa-Xaa-Met motifs results in elevated lipid kinase activity. The p110β isoform can also signal downstream of GPCRs via association with $G_{\beta\gamma}$; however, the mode of interaction is not clear [18–20]. The iSH2 domain acts as the high-affinity binding site for the p110 ubiquitin-like ABD [21]. Quantitative MS analysis shows that, in cell lines and in tissues, the p110 catalytic and the p85 regulatory subunits are present in equimolar amounts, indicating that p85 and p110 form obligate heterodimers *in vivo*. In NIH 3T3 cells, there are 10000–15000 p85–p110 heterodimers per cell, with the p110 catalytic and p85 regulatory subunits present in equimolar amounts, but the relative abundance of the isotypes of catalytic subunits varies [22].

In contrast with the class IA enzymes, the single class IB isotype, p110γ, associates with p101 [23] or the closely related p84–p87 regulatory subunits [24,25]; neither subunit has a recognizable domain organization [26]. The p101 subunit protects cells from apoptosis when overexpressed, and this may explain why it is a common site for insertion in T-cell lymphomas induced by the MoFe2-MuLV retrovirus [27]. The p101 and p84–p87 subunits facilitate activation of p110γ downstream of GPCRs and enable p110γ membrane recruitment [28–30]. The p101 subunit binds both the p110γ catalytic subunit and the $G_{\beta\gamma}$ heterodimer. In addition to enabling translocation of the catalytic subunit, the regulatory subunit influences substrate specificity of the catalytic subunit. *In vitro* lipid kinase assays show that, in the absence of p101, p110γ converts PtdIns into PtdIns3*P* upon $G_{\beta\gamma}$ stimulation. However, in a complex with p101, PtdIns(4,5)P_2 is the preferred substrate that is converted into PtdIns(3,4,5)P_3, which is in accordance with the substrate preference *in vivo* [20]. The N- and C-terminal regions of p101 have been implicated in binding to p110γ and $G_{\beta\gamma}$ respectively. However, the exact binding sites of both regulatory subunits still remain controversial [26,29]. The N-terminal region of the p110γ subunit (residues 1–333) has been reported to be both necessary and sufficient for p101 binding, but both the N- and C-terminal regions of the catalytic subunit are necessary for activation by LPA (lysophosphatidic acid) stimulation [26]. Similarly to p101, the p84–p87 subunits are expressed in dendritic cells, macrophages and neutrophils; however, they are also highly expressed in heart [25]. The p84–p110γ heterodimer is approximately 4-fold less sensitive to G$\beta\gamma$ than p101/p110γ in terms of lipid kinase activation [24].

Class II PI3Ks

Like their class IA counterparts, class II PI3Ks are characterized by the presence of an RBD/C2/helical/catalytic core, but in addition, they possess the unique class II characteristic C-terminal PX and C2 domains. No regulatory subunit has been identified for these enzymes. In mammals, there are three isoforms of the class II enzymes (PI3K-C2α, PI3K-C2β and PI3K-C2γ), and each has a unique N-terminal region with no known domain organization. In comparison with the class I and class III enzymes, relatively little is known about the class II enzymes. Recently, however, there have been considerable advances in understanding the roles of these enzymes [31]. PI3K-C2α has a role in clathrin-mediated vesicle trafficking [32,33], neurosecretory granular exocytosis [34], smooth muscle cell contraction [35] and insulin signalling, where it contributes to the translocation of GLUT4 (glucose transporter 4) to plasma membranes and subsequent glucose uptake [36–38]. PI3K-C2β regulates cell migration [39–41] and contributes to neuronal cell survival through interaction with the protein intersectin [42]. *In vitro*, these enzymes preferentially use PtdIns as a substrate and only poorly act on PtdIns4*P* and PtdIns(4,5)P_2 [31]. *In vivo*, there are emerging lines of evidence that PtdIns3*P* may be an important product of PI3K-C2α and PI3K-C2β. Metabolic labelling showed that PtdIns3*P*, localized on the plasma membrane, is the sole product of insulin-induced PI3K-C2α activation [36]. PI3K-C2β-dependent production of PtdIns3*P* on plasma membrane upon LPA stimulation has also been observed [39]. There is a growing picture of regulation of kinase activity and membrane targeting. The isoform-specific N-terminal regions of both PI3K-C2α and PI3K-C2β appear to play roles that are analogous to those of a regulatory subunit. Clathrin binds to this region of both PI3K-C2α and PI3K-C2β, resulting in increased kinase activity [32,33,43]. This region of PI3K-C2β can also interact with the adaptors Shc and Grb2 (growth-factor-receptor-bound protein 2), resulting in recruitment of PI3K-C2β to activated EGF (epidermal growth factor) receptor as part of the Eps8–Abi1–Sos1 complex, which activates Rac1 [41]. Activation of PI3K-C2α involves translocation to the plasma membrane and activation by the small G-protein TC10. This forms a part of the mechanism that translocates the insulin receptor GLUT4 to the plasma membrane [36]. Ca^{2+} ions regulate activity of PI3K-C2α *in vitro* and in living neurosecretory cells [32,33]. The C-terminal PX domain of PI3K-C2α specifically binds PtdIns(4,5)P_2 and with higher affinity than the C2 domain, which lacks phosphoinositide specificity [44]; however, their biological roles are still unknown.

