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Phosphatidylinositol 3-kinase (PI3K): The Oncoprotein

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Abstract

The catalytic and regulatory subunits of class I phosphoinositide 3-kinase (PI3K) have oncogenic potential. The catalytic subunit p110α and the regulatory subunit p85 undergo cancer-specific gain-of-function mutations that lead to enhanced enzymatic activity, ability to signal constitutively and oncogenicity. The β, γ and δ isoforms of p110 are cell-transforming as overexpressed wild-type proteins. Class I PI3Ks have the unique ability to generate phosphoinositide 3,4,5 trisphosphate (PIP_3). Class II and class III PI3Ks lack this ability. Genetic and cell biological evidence suggests that PIP_3 is essential for PI3K-mediated oncogenicity, explaining why class II and class III enzymes have not been linked to cancer. Mutational analysis reveals the existence of at least two distinct molecular mechanisms for the gain of function seen with cancer-specific mutations in p110α, one causing independence from upstream receptor tyrosine kinases, the other inducing independence from Ras. An essential component of the oncogenic signal that is initiated by PI3K is the TOR (target of rapamycin) kinase. TOR is an integrator of growth and of metabolic inputs. In complex with the raptor protein (TORC1), it controls cap-dependent translation, and this function is essential for PI3K-initiated oncogenesis.

Phosphatidylinositol 3-kinases and cancer

The phosphatidylinositol 3-kinases (PI3Ks) are grouped into three classes (I – III) which differ in structure and function (Fruman et al. 1998; Vanhaesebroeck et al. 1997; Vanhaesebroeck and Waterfield 1999). Class I enzymes have been intensely studied and have emerged as promising drug targets in cancer and in immune disorders (Brachmann et al. 2009; Ghigo and Hirsch 2008). They are heterodimeric enzymes consisting of a catalytic subunit p110 that associates with a regulatory subunit. The vertebrate genome codes for four isoforms of p110 (α-δ). Several regulatory subunits have been identified. For p110α, β, and δ, the regulatory subunit p85 is the most prevalent. p110γ associates with separate, specific regulatory subunits of which p101 is the most common (Stephens et al. 1997). Class I PI3Ks occur as obligatory dimers in the cell (Geering et al. 2007). Regulatory and catalytic subunits show distinct structure-function domains that are illustrated in Figure 1 (Amzel et al. 2008; Huang et al. 2007; Walker et al. 1999). Class I PI3Ks act on three substrates, the non-phosphorylated phosphatidylinositol, PI, the inositol monophosphate (PI(4)P) and the bisphosphate (PI(4,5)P_2) to add a phosphate group in the D-3 position of the inositol ring and generate PI(3)P, PI(3,4)P_2 and PI(3,4,5)P_3 respectively (Carpenter et al. 1990). The latter, also referred to as PIP_3, functions as an important second messenger in the cell and is the predominant mediator of PI3K activity. The phosphatase PTEN (phosphatase and tensin homolog deleted on

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chromosome 10) removes the phosphate group from the D-3 position of phosphatidylinositol, acting as the direct catalytic antagonist of PI3K (Li et al. 1997; Maehama and Dixon 1998).

Class I PI3Ks have a long history of association with cancer (Yuan and Cantley 2008; Zhao and Roberts 2006; Zhao and Vogt 2008a). Extensive studies in the 1980s have documented a tight link of PI3K activity with tyrosine kinase oncoproteins and with the polyoma virus middle T oncoprotein (Sugimoto et al. 1984; Whitman et al. 1985). A representative interaction of this type is the binding of middle T to the Src oncoprotein, leading to an activation of the Src kinase which results in the phosphorylation of several tyrosine residues on middle T and the subsequent recruitment and activation of the p85-p110α dimer (Courtneidge and Smith 1984; Utermark et al. 2007). The cell-transforming activity of tyrosine kinase oncoproteins is correlated with their ability to associate with PI3K (Engelman et al. 2006; Kaplan et al. 1989; Schaffhausen and Roberts 2009). In 1997, the gene encoding the p110α catalytic subunit of PI3K was identified as the cell-derived oncogene in an avian retrovirus and shown to be constitutively activated by N-terminal fusion to viral sequences (Chang et al. 1997). The isolation of this avian retrovirus documented the direct oncogenic potential of p110α.

