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The Regulation of Class IA PI 3-Kinases by Inter-Subunit Interactions

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Abstract

Phosphoinositide 3-kinases (PI 3-kinases) are activated by growth factor and hormone receptors, and regulate cell growth, survival, motility, and responses to changes in nutritional conditions (Engelman et al. 2006). PI 3-kinases have been classified according to their subunit composition and their substrate specificity for phosphoinositides (Vanhaesebroeck et al. 2001). The class IA PI 3-kinase is a heterodimer consisting of one regulatory subunit (p85 α , p85 β , p55 α , p50 α , or p55 γ) and one 110-kDa catalytic subunit (p110 α , β or δ). The Class IB PI 3-kinase is also a dimer, composed of one regulatory subunit (p101 or p87) and one catalytic subunit (p110 γ) (Wymann et al. 2003). Class I enzymes will utilize PI, PI[4]P, or PI[4,5]P₂ as substrates *in vitro*, but are thought to primarily produce PI[3,4,5]P₃ in cells.

The crystal structure of the Class IB PI 3-kinase catalytic subunit p110 γ was solved in 1999 (Walker et al. 1999), and crystal or NMR structures of the Class IA p110 α catalytic subunit and all of the individual domains of the Class IA p85 α regulatory subunit have been solved (Booker et al. 1992; Günther et al. 1996; Hoedemaeker et al. 1999; Huang et al. 2007; Koyama et al. 1993; Miled et al. 2007; Musacchio et al. 1996; Nolte et al. 1996; Siegal et al. 1998). However, a structure of an intact PI 3-kinase enzyme has remained elusive. In spite of this, studies over the past 10 years have lead to important insights into how the enzyme is regulated under physiological conditions. This chapter will specifically discuss the regulation of Class IA PI 3-kinase enzymatic activity, focusing on regulatory interactions between the p85 and p110 subunits and the modulation of these interactions by physiological activators and oncogenic mutations. The complex web of signaling downstream from Class IA PI 3-kinases will be discussed in other chapters in this volume.

1 Structural Organization of p85 α /p110 α

The p85 regulatory and p110 catalytic domains of Class IA PI 3-kinase are both multi-domain proteins (Fig. 1). The crystal structure of p110 α (Huang et al. 2007) shows an N-terminal Adapter Binding Domain (ABD), which binds to the coiled-coil domain of p85 (the iSH2 domain). The ABD has an ubiquitin-like fold that makes a large hydrophobic contact with the iSH2 domain (Huang et al. 2007; Miled et al. 2007). The remainder of p110 α consists of a Ras-binding domain, a C2 domain, a helical domain (found in all PI 3-kinase and formerly referred to as the PIK domain), and a kinase domain with a two-lobed structure typical of protein kinases; this organization is similar to that found in p110 γ (Huang et al. 2007; Walker et al. 1999).

The full-length p85 isoforms, p85 α and p85 β , consist of an SH3 domain, a Racbinding domain (the BCR homology domain) flanked by two proline-rich domains (nPRD and cPRD), and two SH2 domains (nSH2 and cSH2) linked by the inter-SH2 (iSH2) domain (Vanhaesebroeck et al. 2001). Three shorter regulatory subunits, p55 γ and the p85 α splice variants p55 α and p50 α , lack the SH3, nPRD, and BCR domains. The two p85 nSH2 and cSH2 domains share a similar specificity for tyrosine-phosphorylated motifs of the form

YxxM (Songyang et al. 1993). While individual domains of p85 α have been structurally characterized, as yet there is no structural data concerning the spatial organization of these domains within p85.

The iSH2 domain was predicted to be a long antiparallel coiled coil, based on sequence analysis (Dhand et al. 1994a) and subsequent site-specific spin labeling/electron paramagnetic resonance spectroscopy and molecular modeling of the nSH2-iSH2 fragment of p85 α (Fu et al. 2003). A crystal structure of the isolated iSH2 domain (residues 431–600) bound to the ABD of p110 α (Miled et al. 2007) shows a 115-Å long rod comprising two major helices (440–515 and 518–587). The C-terminal end of the iSH2 domain forms a short third helix (588–599); the extent of this third helix in intact p85 α is not yet known. The presence of the p85 α N- and C-terminal SH2 domains at the ends of an antiparallel coiled-coil suggests that the domains are relatively close to each other. The linker between the nSH2 domain and the iSH2 domain is only 10 amino acids long, and recent EPR data suggest that this short tether limits the range of mobility of the nSH2 domain relative to the iSH2 domain (Sen et al. 2010; see below). In contrast, the linker region between structurally defined helical regions of the iSH2 domain and the cSH2 domain (23 amino acids) is long enough to allow for considerable uncertainty as to their relative orientation. Isothermal calorimetry studies showed that the two SH2 domains of p85 α can bind simultaneously to a peptide containing phosphotyrosine residues separated by 11 amino acids (O'Brien et al. 2000), and the nSH2 domain can bind to a phosphorylated Tyr688 in the C-terminal SH2 domain in the context of a p85/p110 α dimer (Cuevas et al. 2001). While these studies suggest that the phosphotyrosine binding sites of the nSH2 and cSH2 domains can in some cases be close together, the range of motion and orientation of the two domains in the p85 α /p110 dimer is not known.

The crystal structure of p110 α bound to an nSH2-iSH2 fragment of p85 α (Huang et al. 2007) is shown in Fig. 1 in both space-filling and schematic views, from two orientations. Structures of the p110 α ABD or intact p110 α bound to the iSH2 domain of p85 α show extensive hydrophobic interactions between the ABD and the iSH2 domain, involving seven helical turns in iSH2 helix 1 and three turns of helix 2 (Miled et al. 2007). The C2 domain and kinase domains of p110 α drape over the iSH2 domain, with predicted hydrogen bonding between residue N345 in the C2 domain with residues D560 and N564 in helix 2 of the iSH2 domain. The helical domain contacts the kinase and C2 domains, and is located near the p85 SH2 domains and away from the ABD-iSH2 domain contacts. In the orientation shown in Fig. 2a, b, the Ras-binding domain is on top, separated from the iSH2 domain and making contacts with the kinase domain. The position of the p85 α nSH2 domain is not seen in the crystal structure of wild-type p110 α , but is visible in the structure of the p85 nSH2-iSH2 fragment dimerized with the H1047R mutant of p110 α (Huang et al. 2007; Mandelker et al. 2009). The nSH2 domain makes contacts with the helical domain of p110 α , as previously predicted (Miled et al. 2007), as well as with the kinase and C2 domains.