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Class III PI3Ks

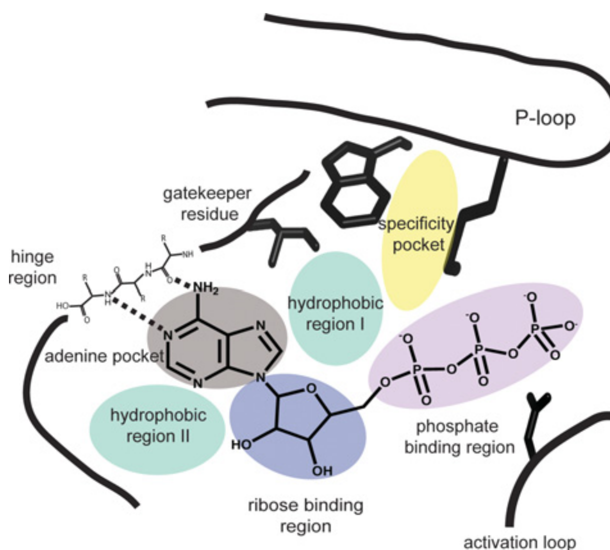
The class III enzymes have only the core C2, helical and catalytic domains. Vps (vacuolar protein sorting) 34 represents the primordial PI3K and there is a gene for it in all eukaryotic organisms [45]. This enzyme uses only PtdIns as a substrate and associates with a putative serine/threonine protein kinase hVps15 (human Vps15)/p150 regulatory subunit. It has an important role in a variety of intracellular trafficking pathways and is essential for vesicular traffic of membrane proteins to the lysosome [46,47]. More recently, mammalian Vps34 has been implicated in the activation of mTOR (mammalian target of rapamycin) [48–51], although the components of the signalling pathway upstream and downstream of Vps34 in this context have not been elucidated. In addition to its role in vacuolar protein sorting, Vps34 is also involved in autophagy. In yeast, these two roles of Vps34 are paralleled by the complexes II and I respectively in which Vps34 is found. The Vps34–Vps15 complex associates with Vps30/Atg6 and Vps38 in the complex II required for endosomal traffic and with Vps30 and Atg14 for the complex I that is essential for autophagy [52]. In mammals, the Vps30 orthologue Beclin1 associates with Atg14/Barkor in an early stage of autophagy [53,54], and with UVRAG (UV irradiation resistance-associated gene) [55] and Bif-1 (Bax-interacting factor-1) in a later stage [56]. UVRAG is an orthologue of Vps38 and also has been proposed to function in endosomal sorting. Recently, UVRAG was shown to interact with the class C Vps HOPS (homotypic fusion and vacuole protein sorting) complex, required for fusion of autophagosomes/endosomes (amphisomes) with lysosomes [57] and has been proposed to function in endosome to *trans*-Golgi network retrograde transport [53].

The active site of class I PI3Ks

All of the PI3Ks have a catalytic domain that shows homology with protein kinases. Like protein kinases, the catalytic domain of PI3Ks exhibits a bilobal organization with the ATP-binding pocket in a cleft between the N- and C-lobes, and PI3Ks share the same conserved catalytic residues in the phosphate-binding subsite of protein kinases [1]. Loop-swapping studies have shown that phosphoinositide-specificity is determined by basic residues in a loop analogous to the activation loop of the protein kinases [58,59]. Unfortunately, despite considerable effort in structural studies of PI3Ks, the details by which they are able to achieve phosphoinositide substrate specificity remain obscure, because it has not been possible to obtain structures in the presence of phosphoinositides or their headgroup analogues.

Nearly two decades of structural studies on protein kinases have shed considerable light on how these enzymes recognize their substrate proteins [60]. Studies of protein kinases in complexes with small-molecule ATP-competitive inhibitors have suggested that conformational changes are often a part of the catalytic cycle of these enzymes. Most protein kinase inhibitors that have been developed target the active conformation of the enzymes so that the inhibitor-bound conformation is very similar to the ATP-bound conformation. These have been referred to as the type I inhibitors [61]. In contrast, a few inhibitors, the type II inhibitors such as imatinib, preferentially stabilize and bind to an inactive conformation of the enzyme [61]. Given that all of the kinases have to recognize ATP in the active conformation, it is not surprising that kinase-specific inhibition can be achieved by taking advantage of accessible protein kinase conformations that are not under selective pressure to recognize the ATP substrate. For example, specific protein kinase inhibition has been achieved by targeting the inactive ‘DFG-out’ conformation (see below) [61]. A pharmacophore model often used to describe binding by the type I protein kinase inhibitors divides the ATP-binding site into the hinge region, the hydrophobic regions I and II, the adenine region, the ribose region and the phosphate region (Figure 1). Inhibitor compounds often have a central scaffold occupying the adenine pocket and form hydrogen bonds with the hinge region, with substituents sampling adjacent hydrophobic regions to gain affinity and selectivity.