Cancer-specific mutations in PI3K

In 2004, the discovery of cancer-specific mutations in *PIK3CA*, the gene encoding the catalytic subunit p110 of PI3K put PI3Ks in the limelight as clinically relevant oncoproteins and as drug targets (Liu and Roberts 2006; Samuels and Velculescu 2004; Samuels et al. 2004; Stephens et al. 2005). Mutations have now been identified in the genes coding for both subunits of PI3K, in *PIK3CA* and in *PIK3R1*, the gene encoding p85 (Figure 2) (Cancer Genome Atlas Research Network 2008; Samuels et al. 2004). These mutations occur at frequencies extending from 5–25 % in several common cancers, including cancers of the breast, endometrium and the large intestine (http://www.sanger.ac.uk). The PI3K antagonist PTEN functions as an important tumor suppressor and is frequently inactivated by mutation or deletion in cancer (Di Cristofano and Pandolfi 2000).

The *PIK3CA* mutations are concentrated in three hot spots in the coding sequence (Samuels et al. 2004). Two of these hot spots are located in the helical domain of p110α, and one is situated in the catalytic domain. These hot spot mutations are single nucleotide substitutions that lead to the amino acid substitutions: E542K, E545K and H1047R (Samuels et al. 2004). The preferential mapping of cancer-specific mutations to hot spots suggested immediately a strong positive selection for such mutations, possibly reflecting a powerful replicative advantage of mutant-carrying cells. Studies of the mutant proteins rapidly revealed a mutation-induced gain of function as compared to the wild-type enzyme. The PIP_3-generating lipid kinase activity of the mutants is increased several fold (Carson et al. 2008; Ikenoue et al. 2005; Kang et al. 2005; Sugita et al. 2008; Zhao et al. 2005). Downstream signaling is no longer dependent on upstream stimulation by growth factors. It is constitutive and operates in serum-starved cells. This downstream signaling manifests itself in the phosphorylation of AKT (murine thymoma viral oncoprotein homolog) at T308 and S473, of the eukaryotic initiation factor 4E-binding protein (4E-BP) at T37 and T46 and of p70 S6 kinase (S6K) at T389. The three hot spot mutations activate the oncogenic potential of p110α. Expression of wild-type p110α does not detectably affect the growth behavior and the morphology of the cell. In contrast, expression of the hot spot mutants induces oncogenic transformation in avian and in mammalian cell culture (Ikenoue et al. 2005; Isakoff et al. 2005; Kang et al. 2005; Zhang et al. 2008; Zhao et al. 2005). The transformed cells are also tumorigenic in animal model systems (Bader et al. 2006; Zhao et al. 2005). In a mouse model, transgenic expression of the H1047R mutant p110α in the lung induces adenocarcinomas (Engelman et al. 2008). These data on enhanced enzymatic activity, constitutive downstream signaling and oncogenic potency strongly suggest

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that the hot spot mutations function as “drivers” in human cancer, responsible for at least part of the oncogenic phenotype of the cancer cell.

The hot spot mutations account for about 80% of the mutated *PIK3CA* genes in cancer. But there are also numerous cancer-specific mutations that occur at lower frequencies. Most of these are again single nucleotide substitutions, but recently two in-frame deletions have also been identified (Cancer Genome Atlas Research Network 2008). An investigation of seventeen rare point mutations led to the surprising finding that most of these (16 out of 17) also show a gain of function (Gymnopoulos et al. 2007). However, compared with the hotspot mutants, the rare mutations induce smaller gains of function. The mutant proteins show lower enzymatic activity, mediate lower levels of downstream phosphorylation and induce decreased oncogenic transformation in cell culture as measured by the number of transformed cell foci per ng of DNA. These lesser gains of function may explain the low frequencies at which such mutants are found in cancer. The broad distribution of rare, cancer-specific mutations over almost the entire coding sequence of p110α, with the notable and so far unexplained exception of the RAS-binding domain, raised the possibility that any random mutation may induce a gain of function, perhaps by triggering a conformational change. However, several random mutations introduced into *PIK3CA* had no phenotype, suggesting that cancer-specific mutations, no matter how rare, are still the result of positive selection (Gymnopoulos et al. 2007).