2 p85 α Inhibits and Stabilizes p110

The p85 α and p85 β regulatory and p110 α catalytic subunits of Class IA PI 3K were cloned in 1991 and 1992 (Escobedo et al. 1991; Hiles et al. 1992; Otsu et al. 1991; Skolnik et al. 1991). There was initially some confusion as to whether p85 α activated or inhibited p110 α . The catalytic subunit is active as a monomer when expressed in insect cells (Hiles et al. 1992). In contrast, p85 α is required for p110 α activity in mammalian cells; based on these latter data, Williams and coworkers proposed that p85 α is a p110 α activator (Klippel et al. 1994). Studies in yeast suggested that p85 α is a p110 α inhibitor, as expression of p85 α rescues the lethal effects of p110 α expression in *S. pombe* (Kodaki et al. 1994). Similarly, using recombinant monomeric p110 α produced in insect cells, it was shown that p85 α

binding inhibits the activity of monomeric p110 α by as much as 80% (Yu et al. 1998a). These data were reconciled with the discovery that monomeric p110 α is heat labile, and is stabilized by dimerization with p85 α (Yu et al. 1998a). Monomeric p110 α rapidly loses activity when incubated at 37°C, and it undergoes rapid degradation when expressed as a monomer in mammalian cells. However, the p110 α monomer is active when expressed in insect cells, which grow at 25°C. Similarly, the specific activity of overexpressed monomeric p110 α in mammalian cells is increased 15-fold by culturing the cells at reduced temperature. These data explain the discrepancies in the earlier literature: the apparent activation of p110 α by its co-expression with p85 α in mammalian cells in fact reflects the stabilization of p110 α in an inhibited, low activity state. The heat labile nature of monomeric p110 α also explains the later observation that the homozygous deletion of p85 α and p85 β in MEFs leads to parallel losses of p110 expression (Fruman et al. 2000).

The stabilization of the p110 α subunit by binding to p85 α is not as yet understood. Several groups showed that the N-terminus of p110, the adapter-binding domain or ABD, binds to the coiled-coil domain of p85 α , the iSH2 domain (Holt et al. 1994; Hu et al. 1993; Hu and Schlessinger 1994; Klippel et al. 1993). This interaction is necessary and sufficient for p110-p85 α dimerization and for stabilization of p110 α in mammalian cells, although it does not replicate physiological regulation of p110 α (see below). Surprisingly, the role of regulatory subunit binding in p110 α stabilization is supplanted by the linkage of epitope tags to the N-terminus of p110 α ; the degree of stabilization correlates with the size of the tag (Yu et al. 1998a). This explains the finding that a fusion of the p85 α iSH2 domain with the N-terminus of p110 α (the commonly used p110*) is constitutively active in mammalian cells (Hu et al. 1995). Based on more recent biochemical and structural data, this construct is unlikely to replicate ABD-iSH2 interactions. The ABD of p110 α binds to residues near the hinge region of the rod-like iSH2 antiparallel coiled-coil, at the end furthest away from the two SH2 domains (Huang et al. 2007; Miled et al. 2007) (Fig. 2). In contrast, the p110* chimera links helix 3 of the iSH2 domain to the N-terminus of p110 α , placing the p110 α ABD at some distance from its normal binding site in the iSH2 domain. Thus, the iSH2 domain in p110* presumably stabilizes p110 α by acting as a bulky N-terminal tag, and not by replicating ABD-iSH2 domain interactions. Consistent with this idea, Vogt and colleagues have found that an oncogenic mutant of p110 α , p110 α -H1047R, is not stabilized by a p110*-like linkage to the iSH2 domain (Zhao and Vogt 2008), whereas we find that p110 α H1047R is stabilized by co-expression with the iSH2 domain in trans (J.M. Backer, unpublished observations).

The stabilization of p110 α catalytic subunits (and presumably also p110 β and p110 δ) poses a problem for overexpression studies, since N-terminally tagged p110 will show a higher stability, and hence a higher constitutive activity, than wild-type p110 (Yu et al. 1998a). Whereas expression levels of wild type p110 α are limited by the amount of available p85, this is not true for N-terminally tagged p110 α . Unfortunately, recent data suggest that some C-terminal tags may inhibit the activity of p110 α toward PI[4,5]P2 *in vivo* (Bart Vanhaesebroeck, personal communication). Thus, the definition of an activity-neutral tag for the study of p110 isoforms continues to pose a significant experimental problem.

The inhibition of p110 by p85 is an example of a widely used regulatory scheme in eukaryotic cells, in which regulatory subunits of kinases maintain enzyme activity at a low level, with subsequent activation of the enzyme by a release of inhibition. The best-studied example of this scheme is PKA, where R1 or R2 subunits inhibit the activity of the C subunits (Taylor et al. 2005). In PKA, the mechanism of disinhibition is dissociative: in the presence of cAMP, the binding between the regulatory and catalytic subunits is disrupted. However, for Class IA PI 3-kinase, this mechanism of disinhibition is impossible for two reasons. First, as discussed above, monomeric p110 is heat labile and would rapidly lose activity should p85 dissociate. Second, p85-p110 α binding is extremely tight, and is

essentially irreversible under cellular conditions (Woscholski et al. 1994). Thus, activation of p85/p110 dimers by upstream regulators must occur by an intramolecular conformational change, in which the subunits remain bound together and activator binding to p85 causes a conformational change in p110.

3 Activation of p85 α /p110 α Dimers by Phosphopeptides

Early studies on Class IA PI 3-kinases demonstrated an increase in PI 3-kinase activity in anti-phosphotyrosine or anti-receptor tyrosine kinase immunoprecipitates upon growth factor stimulation (reviewed in Cantley et al. 1991). With the demonstration that SH2 domains are binding sites for tyrosine phosphorylated proteins (Matsuda et al. 1991; Mayer et al. 1991; Moran et al. 1990), as well as the identification of relatively PI 3-kinase-specific phosphotyrosine motifs in receptor tyrosine kinases (Fantl et al. 1992; Songyang et al. 1993), it became clear that the production of PIP3 *in vivo* is mediated at least in part by the recruitment of PI 3-kinases to activated receptor tyrosine kinases or their substrates. This led to the demonstration that constitutive targeting of p110 α to cell membranes by N-terminal myristylation or C-terminal isoprenylation is sufficient to produce constitutive increases in PIP3 production (Klippel et al. 1996). The preferred tyrosine phosphorylated peptide binding site for the p85 α SH2 domains was defined by degenerate peptide library binding studies to be (P)YxxM (Songyang et al. 1993). A crystal structure of a phosphopeptide-bound p85 α nSH2 domain shows a two-pronged socket binding mechanism, similar to that seen in the Src SH2 domain, in which the negatively-charged phosphotyrosine fits into a positively charged pocket, and the bulky methionine fits into a hydrophobic pocket (Nolte et al. 1996; Waksman et al. 1993).

With the development of antibodies against endogenous p85 α , as well as methods to produce recombinant p85 α and p110 α in baculovirus (Gout et al. 1992; Woscholski et al. 1994), it was possible to directly examine the effect of p85 α SH2 domain occupancy on the specific activity of PI 3-kinase, independently of effects on PI 3-kinase membrane recruitment. Initial studies showed that binding of p85 α /p110 α dimers to tyrosine phosphorylated IRS-1 or tyrosine phosphopeptides peptides from IRS-1, Polyoma middle T or the PDGF receptor causes a two- to threefold activation *in vitro* (Backer et al. 1992; Carpenter et al. 1993). Bisphosphopeptides activate PI 3-kinase with higher potency than corresponding monophosphopeptides (Carpenter et al. 1993; Herbst et al. 1994). p85 α contains two SH2 domains, and a number of tyrosine-phosphorylated proteins that bind p85 α are known to have two or more tyrosine phosphorylated YXXM motifs (e.g., PDRF-R, IRS-1). This suggested that dual occupancy of both domains might lead to increased PI 3-kinase activation. Consistent with this model, individual mutation of the conserved FLRVES motif in the N-terminal or C-terminal SH2 domains of p85 α leads to partial loss of phosphopeptide activation; mutation of both SH2 domains leads to a complete loss (Rordorf-Nikolic et al. 1995).