Figure 1 | Schematic representation of a canonical PI3K/protein kinase ATP-binding pocket



All PI3K–inhibitor complexes that have been reported are type I inhibitors, occupying the adenine region and making characteristic hydrogen bonds to the hinge of region of the PI3K (Table 1a). A region that has been referred to as hydrophobic region I forms a deeper pocket behind the binding site for the ribose moiety of the ATP. In protein kinases, the ‘gatekeeper’ residue, which forms part of the wall of hydrophobic region I, partially restricts access to this region. Kinases with a large residue at the gatekeeper position cannot accommodate inhibitors with large substituents in hydrophobic region I, whereas kinases with a less bulky gatekeeper can. Among the most common resistance mutations of protein kinases is the mutation of a small gatekeeper residue into a larger more restrictive residue. The residue in PI3Ks analogous to the gatekeeper residue of the protein kinases is

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Table 1 | Definitions of functionally important regions in the p110 subunit of the four isotypes

(a) Catalytic domain regions

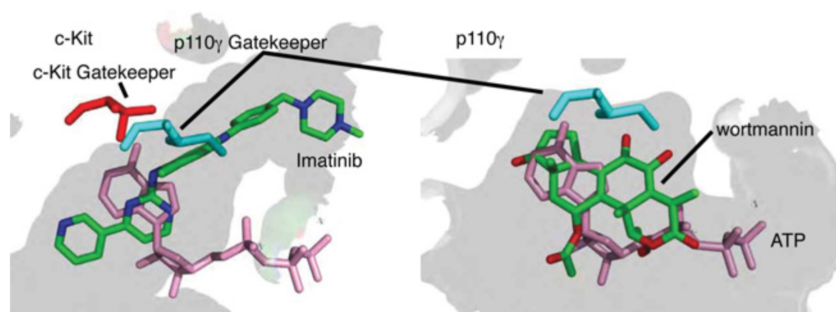
Region	p110α	p110β	p110δ	p110γ
P-loop	771–777	778–784	751–757	803–809
Helix αC	808–827	811–830	785–804	839–858
Hinge	Val^{851}, Glu^{849}	Val^{854}, Glu^{852}	Val^{828}, Glu^{826}	Val^{882}, Glu^{880}
Catalytic loop	909–920	913–924	887–898	940–951
Activation loop	933–958	937–962	911–936	964–989
(DFG motif)	(933–935)	(937–939)	(911–913)	(964–966)
Adenine pocket	Ile^{800}, Tyr^{836}, Phe^{930}, Met^{922}	Ile^{803}, Tyr^{839}, Met^{926}, Phe^{935}	Ile^{777}, Tyr^{813}, Met^{900}, Phe^{908}	Ile^{831}, Tyr^{867}, Met^{953}, Phe^{961}
Gatekeeper	Ile^{848}	Ile^{851}	Ile^{825}	Ile^{879}
Hydrophobic region I (affinity pocket)	Tyr^{836}, Ile^{848}, Ile^{932}, Asp^{810}	Tyr^{839}, Ile^{851}, Ile^{936}, Asp^{813}	Tyr^{813}, Ile^{825}, Ile^{910}, Asp^{787}	Tyr^{867}, Ile^{879}, Ile^{963}, Asp^{841}
Specificity pocket	Met^{772}, Trp^{780}	Met^{779}, Trp^{787}	Met^{752}, Trp^{760}	Met^{804}, Trp^{812}
Ka12 (α) Ka11 (γ)	1032–1047	1034–1049	1008–1023	1061–1076

(b) Residues analogous to cancer-associated somatic mutations of p110α

Region	p110α	p110β	p110δ	p110γ
ABD	Arg^{38}, Arg^{88}	Arg^{48}, Arg^{98}	Arg^{38}, Arg^{88}	–
Helical domain	Glu^{542}, Glu^{545}	Gln^{549}, Glu^{552}	Glu^{522}, Glu^{525}	Pro^{566}, Ala^{569}
Kinase domain	His^{1047}	Leu^{1049}	Leu^{1023}	Arg^{1076}
Kinase domain near ABD mutations	Gln^{738}, Asp^{743}, Asp^{746}	Cys^{745}, Ala^{750}, Glu^{753}	Cys^{718}, Ala^{723}, Glu^{726}	–
C2 contacts to iSH2	Asn^{345}, Asp^{560} and Asn^{564}	Asn^{344}	Asn^{334}	–

Figure 2 | Comparison of the PI3K and c-Kit ATP-binding pockets

A semi-transparent surface rendering of the ATP-binding pockets is shown. The left-hand panel illustrates imatinib bound in the active site of the protein kinase c-Kit. The c-Kit gatekeeper residue is shown as red sticks. The p110γ ATP-binding pocket was superimposed on c-Kit to illustrate the location of the PI3K gatekeeper (cyan sticks) and ATP (pink sticks). The right-hand panel illustrates the p110γ ATP-binding pocket with bound wortmannin. The ATP from the p110γ–ATP complex is shown superimposed for comparison.



an isoleucine residue (Ile^{871} in PI3Kγ), and this residue forms part of the wall of the very small PI3K hydrophobic region I. Many PI3K inhibitors such as pan-specific LY294002 and PI3Kγ-selective AS-604850 occupy this region (Table 1a and Figure 2). This region has also been referred to as the 'affinity pocket' in the PI3Kγ–inhibitor complexes [5].