The mutations in *PIK3R1* are also clustered (Cancer Genome Atlas Research Network 2008). Most occur within a stretch of six residues (560 – 565) located in the inter-SH2 domain of p85 (Cancer Genome Atlas Research Network 2008). This portion of p85 includes the contact points with residues in the C2 domain of p110α. The mutations in *PIK3R1* are likely to interfere with the proper binding to p110α, relieving an inhibitory interaction. A cancer-derived *PIK3R1* mutation in the N-terminal SH2 domain of p85 (G376R) may reduce the inhibitory interaction with the helical domain of p110α as is the case with the engineered p85 mutation K379E (Sun et al. 2010, in press). Thus, the mutations in the inter-SH2 domain of p85 may be functionally equivalent to the mutations in the C2 domain of p110α, and the p85 mutations in the N-terminal SH2 domain may have the same effect as the helical domain mutations in p110α.

The map of the gain-of-function mutations on the structure of p110α (Amzel et al. 2008; Huang et al. 2008; Huang et al. 2007; Miled et al. 2007) reveals two properties that are shared by several mutants: location on the surface of the protein and a change from an acidic to a basic amino acid. This observation suggests that many of the gain-of-function mutations change the surface properties of the enzyme, probably affecting the interaction with other proteins or with membranes. In fact, of three engineered mutants inducing an acidic to basic change on the protein surface, two showed oncogenic activity (Gymnopoulos et al. 2007).

Several molecular mechanisms can induce a gain of function in p110

The occurrence of gain-of-function mutations distributed over several domains of p110α raises the question of the molecular mechanism responsible for increased activity. Do all these mutants operate by the same mechanism, or are there several distinct ways of enhancing p110α function? The available evidence strongly favors the existence of several molecular mechanisms leading to a gain of function. The combination of two hot spot mutations, one from the helical and the other from the kinase domain, in one protein has a strong synergistic effect on p110α activity. In contrast, introducing both helical domain mutations into the same molecule results in an only moderately additive effect (Zhao and Vogt 2008b). These results suggest that helical and kinase domain mutations work by different mechanisms that can cooperate. Several additional mutant combinations have been studied. Combinations of mutations located in different domains of p110α often show a synergistic effect, but

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combinations of mutations in the same domain are merely additive. There is also one pair of mutations, E545K/Y1021C, that shows loss of function when introduced in the same molecule, indicating incompatibility of the combined mutation-induced changes with p110α function (Gymnopoulos and Vogt, to be submitted).

The distinction between helical and kinase domain mutations is further illuminated by investigations that explore the interactions of the mutant proteins with the p85 regulatory subunit and with RAS (Zhao and Vogt 2008b). An N-terminal deletion of p110α that still permits expression of the protein but eliminates binding to p85, has contrasting effects on helical and kinase domain mutants. The oncogenic activity of the kinase domain mutant that lacks p85 binding is completely inactivated, and its downstream signaling is greatly reduced. The two helical domain mutants are much less affected by a lack of p85 binding. Their oncogenic activity in cell culture is only moderately reduced, and the effect on signaling is also minor. For wild-type p110α, deletion of the p85-binding domain has an activating effect, resulting in constitutive signaling and oncogenicity (Zhao et al. 2005). This somewhat surprising observation is explained by the fact that in cells devoid of upstream signaling, the p85-p110α interaction is both inhibitory and stabilizing for p110α. Upon growth factor stimulation, the SH2 domains of p85 interact with phosphorylated tyrosine on upstream signaling molecules, relieving the inhibition on p110α. Deletion of the p85-binding domain has a similar disinhibitory effect on p110α and reveals its latent oncogenic activity and signaling potential.