While bis-phosphopeptides lead to more potent activation of p85 α /p110 α dimers, the idea that the N- and C-terminal SH2 domains of p85 α bind simultaneously to a single tyrosine-phosphorylated protein has been challenged. Biochemical studies showed that a bis-phosphorylated peptide from Polyoma MT binds to the p85 α nSH2 domain, but not to the cSH2 domain, with enhanced affinity relative to a monophosphopeptide; a second noncanonical binding site for phosphotyrosine in the nSH2 domain was defined using NMR (Günther et al. 1996). Bis-phosphopeptides were also found to induce formation of higher order dimers between p85 α /p110 α holoenzymes; dimerization is independent of peptide concentration, suggesting that it does not involve direct bridging of SH2 domains in distinct p85 α /p110 α dimers (Layton et al. 1998). Finally, isothermal calorimetry experiments suggested that the mode of p85 α binding to receptors containing two potential binding sites

(e.g., the PDGF-R receptor) is concentration dependent: two distinct p85 α molecules bind to a single bis-phosphopeptide when p85 α is in excess, whereas both SH2 domains from a single p85 α molecule bind to a single bis-phosphopeptide when peptide is in excess (O'Brien et al. 2000). It remains unclear whether PI 3-kinase binding to ligands like the tyrosine bis-phosphorylated PDGF receptor *in vivo* involves divalent interactions.

4 A Mechanism for Phosphopeptide Activation

A model for the regulation of p85 α /p110 α dimers by phosphopeptides emerged from biochemical studies, in which active monomeric p110 α (produced in insect cells) was reconstituted with fragments of p85 α , and its activity was measured in the absence and presence of tyrosine phosphopeptides (Yu et al. 1998b). These studies showed that although the iSH2-ABD contact is the major interaction responsible for formation of the p85 α /p110 α dimer, as previously shown by several groups (Holt et al. 1994; Hu et al. 1993; Hu and Schlessinger 1994; Klippel et al. 1993), the iSH2 domain is not involved in the regulation of p110 α activity. First, although the iSH2 domain is sufficient to bind the p110 α ABD, this binding has no effect on p110 α activity. Regulation of p110 α by tyrosine phosphopeptides was instead shown to specifically require the presence of the N-terminal SH2 domain: nSH2-iSH2 fragments of p85 α (p85ni) inhibit p110 and the p85ni/p110 α dimer is activated by phosphopeptide. In contrast, iSH2, iSH2-cSH2, and cSH2-iSH2 fragments bind but do not inhibit p110. Thus, p85ni is the minimal fragment of p85 α that confers phosphopeptide regulation of p110 (Yu et al. 1998b).

Second, NMR and EPR studies on the structure and mobility of the iSH2 domain showed that the iSH2 domain is rigid and conformationally unresponsive to SH2 domain occupancy (Fu et al. 2003, 2004; O'Brien et al. 2000). Based on these data, it was proposed that the iSH2 domain is a rigid tether for p110, holding the subunits together and enabling a second regulatory contact between the nSH2 and an unidentified region of p110 α (Fu et al. 2004). Subsequent activation of the p85 α /p110 α dimer would involve a disruption of this inhibitory interface by binding of a phosphoprotein to the SH2 domain.

While these data suggested an inhibitory nSH2-iSH2 contact, the site of this contact was not known. A potential clue to the site of interaction emerged from studies by Samuels et al., which identified oncogenic mutants of p110 α from human tumor samples (discussed in more detail below) (Samuels et al. 2004). Sporadic mutations are scattered over the length of p110 α , but two hotspots account for nearly 80% of the mutations: an H1047R mutant in the C-terminus of p110 α , and a cluster of 3 change-of-charge mutations (E542K, E545K, Q546K) in the helical domain of p110 α . Could these hotspots indicate the site of the inhibitory nSH2-p110 α contact? If so, then it seemed likely that the mutations would be at residues conserved between Class IA catalytic subunit isoforms (p110 α /p110 β /p110 δ), since p85 α binds to and inhibits all three isoforms. However, whereas the acidic cluster in the helical domain is conserved in all three p110 isoforms, H1047 is not found in the kinase domains p110 β or p110 δ , where residues corresponding to H¹⁰⁴⁶H¹⁰⁴⁷ are replaced with LR. Thus, the helical domain acidic cluster seemed the more likely site of nSH2-p110 α contact.

This hypothesis was tested using an *in vitro* reconstitution approach (Miled et al. 2007). Helical domain mutants of p110 α (E542K, E545K, E546Q) bind p85ni but are not inhibited, nor are p85 α /p110 α -E545K dimers additionally activated by phosphopeptide. In contrast, although the H1047R mutant has increased specific activity as compared to wild-type p110 α , p85 α /p110 α -H1047R dimers are additionally activated by phosphopeptide (Carson et al. 2008), consistent with the observation that p110 α -H1047R is inhibited by p85ni (J.M. Backer and R.L. Williams, unpublished observations). Thus, the helical domain appeared to be the site of the inhibitory nSH2 domain contact. Miled et al. next reasoned that the acidic

patch in the helical domain was likely to interact with basic residues on the surface of the nSH2 domain. If so, then a basic-to-acidic mutation in the nSH2 domain might reduce the inhibition of wild-type p110, but restore the inhibition of the helical domain mutants. In fact, it was shown that K379E and R340E mutants of p85 α exhibited a loss of inhibition of wild-type p110. Additional experiments showed that the K379E mutant of p85 α restores the inhibition of the E545K mutant of p110, indicative of contacts between basic residues in the nSH2 domain and the acidic patch in the helical domain.

These data shed light on both the physiological activation of p85 α /p110 α by tyrosyl phosphoproteins, as well as the mechanism of oncogenic activation in the helical domain mutants (Fig. 3). The nSH2 basic residues whose mutation to Glu reduce the inhibition of wild-type p110 α (K379 and R340) lie adjacent to the phosphopeptide-binding site of the nSH2 domain. One can readily see how the binding of the tyrosine-phosphorylated proteins to the nSH2 domain would disrupt contacts between these residues and the helical domain of p110 α . In the cancer-related p110 α helical domain mutations, this contact is already disrupted. Thus, the helical domain mutants of p110 α found in human cancer mimic the physiological disruption of p110 α inhibition caused by phosphotyrosine protein binding to the nSH2 domain of p85 α . More recent data suggest that helical domain mutations may have additional effects in the context of transformation (Zhao and Vogt 2008) (discussed below).