The type II inhibitors of protein kinases bind to a very different conformation of the active-site pocket from the conformation that binds ATP. One common type of large conformational change found associated with type II inhibitors involves the 'activation loop', which begins with a DFG (Asp-Phe-Gly) motif. This conformational change lifts the phenylalanine residue of the DFG motif to disrupt its interaction with helix αC, thereby creating a new hydrophobic pocket deep behind the ATP-binding site that has been referred to as the 'allosteric site' [61]. This site is occupied by

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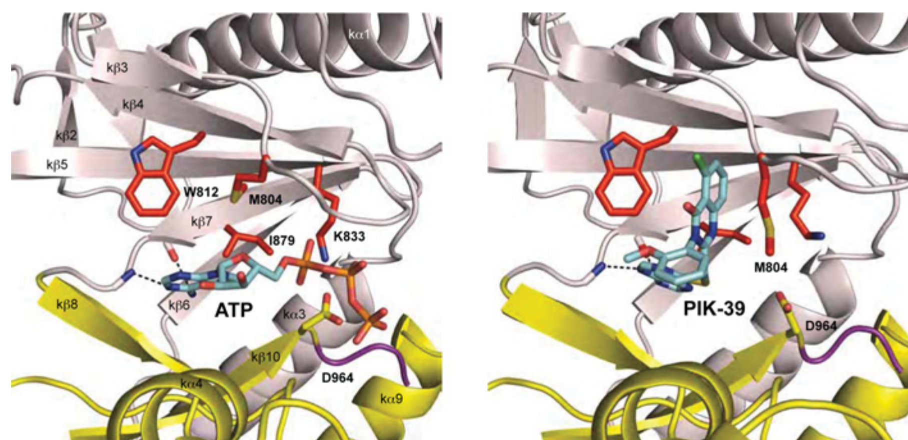
Figure 3 | Comparison of p110γ bound to ATP (left panel) with the p110γ–PIK-39 complex (right)



imatinib in the structure of the Abl kinase–imatinib complex. Although much of the region analogous to the activation loop of protein kinases is disordered in all of the PI3K structures, the DFG motif is ordered, and the observed conformation is essentially very similar to the ATP-bound conformation, so that inhibitors analogous to the type II protein kinase inhibitors have not yet been developed for PI3Ks. However, inhibitor-stabilized conformational change has been observed for one class of PI3Kδ-specific inhibitors (see below). For these inhibitors, a new pocket is formed between the adenine pocket and the P-loop, and has been referred to as the 'specificity pocket' (Figure 1, Table 1a). The transition from a 'DFG-in' to a 'DFG-out' conformation of the protein kinases that bind to type II inhibitors appears to be part of a fundamental regulatory mechanism of some enzymes, in that the inactive conformation appears to be accessible to kinases even in the absence of inhibitor when the enzyme is in its autoinhibited state [62]. It is not yet clear whether this is true of the specificity pocket of the PI3Ks.

Structural insights into isotype specificity of class I PI3K inhibitors

Efforts to develop isotype-specific inhibitors have resulted in inhibitors with some selectivity for p110α [5], p110β [6], p110δ [5,63,64] and p110γ [65,66]. This selectivity is surprising given that they are ATP-competitive inhibitors and that structural studies of the p110γ–LY294002 complex show that the ATP-binding pockets of PI3Ks are completely conserved among the PI3K isotypes [67]. In order to facilitate development of isotype-selective inhibitors, it would be advantageous to have structures of all of the p110 isotypes in complexes with a range of inhibitors of various specificities. This approach has been confounded because only the structure of p110γ has been reported in complexes with ATP or ATP mimetics [1,5,65,68–71]. A structure of p110α in a complex with the iSH2 domain of the p85α subunit was unfortunately of limited use for inhibitor design because the ATP-binding pocket is blocked by a crystal contact [72,73].

On the basis of the structure of p110γ in complexes with the pan-PI3K inhibitors LY294002 and wortmannin as well as with several broad-spectrum protein kinase inhibitors, efforts have been made to model other isotypes of the catalytic subunit and to mechanistically account for isotype specificity of inhibitors [74–76]. The portions of the ATP-binding site where some sequence variation occurs can be divided into four regions [67,74], which are summarized in Table 2. It was proposed that isotype-specificity might be enhanced by taking advantage of these sequence differences [67,74,76]. Using a model of p110δ based on the structure of p110γ along with estimations of the energy of inhibitor–protein interactions, it was proposed that p110δ-specific inhibitors achieve their specificity by interacting with the side chains of residues in regions 1–3. However, a study that examined crystallographic structures of complexes of p110γ with a range of compounds, including some isotype-specific inhibitors, showed an unexpected flexibility-based mechanism of eliciting p110δ-specific inhibition [5]. PIK-39, a p110δ-specific compound (similar to IC87114), causes a conformational change in the ATP-binding pocket. This compound having two large orthogonal ring systems forces the side chain of Met^{804} (p110γ numbering) to rotate away from the bottom of the pocket to create a new hydrophobic pocket between Trp^{812} and Met^{804}. This induced specificity pocket accommodates the quinazolinone moiety of the PIK-39 (Figure 3). Accompanying this shift of the Met^{804} side chain is a shift of the N-terminal lobe of the catalytic domain relative to the C-lobe and conformational changes in the loop encompassing region 1 (see Table 2) (this includes a loop that has been referred to as the 'P-loop' [1]). In contrast, changes in the conformations of residues in regions 2 and 3 were minimal. On the basis of the p110γ crystallographic study, it was proposed that p110δ specificity arises from differences in the flexibility of region 1. In contrast with what had been proposed on the basis of modelling/energy calculations [74], none of the isotype-specific inhibitors interacts directly with the side chains of residues that vary among the class I isoenzymes. Instead, it appears that the

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Table 2 | **Regions near the ATP-binding pocket having sequence variation among the four p110 isotypes**

Residues 802, 805, 886, 887, 890 and 950 are at the periphery of the pocket [67].