A mutational inactivation of the ability of p110α to interact with RAS has the opposite effect on the hot spot mutants. Interference with RAS binding decreases the oncogenicity of the helical domain mutants and drastically diminishes their downstream signaling. The kinase domain mutant is largely independent of RAS binding. Its oncogenicity is preserved in the absence of RAS binding, and signaling to AKT is only mildly affected. Complementary data have emerged from a study of mutant enzyme kinetics (Chaussade et al. 2009). The V_{max} values of the hot spot p110α mutants are significantly above that of the wild-type. Wild-type p110α can be activated by a PDGFR (platelet-derived growth factor receptor)-derived diphosphoryl peptide (Cuevas et al. 2001; Shekar et al. 2005). The phosphorylated tyrosines of this peptide interact with the N-terminal SH2 domain of p85 and thereby alleviate p85-mediated inhibition of p110α. Significantly, this PDGFR-derived peptide has no effect on the activity of the helical domain mutants, but it inhibits the kinase domain mutant (Chaussade et al. 2009). These data on the functional differences between helical and kinase domain mutations suggest that the kinase domain mutant is independent of activation by RAS; the mutation appears to have induced the same or a similar activating change that in the wild-type enzyme and in the helical domain mutants is achieved by the interaction with RAS. However, the kinase domain mutant still remains critically dependent on an interaction with p85, and this dependence requires further clarification. One possibility is suggested the crystal structure of the p110α-p85 complex which reveals an unexpected interaction between the p85-binding domain and the kinase domain (Huang et al. 2007) (Huang et al. 2008). This interaction might be important for the active conformation induced by H1047R and could explain the sensitivity of H1047R to a loss of p85 binding. Interactions between p85 and p110α that are relevant to the properties of the mutant proteins are summarized in Figure 3.

The kinase domain mutation H1047R maps to the hinge region of the activation loop. It could affect the position and the mobility of the activation loop. RAS also interacts with the kinase domain and could induce a change that is similar to the one caused by the H1047R mutation. The helical domain mutants remain dependent on binding to RAS, but are largely independent of p85-binding, probably because they mimic the activating event that follows the growth factor-induced relief of the helical domain-p85 interaction. The same effect of disrupting the helical domain-p85 binding probably also results from the cancer-specific mutation in the N-

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terminal SH2 domain of p85; it can also be achieved experimentally by mutating critical helical domain-interacting residues R340E and K379E in the p85 which are postulated to interact with E542 and E545 residues of p110α (Miled et al. 2007). Overexpression of such experimentally mutated p85 induces oncogenic transformation in cell culture (Sun et al. 2010, in press).

The molecular mechanisms for the mutation-induced gain of function in helical and in kinase domain mutations are complementary and reciprocal. The helical domain mutations have gained activation through p85-independence, but still need the interaction with RAS. The kinase domain mutation is in a state of constitutive RAS activation, but still requires the interaction with p85. The helical-kinase domain double mutant is both independent of RAS and of p85.

Since the hot spot mutations account for about 80 % of all cancer-specific mutations in p110α, inhibitors specific for these mutants could benefit the majority of the affected patients. Mutant-specific inhibitors would not induce side effects that can result from interfering with the life-sustaining functions of PI3K. The identification of small molecules that discriminate between mutant and wild-type is a challenging task for drug discovery. It would be greatly facilitated by structural information that is specific for the mutants.