Given that the nSH2 phosphopeptide binding site contacts the E542/E545/Q546 acidic patch in the helical domain of p110 α , the nSH2 domain presumably must move away from the helical domain to accommodate the binding of tyrosine phosphorylated activators. Recent studies suggest that the nSH2 domain shows considerable mobility relative to the iSH2 domain, at least in the context of an isolated p85 α (Sen et al. 2010; Wu et al. 2009; see below). Biochemical data also suggests that the ability of nSH2 domain to move about the proximal end of the iSH2 domain is necessary for PI 3-kinase activation, as introduction of an engineered disulfide bond between the nSH2 and iSH2 domains produces a mutant that inhibits p110, but cannot be activated by phosphopeptides (H. Wu and J.M. Backer, unpublished observations). A similar model has been proposed by Hale et al. (2008) for the activation of p85 β /p110 α dimers by the NS1 protein from Influenza A. NS1 binds to the C-terminal end of the p85 β iSH2 domain, including helix 3, and the authors propose that binding of NS1 to this region may sterically force the nSH2 domain into a conformational state that disrupts its inhibitory contacts with the helical domain of p110.

As noted above, maximal activation of intact p85 α /p110 α dimers by phosphopeptides requires functional nSH2 and cSH2 domains (Rordorf-Nikolic et al. 1995). Given that an iSH2-cSH2 fragment of p85 α binds but does not inhibit p110 α (Yu et al. 1998b), the mechanisms of activation by the C-terminal SH2 domain must be different. Interestingly, phosphopeptide binding to the cSH2 domain contributes to p110 α activation only within the context of full-length p85 α /p110 α dimers, but not when p110 α is dimerized with the nSH2-iSH2-cSH2 construct; a disabling mutation of the nSH2 domain only partially abolishes phosphopeptide activation of p85 α /p110, whereas it completely abolishes activation of p110 α bound to the nSH2-iSH2-cSH2 construct (Yu et al. 1998b). This means that activation of p110 α dimers by occupancy of the cSH2 domain requires the presence of the N-terminal domains of p85 α (the SH3, proline rich and BCR-homology domains). Presumably, these domains position the cSH2 domain so that it makes direct contacts with p110, or affect inhibitory contacts between p110 α and other domains of p85 α . An example of this latter mechanism was described by Cuevas et al. (2001), who suggested that the tyrosine phosphorylation of a site in the cSH2 domain (Y688) could interact with the nSH2 domain, leading to PI 3-kinase signaling to Akt activation in cultured cells. However, in this case the activation occurs through phosphorylation rather than through cSH2 domain occupancy.

A mechanistically independent role for the cSH2 domain was also suggested by Chan et al. (2002), who showed that Ras-dependent Akt activation is blocked by expression of a p85 α fragment consisting of helix 3 of the iSH2 domain, the iSH2-cSH2 linker, and the cSH2 domain. A truncation mutant identified in a Hodgkin's disease patient (a frame shift that truncates p85 α at residue 635 in the cSH2 domain, and adds 25 unrelated amino acids) also leads to increased Akt activation and transformation in cultured cells (Jucker et al. 2002), despite the fact that this construct appears to inhibit p110 α *in vitro* to the same extent as wild type p85 α (JM Backer, unpublished observations). These data suggest that the deletion of the cSH2 domain may activate PI 3-kinase signaling *in vivo* by a mechanism that does not involve the inhibition of p110 by p85 α .

5 Activation of p85/p110 by GTPases

Class I PI 3-kinases are activated by the binding of activated (GTP-bound) small GTPases. Activated Ras binds directly to the RBD of the catalytic subunit of both Class IA and IB PI-3 kinase. While all four p110 isoforms can bind Ras, only p110 α and p110 γ have been clearly shown to be activated by Ras *in vitro* (Jimenez et al. 2002; Pacold et al. 2000; Rodriguez-Viciana et al. 1996; Rubio et al. 1997). This does not mean that Ras binding is not involved in signaling by p110 β and p110 δ , since an increase in membrane association could lead to increased PIP3 production *in vivo* that might not be detected *in vitro*. For example, p110 β and p110 δ but not p110 α are recruited to Ras in redox-stimulated cells (Deora et al. 1998). In the case of p110 γ , the crystal structure of the GTP-Ras-p110 γ complex shows that activated Ras binding to the RBD involves both the Switch I and II domains of Ras. Ras binding causes a substantial shift in the position of a number of helices near the activation loop, consistent with an allosteric activation mechanism of p110 γ activation (Pacold et al. 2000). A study of knock-in mice expressing RBD mutants of p110 α has clearly shown that Ras-p110 α binding is required for normal lymphatic development, and for tumorigenesis by activated mutants of K-ras (Gupta et al. 2007; Ramjaun and Downward 2007).

Binding of activated (GTP-bound) forms of the small GTPases Cdc42 or Rac activates p85 α /p110 α dimers *in vitro* (Bokoch et al. 1996; Tolias et al. 1995; Zheng et al. 1994); activation of p85 α /p110 β or p85 α /p110 δ dimers has not been assessed. Rac and Cdc42 bind to the BCR homology domain of p85 α (Beeton et al. 1999), but the mechanism by which this binding affects p85 α /p110 α activity is not known. The BCR homology domain is so named because of homology to the Rho-GAP domain of the BCR gene product (Diekmann et al. 1991). A mutagenesis analysis of the BCR homolog β -chimaerin identified a number of residues necessary for Rho-GAP activity that are not conserved in the p85 α BCR domain (Ahmed et al. 1994). Alternatively, another group has found that p85 α does have GTPase activity toward Rac and Cdc42, as well as toward Rab5 and Rab4, but not Rab 11 (Chamberlain et al. 2004).

The original classification scheme for Class I PI 3-kinases suggested that p85 α dimers with p110 α , p110 β , or p110 δ are regulated primarily by receptor tyrosine kinases, whereas dimers of p101 or p87 with p110 γ are regulated by G-protein coupled receptors (GPCRs). This division of labors has been recently questioned, with the demonstration that p85 α /p110 β dimers are also regulated primarily by GPCRs (Guillemet-Guibert et al. 2008).

The activation of p85/p110 β dimers by G $\beta\gamma$ -subunits was first noted in the 1997 by Kurosu et al. (1997), who identified a G $\beta\gamma$ -stimulated PI 3-kinase from rat liver as p85/p110 β . The *in vitro* activation of p110 β by G $\beta\gamma$ occurs in the absence or presence of p85; in the latter case, G $\beta\gamma$ activation is synergistic with phosphopeptide activation (Maier et al. 1999). In a strain of NIH 3T3 cells expressing undetectable levels of p110 β , expression of p110 β is

required for LPA-stimulated activation of Akt (Murga et al. 2000). While these early studies suggested that p85/p110 β dimers could respond to G $\beta\gamma$, recent evidence suggests that p85/p110 β dimers may in fact be unresponsive to receptor tyrosine kinases and primarily regulated by GPCRs. In MEFs expressing a kinase-dead p110 β knock-in, or in the livers of conditional p110 β knockout animals, signaling is normal in response to ligands for receptor tyrosine kinases, such as insulin or IGF-I, but defective in response to GPCR ligands such as LPA or S1P (Ciraolo et al. 2008; Jia et al. 2008). In macrophages, which also express the GPCR-regulated p110 γ , both p85/p110 β and p101/p110 γ dimers respond to a similar range of GPCR ligands, and knockout or inhibition of both isoforms is required to completely inhibit GPCR-mediated activation of Akt (Guillermet-Guibert et al. 2008). The finding that p85/p110 β dimers are responsive to GPCRs explains a number of data in the recent literature using knockouts, kinase-dead knock-ins, or isoform-specific PI 3-kinase inhibitors to evaluate specific signaling by distinct p110 isoforms. For example, in myotubes or adipocyte cell lines, similar amounts of p110 α and p110 β are bound to IRS-1, but pharmacological inhibition of p110 β has minimal affect on IRS-1-associated PI 3-kinase activity or insulin signaling (Knight et al. 2006). Similarly, p110 α is recruited preferentially to IRS-1 as compared to p110 β in insulin-stimulated fat, muscle and liver (Foukas et al. 2006). These data are consistent with the idea that p110 β is primarily regulated by GPCRs.