Region	P110α	P110β	P110δ	P110γ
1	770–778	797–804	750–758	802–810
2	855–856	858–859	832–833	886–887
3	859	862	836	890
4	919	923	897	950

sequence variations contribute to forming interactions that rigidify the protein, and isotype specificity derives from the relative ease by which inhibitor-induced conformational changes are accommodated by the various isoenzymes. It has been shown that when more rigid β-branched amino acids such as valine and isoleucine are substituted for Met^{804}, the mutants are resistant to inhibition by PIK-39 and IC87114, but they remain sensitive to flat multi-targeted inhibitors [5]. Sequence alignments suggest that the induced selectivity pocket of the PI3Ks may be pre-formed in the PI3K-related enzyme ATM (ataxia telangiectasia mutated), which has an alanine residue at the position analogous to Met^{804}, so that the ATM-selective KU-55933 might project an aromatic substituent into this pre-formed pocket [5].

Oncogenic mutations of p110α and p85α

The gene for p110α, *PI3KCA*, is commonly amplified and mutated in many human cancers (reviewed in [77–80]), and expression of mutated p110α is oncogenic in cell culture [81]. Recently, somatic mutations in *PI3KR1*, the gene that encodes the p85α regulatory subunit, have been described in human glioblastomas [82]. The crystal structure of the p110α–iSH2 complex [72] provides a framework for rationalizing the mechanism of gain-of-function for some of these mutations [82]. It shows how the high-affinity iSH2–ABD interaction that had been reported previously fits into the context of the p110α–iSH2 complex, confirming the model proposed earlier that the rod-like iSH2 domain protrudes through a slot between the catalytic and the C2 domains [21]. The location of the iSH2 domain surface in the p110–iSH2 complex suggests that the surface of the iSH2 may be in contact with the membrane.

The p110α somatic missense mutations are not uniformly spread throughout the *PI3KCA* gene. Instead, the vast majority are concentrated in a few small ‘hotspots’ in the catalytic and helical domains (Figure 4) (http://www.sanger.ac.uk/genetics/CGP/cosmic/). Lower-frequency mutations are also found in the ABD and C2 domains. The only domain for which no mutations have been reported is the RBD. In addition, there is a distinct lack of mutations in the N-terminal lobe of the catalytic domain and the surface of the helical domain facing away from the iSH2–phosphoinositide-binding slot (Figure 4). These results suggest that the gain-of-function mutations associated with oncogenesis are unusual and occur in tumours because they confer a selective growth advantage. Following this reasoning, it would seem that mutations in the RBD are only inhibitory of PI3K activation.

Mutations at the ABD–catalytic domain interface

The p110α–iSH2 complex showed that the conserved Arg^{38} and Arg^{88} from the ABD interact with catalytic domain residues Gln^{738}, Asp^{743} and Asp^{746}. These catalytic domain residues in helix kα2 are at the base of a helical hairpin, which is unique to the PI3Ks relative to protein kinases and rests on the β-sheet of the N-lobe of the domain. It is this same helical hairpin that interacts with the P-loop above the ATP-binding pocket and appears to be an important determinant of the rigidity of the ATP-binding pocket. Oncogenic mutations of the ABD residues Arg^{38} and Arg^{88} that increase the catalytic activity of the enzyme may lose contact with the catalytic domain, thereby increasing the ability of the ATP-binding pocket to open and accommodate ATP substrate.

Mutations at the C2 and iSH2 interface

Whereas the ABD–iSH2 interactions form the primary high-affinity binding site in the p110–p85 complex, the C2–iSH2 interactions form the secondary binding site. The presence of the iSH2 appears to order some of the loops of the C2 domain, and the structure of the p110α–iSH2 complex also showed that the iSH2 is closely positioned against the C2’s side of the slot in the catalytic core. Asn^{345}, which is among the oncogenic mutation sites of p110α, is in direct contact with two residues of the p85α iSH2, Asp^{560} and Asn^{564}, somatic missense mutations of which have been found in glioblastomas [82]. Two other residues from the C2 domain involved in rare oncogenic mutations are Cys^{420} and Glu^{453}. Mutation of these residues may also affect the interactions with the iSH2. It is not known how these contacts between the iSH2 and the C2 domain influence catalytic activity; however, one consequence of a mutation-induced loss of the interaction with the C2 domain may be to relieve the inhibitory influence of the nSH2–iSH2 unit on the catalytic subunit. The mutations may free the nSH2–iSH2 entity, allowing the catalytic domain to shift to an active conformation that can more readily accommodate ATP or membrane-associated phosphoinositide. Cancer-linked deletion mutations have also been identified at the N- and C-terminal regions of iSH2 [82,83], which are not in direct contact with p110. These mutations could up-regulate kinase activity by removing inhibitory interactions with the SH2 domains.

Helical and catalytic domain mutations

The residues involved in helical domain hotspot mutations, Glu^{542} and Glu^{545}, have been shown to be involved in inhibitory interactions with the phosphopeptide-binding site of the N-terminal SH2 domain of p85 [21]. The most commonly mutated residue in the p110α subunit is His^{1047}, which is one of a cluster of residues that are commonly mutated in the C-terminal lobe of the catalytic domain. These residues are in the vicinity of the putative phosphoinositide-binding pocket as delineated by the activation loop (Figure 4).