Non-alpha isoforms of class I PI3K in cancer

There are four isoforms of the catalytic subunit of class I PI3Ks: p110α, p110β, p110γ and p110δ (Deane and Fruman 2004; Engelman et al. 2006; Fruman and Bismuth 2009; Hawkins et al. 2006; Stephens et al. 1996; Vanhaesebroeck et al. 2001). They are encoded by different genes, but share a basic domain structure. The α, β and δ isoforms use the same regulatory subunits. The α and δ isoforms are linked primarily to upstream receptor tyrosine kinases, the upstream activation of p110β appears to be context-dependent, involving receptor tyrosine kinases and G-protein-coupled receptors (Ciraolo et al. 2008; Guillermet-Guibert et al. 2008). The p110γ isoform interacts with separate distinct regulatory subunits and is linked to G-protein-coupled receptors (Stoyanov et al. 1995; Yart et al. 2002). The α and β isoforms are expressed ubiquitously. Expression of γ and δ isoforms is restricted mainly to lymphocytes. Genetic inactivation of the α and β isoforms causes early embryonic lethality (Bi et al. 2002; Bi et al. 1999), whereas γ and δ knockouts are viable but suffer from immune deficiencies (Ali et al. 2004; Cantley 2002; Clayton et al. 2002; Hirsch et al. 2000; Ji et al. 2007; Jou et al. 2002; Laffargue et al. 2002; Okkenhaug et al. 2002; Rodriguez-Borlado et al. 2003; Sasaki et al. 2000). The functions of the p110 isoforms are overlapping, but they are clearly not redundant. Conditional and tissue-specific mutations have defined isoform-specific roles in cellular signaling. The principal roles of p110γ and p110δ are in the immune system (Alcazar et al. 2007; Ali et al. 2008; Ji et al. 2007; Okkenhaug et al. 2004; Patton et al. 2007), and p110α and p110β have distinct, complementary, context-dependent and cell type-dependent roles in the control of cell growth and metabolism (Graupera et al. 2008; Marone et al. 2008; Vanhaesebroeck et al. 2005). The functions of specific p110 isoforms are explored in greater detail in other chapters of this volume.

The oncogenic potential of p110α is well documented in experimental systems; the gain-of-function mutations in human cancer add significance to this activity (Samuels et al. 2004). For the non-alpha isoforms of class I PI3K, the connection to cancer is more tenuous. There are no cancer-specific mutations in these isoforms, but differential expression is observed in several cancers. The p110δ isoform is consistently overexpressed in acute myeloblastic leukemia, and inhibitors of p110δ specifically interfere with the growth of these leukemic cells, suggesting a role of p110δ in leukemogenesis (Samuels et al. 2004; Sujobert et al. 2005). Specific inhibitors of p110δ are in clinical trials for hematopoietic malignancies (http://clinicaltrials.gov/ct2/show/NCT00710528). Increased expression of p110γ is seen in

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chronic myeloid leukemia (Hickey and Cotter 2006; Knobbe et al. 2005). There are also data that suggest involvement of non-alpha isoforms of class I PI3K in solid tumors (Bénistant et al. 2000; Knobbe et al. 2005; Mizoguchi et al. 2004). The wild-type non-alpha isoforms have the ability to induce oncogenic transformation when overexpressed in cell culture, whereas wild-type p110α lacks this ability (Kang et al. 2006). The elevated expression of non-alpha isoforms in some cancers may therefore be a determinant of the oncogenic cellular phenotype. The oncogenic activity of wild-type non-alpha isoforms has so far been shown only in chicken embryo fibroblasts which are exquisitely sensitive to transformation by single oncoproteins. In this cell culture system, the various isoforms reveal distinctly different characteristics in their signaling through AKT, their interactions with RAS and sensitivity to inhibitors of the MAP kinase pathway (Denley et al. 2008). Overexpression of p110δ induces strong phosphorylation of AKT at T308 and S473 and of the downstream targets S6K, 4E-BP and GSK3β (glycogen and synthase kinase 3β). FOXO1 (forkhead box transcription factor O1) becomes undetectable in these cells. This signaling pattern closely resembles that of the p110α mutant H1047R. Expression of p110β and p110γ induces much lower levels of phosphorylation of AKT, S6K, 4E-BP and GSK3β. The levels of FOXO1 are not significantly reduced in these cells. The contrasting properties of p110δ versus p110β and p110γ are also seen in their responses to activation by RAS and to inhibition of the MAP kinase pathway. Introducing point mutations into the RAS binding domain that are designed to abolish RAS binding interferes with oncogenic transformation and signaling induced by p110β and p110γ, but does not affect p110δ. The activities of the mutated p110β and p110γ proteins can be restored by adding a myristylation signal to the N-terminus of the proteins, suggesting that the interaction with RAS mediates recruitment to the plasma membrane. The apparent independence of p110δ from RAS requires further examination and confirmation. Although the mutated residue in the RAS-binding domain of p110δ is conserved among the isoforms and is known to control RAS binding in p110α and in p110γ (Pirola et al. 2001; Rodriguez-Viciana et al. 1996), the effect of this mutation in p110δ on RAS binding has not been verified. Sensitivity to inhibitors also sets p110β and p110γ apart from p110δ. The MEK1/2 inhibitor U0126 effectively interferes with cellular transformation induced by p110β and p110γ, but does not affect the oncogenic activity of p110δ and of the H1047R mutant of p110α. The inhibitor of RAF, BAY 43-9006, shows a similar preferential effect on p110β and p110γ, but does not interfere with cellular transformation caused by p110δ. These observations single out p110δ as an exceptionally potent signaling protein, resistant to MAP kinase inhibition and capable of functioning preferentially through AKT. The similarities between p110β and p110γ are unexpected, because these isoforms respond to different sets of upstream signaling: p110β has been linked to receptor tyrosine kinases and G-protein coupled receptors and p110γ exclusively to G-protein coupled receptors (Roche et al. 1998); (Guillermet-Guibert et al. 2008). The similarities between p110β and p110γ revealed by the studies on oncogenicity and signaling in chicken embryo fibroblasts may extend to mammalian cells. An important role of non-alpha isoforms of PI3K in cancer is also emerging from new animal models and from studies in cell culture (Ciraolo et al. 2008; Jia et al. 2008; Torbett et al. 2008; Wee et al. 2008). These reveal isoform-specific enzymatic and non-enzymatic functions of p110β and are discussed in other chapters of this volume.