From a mechanistic point of view, however, we have very little insight into why p110 β is regulated differently from p110 α , or presumably p110 δ . p110 β is inhibited by p85 *in vitro* (H. Dbouk and JM Backer, unpublished observations), and p85/p110 β dimers are activated by tyrosine phosphopeptides *in vitro* (Maier et al. 1999). The helical domain acidic patch required for regulation of p110 α (Miled et al. 2007) is conserved in p110 β . Thus, all the elements for SH2-mediated regulation of p110 β would seem to be in place. This suggests that p110 β is inhibited by nSH2-helical domain contacts, making it surprising that p85/p110 β dimers are not activated by binding to tyrosine-phosphorylated IRS-1 (Knight et al. 2006). The failure of p110 β -bound p85 to bind to tyrosine phosphorylated IRS-1, even in tissues where p110 β is abundant, is also unexplained (Foukas et al. 2006).

Finally, little is known about how G $\beta\gamma$ interacts with p85/p110 β . As mentioned above, G $\beta\gamma$ activation of Akt has been observed in cells expressing p110 β without p85, and p110 β is activated in the absence of p85 *in vitro*, suggesting that G $\beta\gamma$ can interact directly with the p110 β catalytic subunit (Maier et al. 1999; Murga et al. 2000). p110 β -mediated activation of Akt is blocked by overexpression of a construct comprising residues 34–349 of p110 β , which contains the RBD flanked by truncated ABD and C2 domains (Kubo et al. 2005), but a more thorough definition of the G $\beta\gamma$ -p110 γ binding site is still wanting.

p110 β was identified in a proteomics analysis of proteins that bind to the activated form of Rab5, the early endosomal small GTPases (Christoforidis et al. 1999). The binding site for this interaction in p110 β has not been established, nor has it been shown whether Rab5 binding to p85/p110 β dimers increases PI 3-kinase activity. A possible endosome-specific function for p110 β was suggested by the finding that early endosomal PIP3 can be converted by the sequential action of phosphoinositide phosphatase to the functionally important early endosomal lipid PI[3]P (Shin et al. 2005). In addition, a role in clathrin-mediated endocytosis has been suggested by studies showing a marked internalization defect in MEFs derived from mice in which p110 β expression is knocked out or significantly decreased (Ciraolo et al. 2008; Jia et al. 2008). This may be consistent with the fact that Rab5-associated p110 β is found in clathrin coated vesicles but not early endosomes (Christoforidis et al. 1999). It is intriguing to note that the internalization defect in p110 β -deficient MEFs is rescued by kinase-dead mutant of p110 β (Ciraolo et al. 2008; Jia et al. 2008), suggesting that the Rab5-p110 β interaction may not involve regulation of p110 β lipid kinase activity, but rather the regulation of an as yet undefined scaffolding function of p110 β .

6 Activation by Binding to p85 SH3 and Proline-Rich Domains

The SH3 domain of p85 α binds to proline-rich ligands of the general form RXLP α PRPXX or XXXPPLPXR; the two types of ligand may bind in opposite orientations (Koyama et al. 1993). At present, nothing is known about the position of the p85 α SH3 domain relative to the rest of p85 α or p110, although *in vitro* binding studies show that the N-terminal proline rich sequences from p85 α can interact with the p85 α SH3 domain (Kapeller et al. 1994). This suggests that the SH3 domain may fold forward toward the BCR domain, which is flanked by the two proline-rich domains. If this hypothesis is correct, then the disruption of intramolecular SH3-proline-rich domain interactions might provide an activation mechanism for PI 3-kinase. Several studies have suggested that p85 α /p110 dimers are activated by either binding of proline-rich peptides to the p85 α SH3 domain, including the proline-rich motifs from the FAK tyrosine kinase (Guinebault et al. 1995) and the influenza virus protein NS1 (Shin et al. 2007a, b). However, as discussed above, activation of PI 3-kinase by influenza virus may also involve binding between NS1 and the C-terminal end of the p85 β iSH2 domain (Hale et al. 2008). Similarly, addition of recombinant SH3 domains from Src-family kinases activates p85/p110 dimers *in vitro* (Pleiman et al. 1994), and PI 3-kinase activation by the binding the SH3 domains of Grb2 has been demonstrated in IGF-1-stimulated vascular smooth muscle cells (Imai and Clemmons 1999). These data suggest that binding of either the SH3 or proline rich domains of p85 α lead to PI 3-kinase activation, perhaps by disrupting an inhibitory intramolecular SH2-PRD interaction.

7 Regulation by Autophosphorylation

Class I PI 3-kinases possess protein as well as lipid kinase activity. Ser608 in the iSH2-cSH2 linker region of p85 α is a substrate for p110-mediated phosphorylation, which inhibits PI 3-kinase activity *in vitro* (Dhand et al. 1994b). This inter-subunit phosphorylation presumably represents a form of negative feedback regulation, as Ser608 phosphorylation is increased in response to stimulation by agonists like insulin or PDGF that increase Class IA activity (Foukas et al. 2004). However, evaluation of the role of this residue *in vivo* has been difficult, as mutation to either Ala or Glu decreases PI 3-kinase activity (Foukas et al. 2004). Interestingly, p85 α -Ser608 phosphorylation is more robust in dimers with p110 α as opposed to p110 β (Beeton et al. 2000; Foukas et al. 2004); phosphorylation by p110 δ was not tested. Phosphorylation of the corresponding residue (Ser602) in p85 β has not been studied. In contrast, both p110 β and p110 δ undergo inhibitory C-terminal autophosphorylation, at residues S1070 and S1039, respectively (Czupalla et al. 2003; Vanhaesebroeck et al. 1999), whereas p110 α autophosphorylation is not robust (Boldyreff et al. 2008; Dhand et al. 1994b). Thus, distinct Class IA PI 3-kinase isoforms all undergo inhibitory autophosphorylation, although at different sites and on different subunits.