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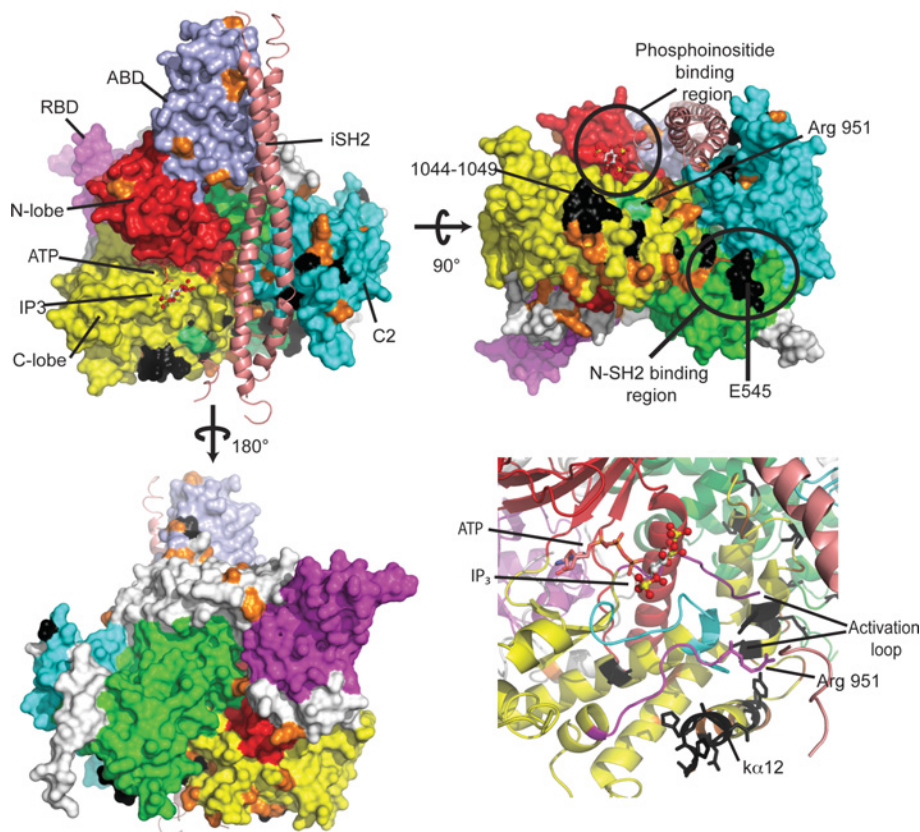
Figure 4 | Location of mutations in the *PIK3CA* gene superimposed on the structure of the p110α–iSH2 complex

Mutations from the Sanger database are shown coloured orange. Residues mutated in more than one individual are coloured black. The hotspot regions in the catalytic and helical domains are highlighted.



The importance of the activation loop for phosphoinositide recognition has been inferred from a study showing that phosphoinositide specificity characteristic of a PI3K class can be interchanged by swapping the activation loops [58,59]. On the basis of loop-swap experiments, it could be inferred that Arg^{949} is likely to be one of the ligands of the 4-phosphate of the phosphoinositide headgroup [59]. Arg^{951} is the nearest activation loop residue ordered in the p110α structure. Its side chain is nestled in a pocket of residues that are mutated in more than one isolate from cancers (Figure 4). The approximate position of the 3-OH of a phosphoinositide substrate can be estimated from the position of the γ-phosphate of the bound ATP in the p110γ structure. It may be that frequent mutations in the C-lobe allow the activation loop to move more freely to accommodate phosphoinositide headgroups (thereby decreasing the activation energy for the phosphate transfer), but this awaits any structural details of phosphoinositide binding.

The helical and catalytic domain hotspot mutations upregulate p110α activity and lead to transformation *in vivo* [84]. A detailed *in vitro* analysis of purified recombinant protein has shown that hotspot oncogenic mutations in the helical and catalytic domains increase the k_{cat} for soluble PtdIns(4,5)P_2 substrate [85]. H1047R and H1047L lead to a gain of function, but the wild-type sequence of p110γ has an arginine residue at the position equivalent to His^{1047} of p110α, whereas p110β and p110δ have a leucine residue at this position (Table 1b). Because both H1047R and H1047L have been reported to be highly oncogenic [86,87], these gain-of-function somatic mutations of p110α may confer a survival benefit on tumours. Simulation of two p110α oncogenic mutations by Langevin dynamics [88] suggests that the H1047R and H1047L mutations at the C-terminal end of helix kα12 make the active site more open, with an increase in the fluctuation and solvent exposure at the C-terminal end of the activation loop (Table 1a). The simulation suggests that the H1047R or H1047L mutation results in a rearrangement of interactions between the catalytic loop (Table 1a) and the N-terminal end of the activation loop, and that this change of conformation results in a rotation of Asp^{933}, leading to exposure of Phe^{934} of the DFG motif. This motion of the DFG region is analogous to the motion that occurs on ATP binding in p110γ (Figure 5). Consequently, it would appear that this mutation results in a conformation that may more loosely resemble the ATP-bound conformation of the enzyme, which could be consistent with the observed increase in k_{cat} for the mutation (i.e. the mutation results in a conformation that more closely resembles the transition state). The simulation