Class II and III PI3Ks

The family of PI3Ks encompasses three distinct classes that differ in structure and function (Vanhaesebroeck et al. 1997; Vanhaesebroeck and Waterfield 1999). One of the defining criteria for each class of PI3Ks is substrate recognition and hence the spectrum of products. Class I PI3Ks can utilize the non-phosphorylated phosphatidylinositol (PI), the monophosphate (PI(4)P) and the bisphosphate (PI(4,5)P_2) phosphatidylinositols, giving rise to PI(3)P, PI(3,4)P_2 and PI(3,4,5)P_3, also referred to as PIP, PIP_2 and PIP_3 respectively. Class II PI3Ks recognize PI and PI(4)P, but not PI(4,5)P_2 as substrates to produce PIP and PIP_2. Class III PI3Ks can use

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only PI and convert it to PIP (Pirola et al. 2001). In addition, class I PI3Ks can function as serine protein kinases (Dhand et al. 1994). One of the protein substrates of class I PI3Ks is the regulatory subunit p85. This phosphorylation represents an autoregulatory mechanism (Foukas et al. 2004). The protein kinase activity of p110 is, however, not sufficient for oncogenic transformation induced by class I p110 mutants and isoforms (Denley et al. 2009; Kang et al. 2006). In the canonical PI3K signaling pathway, PIP_2 and PIP_3 are recognized by the pleckstrin homology domains of PDK-1 (phosphoinositide-dependent kinase) and of AKT (Alessi et al. 1996a; Alessi et al. 1996b; Klippel et al. 1997; Nicholson and Anderson 2002). These interactions recruit PDK-1 and AKT to the plasma membrane, resulting in the phosphorylation of AKT by PDK-1, catalytic activation of AKT and phosphorylation of downstream targets (Alessi et al. 1997; Currie et al. 1999; Filippa et al. 2000; McManus et al. 2004; Milburn et al. 2003; Vanhaesebroeck and Alessi 2000). The structure of the AKT pleckstrin homology domain bound to IP_4, the headgroup of PIP_3, shows critical ionic interactions between basic pleckstrin homology domain residues and the phosphates at the D-3 and D-4 positions of IP_4 (Milburn et al. 2003). The phosphate at the D-5 position does not participate in the interaction with the AKT pleckstrin homology domain. Hence AKT has a lower affinity for PIP_2 (PI(4,5) P2) than for PIP_3.