8 Oncogenic Mutation of p85 α and p110

Studies over the past five years have identified mutations in p85 α and p110 α that clearly define PI 3-kinase as an oncogene in human cancer. The landmark study by Samuels et al. examined lipid kinases in human colon, brain, gastric, breast and lung tumors (Samuels et al. 2004). Mutations were commonly found in p110 α , but not in p110 β , p110 δ or p110 γ . The vast majority of sites involve mutation of H1047 to Arg in the catalytic domain (33%), and E542, E5445 or Q546 to Lys in the helical domain (47%). Less frequent sites of mutation are in the C2 domain and the ABD. Multiple subsequent studies have identified these mutants in p110 α from an ever-widening number of malignancies (reviewed in Liu et al. 2009). In a summary of series examining more than 100 patient samples, p110 α mutations are found in 25% of breast tumors, 22% of endometrial tumors, 17% of urinary tract tumors, 14% of large intestine tumors, 10% of cervical and upper aerodigestive tract tumors,

and at lower frequencies in other tumors types (<http://www.sanger.ac.uk/genetics/CGP/cosmic>).

For the common E545K and H1047R mutants, as well as for the less common G106V, C420R, E453Q, E542K, and M1043I mutants, an increase in catalytic activity has been demonstrated *in vitro* (Carson et al. 2008; Ikenoue et al. 2005). Introduction of p110 α mutants into chick fibroblasts or human mammary endothelial cells causes increased levels of Akt phosphorylation, transformation (Ikenoue et al. 2005; Isakoff et al. 2005; Kang et al. 2005; Zhao et al. 2005), tumor formation in the chick chorioallantoic membrane assay and in mice (Bader et al. 2006; Samuels et al. 2005; Zhao et al. 2005), and growth-factor independent growth in hematopoietic cells (Horn et al. 2008). In chick fibroblasts, a gradient of transforming efficiency was noted, and it was possible to subdivide p110 α mutants into strongly (H1047R, N345K, C420R, P539R, E542K, E545K, E545G, Q546K, Q546P, H1047L, H1047R), moderately (E545A, T1025S, M1043I, M1043V, H1047Y), and weakly (R38H, K111N) transforming (Gymnopoulos et al. 2007).

It is surprising that mutants in other p110 catalytic subunits have not been identified. Two studies on the transforming potential of p110 β , δ , and γ reached divergent conclusions: Kang et al. found that, in contrast to p110 α , overexpression of wild type p110 β , δ or γ is sufficient to cause transformation of avian fibroblasts (Kang et al. 2006). p110 γ already contains an Arg residue at the site homologous to H1047R in p110 α , but its mutation to His does not appear to alter p110 γ activity (Kang et al. 2006). The authors proposed that given the transforming capacity of these isoforms in wild type state, their contribution to oncogenesis might be primarily via changes in expression as opposed to mutation. In contrast, Zhao et al. found that p110 β is poorly transforming in mammary epithelial cells, even when the equivalent of the p110 α E545K hotspot mutant (E552K) is introduced (Zhao et al. 2005). Given the differences in the systems used, the potential for transformation by modulation of p110 β , δ and γ expression deserves further study.

Mutations in the p85 α regulatory subunit have been described in murine and human cancers. The first mutants to be discovered were deletions or truncations in the C-terminal end of the iSH2 domain. Jimenez et al. described the fusion of p85 α with a non-catalytic fragment of the Eph tyrosine kinase, which results in the termination of p85 α at residue 571 (Jimenez et al. 1998). Expression of either the p85 α -Eph fusion or the truncated p85 α (1–571) leads to increased PI 3-kinase signaling and a modest increase in transformation in cultured cells. Philp et al. described the replacement of residues Met582–Asp605 of p85 α with Ile (Philp et al. 2001), and Jucker et al. described a missense mutation resulting in the substitution of 25 unrelated amino acids after residue 635, in the cSH2 domain of p85 α (Jucker et al. 2002); these mutations also lead to increased Akt activation and PIP3 production in cultured cells. Recent sequencing efforts in glioblastoma and colon cancer have identified additional point mutants and small deletions, most clustered in the iSH2 domain and the cSH2 domain (CGART 2008; Jaiswal et al. 2009; Parsons et al. 2008). A number of these mutants lead to increased PI 3-kinase activity, and Akt signaling, survival and transformation in BaF3 cells (Jaiswal et al. 2009).

8.1 Helical Domain Mutants: Disruption of the nSH2-Helical Domain Interface

The mechanism by which helical domain mutants lead to activation of p110 α has been discussed above in the section on phosphopeptide activation of p85 α /p110 α dimers. Charge change mutations in a patch of acidic residues on the helical domain surface (E542K, E545K and Q546K) are proposed to disrupt an inhibitory contact with the nSH2 domain of p85 α , mimicking the disruption of this interface that would occur with physiological SH2 domain occupancy (Miled et al. 2007). Transformation by helical domain is blocked by an RBD mutant (K277E) that disrupts Ras binding (Zhao and Vogt 2008), which is consistent with a

previous study showing that phosphopeptide activation of p85 α /p110 α facilitates subsequent activation by Ras (Jimenez et al. 2002). Surprisingly, the E545K mutant causes increased transformation even in the context of an ABD truncation (residues 1–72) that abolishes p85 α binding, suggesting that this mutation might have some effects independent of the disruption of inhibition by p85 α (Zhao and Vogt 2008). However, it is important to note that the ABD truncation also causes a significant increase in transformation by wild type p110 α , to a level that is only slightly less than that seen with the truncated helical domain mutant. This would suggest that in the context of the ABD truncation, the additional activating effect of the helical domain mutant is small relative to its effect on wild type p110 α .

8.2 The Kinase Domain Mutant H1047R

The H1047R mutant of p110 α is inhibited by p85 α (Y. Yan and J.M. Backer, unpublished observations), and p85 α /p110-H1047R dimers are activated by phosphopeptides (Carson et al. 2008), suggesting that this mutation is mechanistically distinct from helical domain mutants. Consistent with these data, the H1047R mutant is additive with the E545K mutant for Akt activation and transformation in avian fibroblasts (Zhao and Vogt 2008). Unlike the E545K mutant, the H1047R mutant is unaffected by mutation of the Ras-binding domain, and is not activated by Ras-GTP binding (Chaussade et al. 2009; Zhao and Vogt 2008). These data led to the proposal that the H1047R mutant mimics the effect of Ras-GTP binding to the RBD of p110 α . Surprisingly, the transforming effects of the H1047R mutant are abolished by deletion of the ABD, to levels much lower than were seen in a similarly deleted wild type p110 α (Zhao and Vogt 2008). The reason for this requirement for the p85-binding domain, which is not seen with wild type or E545K p110 α , is not clear.

A crystal structure of the H1047R hotspot mutant of p110 α has been recently solved (Mandelker et al. 2009). The R1047 residue is oriented at a 90° angle from the position of H1047 in the wild type enzyme, and leads to changes in the conformation of two loops (864–874 and 1050–1062). The net effect of the mutation is predicted to increase the ability of the kinase to interact with cellular membranes; this is also suggested by biochemical data showing a greater sensitivity to variations in membrane lipid composition than wild type p110 α during *in vitro* kinase assays. If a major effect of Ras binding to p110 α is to enhance its association with cellular membranes (see below), then this model for the enhanced activity of p110 α H1047R would be consistent with its insensitivity to mutations in the Ras binding domain.