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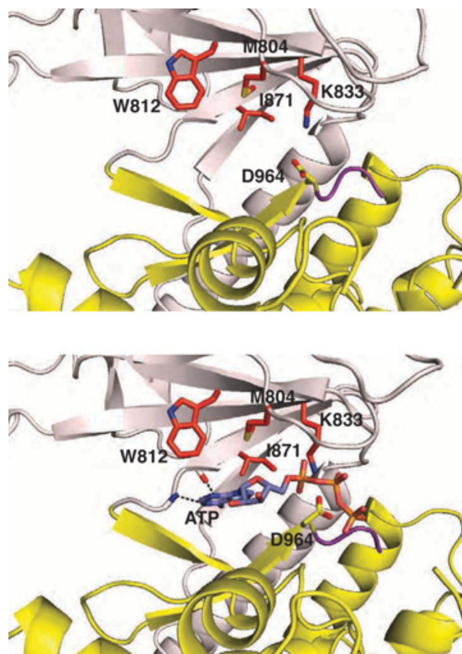
Figure 5 | Views of the p110γ ATP-binding pocket with (lower panel) and without (upper panel) ATP

The N-lobe is coloured red and the C-lobe is coloured yellow. The ordered portion of the activation loop is coloured purple. Asp[964] swings out to accommodate the $Mg^{2+}/\beta\gamma$-pytophosphate moiety.



also suggests a slight shift of the N-lobe relative to the C-lobe that is consistent with a model in which the enzyme undergoes a closed-to-open/inactive-to-active transition as part of its catalytic cycle. The C-terminal end of helix kα11 (Table 1a) of p110γ is displaced relative to the p110α structure, and His[1047] of p110α appears to be more loosely packed against the C-terminal end of the activation loop than for the analogous residue of p110γ. However, it is difficult to interpret these conformational differences. Given the crystal packing that blocks the active site of p110α, it is not clear that the p110α structure represents the apo-conformation of the enzyme.

Context-dependent oncogenic potentials of p110 isoforms in the PI3K pathway

p110α

Most colon, breast and endometrial cancers that have mutated p110α also have mutations in other genes that are either upstream of PI3K or which contribute to the stability of the PtdIns(3,4,5)P_3 [89]. These genes include *Ras*, *ERBB2/ERBB3* and *PTEN* (phosphatase and tensin homologue deleted on chromosome 10). PTEN is a lipid phosphatase which reverses class I PI3K activity by converting PtdIns(3,4,5)P_3 into PtdIns(4,5)P_2, and it is the second most frequently mutated gene in cancers. The p110α mutations appear to be correlated with invasive tumours, whereas the upstream mutations are found in both early- and late-stage tumours [89]. This suggests that the p110α mutations may be late-stage mutations that give the tumours a survival advantage. Mutant p110α augments anchorage-independent growth of cells expressing activated mutant Ras [89,90]. However, the helical domain and kinase domain hotspot mutants do not behave identically. Although the helical domain mutants E542K and E545K can form foci independently of the p85 subunit, they require interaction with activated Ras. In contrast, the H1047R catalytic domain mutation requires an interaction with p85, but does not require interaction with Ras [90]. These results are consistent with those of kinase activity assays performed with purified mutant p110 complexes in that the E545K, but not the H1047R, mutant synergizes with activated Ras [91]. Given that the helical domain mutations have been shown to activate p110α by eliminating an inhibitory contact with the N-terminal SH2 domain of the p85 subunit [21], it is not surprising that the transforming activity would be independent of p85 binding. The requirement for Ras binding by the helical domain oncogenic mutants might suggest that Ras is able to activate the enzyme once the basal inhibition due to the p85 subunit is removed. The indifference of the activity of the H1047R mutant to interaction with Ras may suggest that the H1047R mutation and Ras binding elicit similar allosteric changes in the active site.

p110β and p110δ

The p110δ isotype is overexpressed in AML (acute myeloblastic leukaemia) [92,93]. Although no oncogenic mutations have been reported for the other class I PI3Ks, p110β, p110δ and p110γ isotypes have an oncogenic potential that can be elicited by overexpression in a cell-based transformation model [94]. It has been proposed that the p110β, p110δ and p110γ isoenzymes are not mutated because the wild-type forms of the enzymes have a greater oncogenic potential than the wild-type p110α, and that these isoforms may contribute to oncogenesis by mechanisms that increase expression of the enzymes [94]. Recent reports have led to a re-examination of the roles of p110β and p110δ in cancer [7,95,96]. It was established that mice with a prostate epithelium-specific deletion of PTEN develop high-grade neoplasias. *Pten*$^{-/-}$ mice were protected from tumour formation by ablation of p110β, whereas p110α ablation produced no effect [96]. Mouse embryonic fibroblasts with an ablation of p110β were protected from transformation by activated Ras [96]. Surprisingly, partial protection from transformation is conferred even by a catalytically inactive point mutant of p110β. Replacement of p110β by a catalytically inactive variant resulted in mice that survived to adulthood, and expression of the kinase-dead p110β was sufficient to protect from tumour development in an ERBB2-driven model of breast cancer [7]. Although wild-type p110β, p110δ and p110γ have oncogenic potential when overexpressed, these isotypes differ in their requirements for Ras. Whereas the oncogenicity of p110β and p110γ requires Ras, overexpression of the p110δ isotype is strongly oncogenic independently of Ras [94]. One explanation might be that sequence differences between p110γ and p110δ result in p110δ having a conformation similar to the Ras-activated conformation of p110γ. When the C-terminal lobes of p110γ

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and p110α are superimposed, there is close agreement of the backbone conformations in most areas. However, the structures show differences in helix kα11 of p110γ (Table 1a), which may impart differences in the conformations of the adjacent activation loop and the catalytic loop. Nevertheless, it is not clear how the conformation of p110δ might mimic the Ras-activated conformation of the p110γ catalytic domain.