So far, only class I PI3Ks have been firmly involved in cancer, although there are some observations that suggest class II may also play a role (Low et al. 2008). The defining characteristic of class I PI3Ks is the generation of PIP_3 (Vanhaesebroeck et al. 1997; Vanhaesebroeck and Waterfield 1999). This ability may therefore constitute a prerequisite for the oncogenicity of lipid kinases. Emerging evidence supports this suggestion. A short sequence in the activation loop of PI3Ks determines substrate recognition and product specificity (Bondeva et al. 1998; Pirola et al. 2001). Substitution of this sequence in class I PI3K with the corresponding sequence of class II or of class III generates enzymes that produce PIP and $PI(3,4)P_2$ or PIP respectively, but fail to make PIP_3. These constructs do not induce oncogenic transformation in cell culture and show greatly reduced signaling through AKT. Expression of the wild-type or the myristylated form of hVps34 class III PI3K fails to induce oncogenic transformation in cultures of chicken embryo fibroblasts and does not increase the phosphorylation status of Akt, p70 S6 kinase, 4E-BP, and glycogen synthase kinase-3β or cause a change in the level of FoxO1 (Denley et al. 2009). The production of PIP_3 and PI3K signaling are reduced in the presence of the PIPP phosphatase which removes the phosphate from the D-5 position of phosphatidylinositol. Expression of PIPP also interferes with oncogenic transformation induced by the four isoforms of class I PI3K, reduces the levels of AKT phosphorylation and attenuates the degradation of FOXO1 (Denley et al. 2009). These observations support the conclusion that the ability to produce PIP_3 is essential for the oncogenic activity of PI3K.

PI3K-driven oncogenic transformation: mechanistic considerations

PI3K signaling affects numerous downstream targets, not all will be essential for oncogenic transformation. The canonical signaling cascade proceeds through AKT, the TSC (tuberous sclerosis complex), RHEB (Ras homolog enriched in brain) to TOR and from there to additional targets. In this pathway, two components stand out as particularly significant for oncogenicity: AKT and TOR. AKT is an important signal branching point that can direct PI3K signals into many different directions; TOR is of importance because it functions as integrator, receiving input from multiple sources. Thus AKT and TOR link the canonical PI3K pathway to other regulatory activities in the cell.

In the canonical pathway, AKT phosphorylates and thereby inhibits TSC2 (Dan et al. 2002; Inoki et al. 2002; Manning et al. 2002). The TSC complex functions as GTPase-activating protein for RHEB (Castro et al. 2003; Garami et al. 2003; Inoki et al. 2003; Tee et al. 2003;

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Zhang et al. 2003b); reduction of GTPase activation by AKT activates RHEB. The GTP-bound RHEB then directly interacts with TOR and activates this target. AKT and RHEB are oncogenic when constitutively activated (Ahmed et al. 1993; Aoki et al. 1998; Jiang and Vogt 2008). TOR is a PI 3-kinase related protein kinase (PIKK) (Abraham 2004) that fulfills numerous tasks in cell growth and metabolism.

TOR functions in two distinct multiprotein complexes, TORC1 and TORC2 (Jacinto et al. 2004). TORC1 contains the proteins RAPTOR, LST8 and PRAS40 (Kim et al. 2002). TORC2 consists of LST8, RICTOR and SIN1 (Sarbassov et al. 2004). TORC1 and TORC2 are differentially regulated and have distinct functions. TORC1 can be activated by AKT-dependent and AKT-independent signals. AKT stimulates TORC1 by inducing an inhibition of the GTPase-activating protein activity of the TSC complex that targets RHEB and by phosphorylating and thereby inactivating PRAS40, a negative regulator of TORC1. AKT-independent regulation of TOR can also be mediated by the TSC complex. TSC is a sensor and integrator of signals that originate from energy deprivation, hypoxia or stimulation of growth. For instance, AMPK (AMP kinase), activated by an increase of cellular AMP, activates TSC2 and thereby inhibits TOR. Rag (Ras-related small GTP-binding proteins) activate TORC1 in response to the availability of amino acids (Sancak et al. 2008). Another AKT-independent pathway to TORC1 has been identified in glioblastoma and is mediated by PKCα (protein kinase Cα) (Fan et al. 2009). The principal downstream targets of TORC1 are 4E-BP1 (eukaryotic initiation factor 4E-binding protein) and S6K1 (p70 ribosomal protein S6 kinase). They will be considered below.