8.3 p110 α Mutations Disrupting the C2-iSH2 Domain Interface

The crystal structure of p110 α identified a previously unappreciated contact between the C2 domain of p110 α and the iSH2 domain of p85 α (Huang et al. 2007). In particular, hydrogen bonding was predicted between p110 α residues N345 and p85 α iSH2 domain residues D560 and N564. Mutation of p110 α N345 to Lys had previously been shown to induce transformation in avian fibroblasts, suggesting that disruption of the contact would lead to activation of p110 α (Gymnopoulos et al. 2007). More recently, mutations at residues D560 and N564, as well other p85 α residues near the C2-iSH2 domain interface, were discovered in human glioblastoma and colon cancer samples (CGART 2008; Jaiswal et al. 2009; Parsons et al. 2008). Analysis of the naturally occurring D560Y, N564D, D569Y/N564D, and DYQL⁵⁷⁹ mutants show that they bind but fail to inhibit p110 α , β or δ , and lead to enhanced cell survival, Akt activation, anchorage independent cell growth, and oncogenesis (Jaiswal et al. 2009).

These data all point to the C2-iSH2 contact as an important inhibitory interface, whose mutation leads to increased constitutive PI 3-kinase activity and cellular transformation. However, unlike the nSH2-helical domain interface that is modulated during phosphoprotein

regulation of PI 3-kinase, it is not yet known whether the C2-iSH2 domain interface is regulated under normal conditions. Furthermore, it is not yet known whether the loss of inhibition due to mutations at this site are due to direct conformational effects on p110 α , as opposed to indirect effects due to a loosening of the iSH2-p110 α coupling that might secondarily affect nSH2-helical domain interactions.

8.4 p85 α Mutations Disrupting the C2-iSH2 Domain Interface

Interestingly, recent data suggest that the iSH2 truncation and deletion mutants described by Jimenez et al. (1998) and Philp et al. (2001), discussed above, may also exert their effects by disrupting the iSH2-C2 domain interface. A number of hypotheses as to the mechanism of PI 3-kinase activation by these mutations have been proposed. Foukas et al. (2004) noted that these mutants cause the loss of Ser608, whose transphosphorylation by p110 α had been shown to cause an inhibition of PI 3-kinase lipid kinase activity (Dhand et al. 1994b). The authors proposed that the loss of this inhibitory phosphorylation site would lead to constitutive elevated activity *in vivo*. However, Shekar et al. compared the inhibition of p110 α *in vitro* by an nSH2-iSH2 construct (residues 321–600) as opposed to a truncated version of this construct (residues 321–572) (Shekar et al. 2005). Whereas the nSH2-iSH2 construct inhibits p110 α , even though it did not contain Ser608, truncation of the iSH2 domain at residue 572 causes a significant loss of p110 α inhibition. Thus, while the loss of the inhibitory Ser608 site might modulate the level of PI 3-kinase activation *in vivo*, truncation of the iSH2 domain at residue 572 causes a loss of inhibition independent of the removal of Ser608.

A second hypothesis was based on the finding that introduction of spin labels into the C-terminal end of the iSH2 domain, the region that is deleted in the p85 α (1–572) truncation, has pronounced relaxation effects on a single face of the nSH2 domain (Shekar et al. 2005). The authors interpreted the data as showing a contact between the nSH2 domain and the C-terminal iSH2 domain, and proposed that these contacts were required to orient the nSH2 domain so as to make inhibitory contacts with p110 α . However, subsequent studies on the nSH2-iSH2 fragment of p85 α show that the nSH2 domain is in fact highly mobile with respect to the iSH2 domain. ¹⁵N NMR relaxation methods were used to measure the rotational dynamics of the nSH2 domain within the nSH2-iSH2 construct, and defined an apparent rotational correlation time of 12.7 ± 0.7 ns for the nSH2 domain (Wu et al. 2009). These data are similar to experimental measurements for isolated SH2 domains (6.5–9.2 ns) and quite different from hydrodynamic calculations for several proposed nSH2-iSH2 geometries (45–52 ns) (Bernado et al. 2002; Farrow et al. 1994; Fushman et al. 1999; Zhang et al. 1998). Pulsed EPR measurements and molecular modeling of spin-labeled nSH2-iSH2 showed that the motion of the nSH2 domain is restrained to a torus-like distribution around the proximal end of the iSH2 domain (Sen et al. 2010). Thus, the nSH2 domain moves in a hinge-like manner, and not like a ball on a string; this restricted motion presumably explains why relaxation effects from iSH2 spin labels were only seen on one face of the nSH2 domain in the earlier study (Shekar et al. 2005). Similar results were obtained for the motion of the nSH2 domain in the context of full-length p85 α (Sen IK, Wu H, Backer JM and Gerfen GJ, unpublished observations). Taken together, these data do not support a model in which truncation of the iSH2 domain relieves a conformational constraint on the nSH2 domain.

A third hypothesis was proposed by Huang et al., based on the discovery of a close contact between the C2 domain of p110 α and the iSH2 domain of p85 α (discussed above) (Huang et al. 2007). The authors proposed that the loss of residues 572–600 in the truncation mutant described by Jimenez et al. (1998) might destabilize the iSH2 domain coiled-coil at the C2 domain contact sites (near residues D560 and N564). This hypothesis was directly tested by Wu et al. (2009), who showed that wild type p110 α is inhibited efficiently by the wild type

nSH2-iSH2 construct but not by a construct truncated at residue Q572. In contrast, the p110 α -N345K mutant shows the same degree of partial inhibition by either full-length nSH2-iSH2 or a construct truncated at residue Q572. These data suggest that the Q572 truncation has no phenotype when paired with a mutant p110 α that is incapable of forming the C2-iSH2 domain contact. *In vivo* data further supported this hypothesis. Either truncation of the nSH2-iSH2 fragment at Q572, or mutation of D560 and N565 to Lys, leads to constitutive activation of Akt and S6K1 and increased transformation, but no additive effects are seen in a truncated D560K/N565K construct. Similarly, the p110 α -N345K mutation leads to increased rates of transformation as compared to wild type p110 α (Gymnopoulos et al. 2007), but cells co-transfected with p110 α -N345K plus wild-type, truncated, D560K/D565K, or truncated D560K/D565K mutants all show similar rates of transformation. These data suggest that truncation of p85 α at residue Q572, or mutation of p85 α residues D560K/N565K, both lead to constitutive PI 3-kinase activation by disrupting the inhibitory C2-iSH2 domain interface identified by Huang et al. (2007).

9 Regulation of p85/p110 *In vivo*: Activation Versus Translocation

PI 3-kinase must interact with cellular membranes in order to reach their phospholipids substrates. Constitutive membrane association of p110 α catalytic subunits linked to N-terminal myristylation motifs or C-terminal CAAX motifs, leads to large increases in intracellular PIP3, Akt phosphorylation, and cellular transformation (Klippel et al. 1996). Since Class I PI 3-kinase has substantial activity under basal conditions (Beeton et al. 2000), membrane recruitment of PI 3-kinase leads to increased PIP3 production even in the absence of changes in PI 3-kinase specific activity. However, as described above, PI 3-kinases are also directly activated by allosteric interactions with tyrosine phosphorylated proteins and GTP-bound small Rac (Backer et al. 1992; Bokoch et al. 1996; Carpenter et al. 1993; Tolia et al. 1995; Zheng et al. 1994). These activations can be measured *in vitro*, independently of membrane recruitment, and reflect an increase in enzyme specific activity.