PI3K inhibitors in therapy

Because the various p110 isotypes have been associated with specific roles in a range of human diseases, development of PI3K-specific inhibitors has undergone a tremendous surge in activity [84]. Some of these have recently entered Phase I and II clinical trials, targeting mainly cancers, but also a reduction of tissue damage because of inflammatory response after myocardial infarction [97]. Currently, the majority of these drugs are class I PI3K-specific (PX-866, GDC-0941, XL147 and GSK615) with a few also cross-inhibiting protein kinases such as mTOR, DNA-PK (DNA-dependent protein kinase) and ERK2 (extracellular-signal-regulated kinase) (SF1126, NVP-BEZ235 and XL765). These are predominantly used for treatment of various solid tumours. The exceptions are the p110δ-specific CAL-101/IC87114 for treatment of AML, chronic lymphocytic leukaemia and selected B-cell non-Hodgkin's lymphoma and the p110δ/γ-selective TG100-115 for treatment of myocardial infarction. There are two general paradigms of therapeutic strategies: (i) using p110 isoform-selective inhibitors to minimize potential toxicities such as insulin resistance; and (ii) modulating the RTK–Ras–PI3K–mTOR pathway using either drug combination treatment or multi-targeted inhibitors [98–100].

Multi-targeted PI3K inhibitors

The structures of the imidazoquinazoline PIK-90 and the phenylthiazole PIK-93 in complexes with p110γ suggested that multi-targeted high-affinity inhibitors are generally flat and have substituents that occupy hydrophobic region I adjacent to the gatekeeper residue (Table 1a) [5]. This is consistent with models of the p110α/mTOR inhibitor PI-103 binding in the active site of p110γ [5,101]. Similar binding characteristics were apparent for two Src-family tyrosine kinase inhibitors bound to p110γ, and these compounds served as starting points for a diversification aimed at developing inhibitors that would simultaneously target both tyrosine kinases and PI3Ks [102]. One compound from this study, PP121, efficiently inhibits Src-family kinases, p110α and mTOR. Structural analysis suggests that this is achieved by exploiting a hydrophobic region adjacent to the gatekeeper residues of the tyrosine kinases and PI3Ks [102]. Dual binding is achieved through a conformational change in the Src pocket and a rotatable bond in the compound that enables the compound to adjust to the altered orientation of the PI3K gatekeeper residue relative to its Src equivalent. The three targets inhibited by PP121 lie in the same signalling pathway, which is one of those most commonly mutated in tumours, and PP121 blocks proliferation of tumour cells. Multi-targeted inhibitors may prove to be a useful strategy to overcome the development of drug resistance in tumours.

Scaffolding roles of PI3K catalytic subunits

In addition to their catalytic roles, studies involving knockin of catalytically inactive p110 isoforms have suggested that there may be 'scaffolding' roles that do not involve catalytic activity [103]. Germline deletion of p110β has been reported to result in embryonic lethality [104]. Embryonic fibroblasts from a p110β-knockout mouse show decreased proliferation and clathrin-mediated endocytosis, while reconstitution of a kinase-dead p110β in the knockout mouse embryonic fibroblasts restored both functions, suggesting that p110β has a scaffolding role [96]. The interactions that p110β makes in this scaffolding role are not clear, but previous reports have shown that, unlike other class IA isotypes, p110β has a role in GPCR signalling [18] and that it interacts with Rab5 on clathrin-coated endocytic vesicles [105,106].

Several direct interactions between p110γ and cellular proteins other that the p101 and p84 subunits have been reported. P110$\gamma^{-/-}$ mice are protected from cardiac hypertrophy generated by chronic exposure to the Aβ receptor agonist isoprenaline (isoproterenol) [107]. The helical domain of p110γ binds βARK1 (β-adrenergic receptor kinase 1) in a complex that promotes clathrin-mediated endocytosis of Aβ receptor [108,109]. It has been proposed that translocation of p110γ to the receptor by association with βARK1 leads to local PtdIns(3,4,5)P_3 generation, facilitating receptor internalization [108]. Indeed, overexpression of a kinase-dead p110γ in hearts causes a specific defect in Aβ receptor internalization [110]. P110$\gamma^{-/-}$ mice show extensive cardiac necrosis following TAC (transverse aortic constriction). However, mice expressing a kinase-dead variant of the protein showed no such damage. Both basal and post-TAC levels of cAMP are elevated in p110$\gamma^{-/-}$ mice; however, p110$\gamma^{K\Delta/K\Delta}$ mice had wild-type levels of cAMP. P110$\gamma^{K\Delta/K\Delta}$ and wild-type p110γ constitutively associate with and activate PDE3B (phosphodiesterase 3B) through a mechanism that does not require p110γ catalytic activity; however, the nature of this association has not been established.

Conclusions

The emerging picture of PI3Ks is that there are conformational changes leading to allosteric activation by the small G-protein Ras and inactivation by the p85 subunit. It may be that the activation by Ras and activation by oncogenic mutants result in conformational changes that mimic the conformational changes associated with ATP binding and phosphoinositide binding. Only further structural studies of all of the PI3K isotypes both free and in complex with small-molecule inhibitors, Ras and the regulatory subunits will illuminate these mechanisms. Nevertheless, a burgeoning

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body of evidence suggests that inhibitors of these enzymes may have a variety of pharmaceutical applications.

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