The allosteric activation of PI 3-kinases *in vitro* is not large (in the range of two-to fivefold). Given the magnitude of these changes in activity, there has been some question as to whether membrane translocation is sufficient to drive most PI 3-kinase dependent processes, and whether increases in catalytic activity play an important role in PI 3-kinase signaling. In this regard, the oncogenic potential of the helical domain p110 α is informative. The helical domain mutants disrupt the inhibitory interface that is regulated by SH2 domain occupancy, and lead to an approximately twofold increase in activity *in vitro* (Carson et al. 2008). These mutants have normal SH2 domains and still interact with Ras (Zhao and Vogt 2008), and would therefore be expected to undergo normal ligand-stimulated translocation to cell membranes. Nonetheless, these mutations have potent transforming activity. These data strongly suggest that the relatively small increases in Class IA PI 3-kinase specific activity caused by SH2 domain occupancy are critical for function *in vivo*.

The effect of Ras binding on Class IA PI 3-kinase activity is somewhat more complex. As discussed above, Ras binding to p110 γ causes changes in the catalytic cleft, consistent with direct allosteric regulation of activity (Pacold et al. 2000). However, the original studies on Ras activation only saw increases in PI 3-kinase activity with prenylated Ras, despite the fact that non-prenylated GTP-loaded Ras can still bind to p110 α (Rodriguez-Viciano et al. 1996). Thus, Ras is likely to activate by Class I PI 3-kinase by a combination of translocation and activation. This point was directly supported by studies showing that the transforming capacity of overexpressed p110 β and δ is abolished by mutation of the Ras-binding site, but rescued by N-terminal myristylation (Denley et al. 2008). Similarly, the H1047R mutant of p110 α , whose transforming potential is insensitive to mutation of the Ras

binding sites, appears to act by enhancing p110 α interactions with cellular membranes (Mandelker et al. 2009; Zhao and Vogt 2008).

10 Unanswered Mechanistic Questions on p85–p110 Interactions

Despite the concerted efforts of numerous laboratories, many very basic questions about p85–p110 interactions remain unanswered. With regard to structural questions, the structure of the nSH2-iSH2 fragment of p85 dimerized with p110 α has been solved (Mandelker et al. 2009), but we know virtually nothing about how the rest of p85 (the cSH2 domain, as well as the SH3, proline-rich and BCR homology domains) is organized in space. Hopefully, continued advances using biochemical and biophysical methods will lead to new information on these issues in the near future, and will undoubtedly increase our understanding of how interactions between these domains and upstream activators regulate the catalytic activity of p110.

An even more complex question is that of the specificity of p85–p110 pairing and regulation. Mass spectrometry studies have suggested that p85 and p110 expression levels are matched in multiple cell types and tissues (Geering et al. 2007), although this point has been controversial (Brachmann et al. 2005). However, we know very little about whether there is selectivity for the interaction of distinct p110 isoforms with distinct p85/p55 isoforms, since any one of the p110 ABD domains should be able to bind to all the p85/p50/p55 iSH2 domains. Given that knockout, knock-in and transgenic studies in mice have clearly demonstrated distinct physiological roles for the isoforms of p85 and p110 (reviewed in Fruman 2007; Liu et al. 2009; Vanhaesebroeck et al. 2005), one would think that the pairing of different Class IA regulatory and catalytic subunits would be a regulated process with significant physiological consequences. If so, then we have not even begun to figure out the rules for this sorting process, nor are the mechanisms for selectivity apparent. This question is likely to converge on the broader question of the isoform specificity of PI 3-kinase signaling *in vivo*, which has been a major focus of recent work in the field (Marone et al. 2008; Rommel et al. 2007; Stuart and Sellers 2009).

A significant caveat with regard to our current understanding of p85–p110 interactions is that nearly all the *in vitro* experiments have been done with p85 α and p110 α . For example, it will be very important to analyze mutants such as the acidic helical domain mutants of p110 α (e.g., E545K) in the context of p110 β and p110 δ , to see if they have similar effects on the regulation of these isoforms. This is also true for recently described point mutants of p85 α . New biochemical studies on p110 β should be particularly interesting, since this isoform appears to have all the p85-related tools in place for regulation by receptor tyrosine kinases, yet somehow is instead activated downstream of GPCRs. Finally, very little biochemical analysis has been done on the shorter splice variants and isoforms of p85, p55 α , p50 α and p55 γ . These variants contain the same nSH2-iSH2-cSH2 organization found in the so-called p85 α construct that has been extensively studied *in vitro* (Wu et al. 2007), but p55 γ and p55 α contain homologous 30 amino acid N-terminal extensions that contains a YXXM motif (Dey et al. 1998; Inukai et al. 1996) and has been suggested to associate with tubulin and with the retinoblastoma gene product Rb (Inukai et al. 2000; Xia et al. 2003). The potential role of these additional sequences in the regulation of Class IA dimers is not known. A better understanding of the enzymatic regulation of p110 catalytic subunits by these isoforms will be important in deciphering their differential regulation and physiological functions *in vivo*.

The biochemical analysis of these large and complex enzymes remains a challenging enterprise. Given the important role of the PI 3-kinases in human physiology, it is to be

hoped that an enhanced understanding of how the enzymes work will lead to new ideas about the pharmacological modulation of their activity.

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Fig. 1. Domain structure of p85 α and p110 α

The p85 α regulatory subunit contains Src-homology 3 (SH3), BCR-homology (BCR), Proline rich (PRD), and Src homology 2 (SH2) and domains. The inter-SH2 domain (iSH2) is an antiparallel coiled-coil that links the two SH2 domains. The p110 α catalytic subunit contains the adapter binding domain (ABD), which binds to the iSH2 domain of p85, a Ras-binding domain (RBD), a C2 domain, a helical domain (formally called the PIK homology domain), and a kinase domain

Fig. 2. Space-filling views of the nSH2-iSH2-p110α[H1047R] crystal structure (Huang et al. 2007; Mandelker et al. 2009), facing the iSH2 domain from the side (a) or down its barrel with the nSH2 domain removed (b). (c, d) Schematic models of the same orientations. In (d), the nSH2 domain is shown only in outline, to allow the rest of the iSH2 domain and p110α to be seen. The sites of the oncogenic mutations in p85α (D560, N564 and Q572) and p110α (E542/E545/Q546 and H1047) are shown, as is the phosphopeptide binding site of the nSH2 domain

Fig. 3. Models of physiological and oncogenic activation of p85/p110 dimers

The models show a schematic of p110α bound to the nSH2-iSH2 fragment of p85. In normal cells, phosphoprotein binding to the nSH2 domain disrupts the inhibitory interface with the helical domain of p110α. In transformed cells, mutations of residues in the acidic patch in the helical domain (for example E545K) constitutively disrupt nSH2-helical domain interactions, leading to the deregulation of the enzyme