In contrast to the various AKT-dependent and AKT-independent ways that have been identified for the regulation of TORC1 (Cheng et al. 2009; Memmott and Dennis 2009; Vasudevan et al. 2009), the regulation of TORC2 is not fully understood. A distinguishing mark of TORC2 activation is the requirement for an active TSC complex, one that is not phosphorylated by AKT and opposite to the requirement for TORC1 activation (Huang and Manning 2009). Several targets have been identified for TORC2 (Huang et al. 2009). Among these, the phosphorylation of AKT at S473 appears potentially relevant to PI3K signaling (Sarbassov et al. 2005). This phosphorylation event achieves maximal activation of AKT, and it also expands the spectrum of AKT targets to include PRAS40 and FOXO (Guertin et al. 2006). However, it is doubtful whether the additional targets that can be addressed by the S473-phosphorylated AKT play an important role in transformation (see below).

TOR is essential for the oncogenicity of PI3K and AKT (Jiang et al. 2000; Neshat et al. 2001; Podsypanina et al. 2001). The TOR inhibitor rapamycin strongly and specifically interferes with PI3K- and AKT-induced cellular transformation; yet it does not reduce transformation caused by fourteen other oncogenes (Aoki et al. 2001). Exposure to resistance-inducing concentrations of rapamycin does not significantly affect cell replication. Whereas short-term treatment with rapamycin selectively inhibits TORC1, cells treated over prolonged periods of time (24 hours or more), also show inhibition of TORC2 (Sarbassov et al. 2006). Since interference with oncogenic transformation by rapamycin results from long-term treatment with the drug, both TORC1 and TORC2 would be affected and could play an essential role in oncogenicity. However, at least one TORC2 activity, the phosphorylation of AKT on S473, appears to be dispensable for oncogenic transformation (Aoki et al. 1998).

The available data are compatible with the idea that TORC1, but not TORC2, plays the predominant role in oncogenesis (Guertin and Sabatini 2007). TORC1 functions as a positive regulator of protein synthesis. It phosphorylates and thereby activates S6K. It also phosphorylates 4E-BP1, releasing eIF4E (eukaryotic initiation factor 4E, the cap-binding protein) to become available for the assembly of the translation initiation complex. The TOR-dependent stimulation of protein synthesis preferentially affects mRNAs that have complex

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secondary structures in their 5′ untranslated regions (Culjkovic et al. 2005). These secondary structures require unwinding performed by the eIF4A (eukaryotic initiation factor 4A) helicase that together with the eukaryotic initiation factors eIF4E and eIF4G forms the eIF4F initiation complex. Numerous mRNAs that encode growth-promoting proteins are characterized by 5′ untranslated sequences with complex secondary structures, and their efficient translation is highly dependent on an abundance of eIF4E and the eIF4A helicase (Culjkovic et al. 2006). The enhancement of this activity by TORC1 could be a critical factor in the transformation process.

The importance of high efficiency translational initiation in PI3K- and AKT-induced oncogenicity is also documented by the effects of the YB-1 (Y Box binding protein) on the transformation process. The YB-1 protein is highly conserved in evolution with close relatives throughout prokaryotic and eukaryotic forms of life. It is abundantly and ubiquitously expressed, and with its cold shock domain, it binds both DNA and RNA, affecting transcription and translation (Evdokimova et al. 2006a; Evdokimova et al. 2009; Izumi et al. 2001; MacDonald et al. 1995; Mertens et al. 1997; Zasedateleva et al. 2002). By binding to mRNA, YB-1 has the capacity to inhibit translation (Evdokimova et al. 2006a). In cells transformed by PI3K or AKT, YB-1 is downregulated transcriptionally and posttranscriptionally (Bader et al. 2003; Bader and Vogt 2008; Bader and Vogt 2005; Evdokimova et al. 2006b; Sutherland et al. 2005). This downregulation appears to be a necessary facilitator of transformation, because re-expression of YB-1 causes a strong and specific cellular resistance to PI3K- and AKT-induced oncogenic transformation, yet does not interfere with transformation induced by other oncogenes (Bader et al. 2003). These YB-1-expressing cells do not show a detectable reduction in the rate of replication. Phenotypically, YB-1 therefore acts like a rapamycin mimic, but it intervenes in transformation downstream of TORC1, at a level of mRNA (Bader and Vogt 2008). Interference with transformation depends on cytoplasmic localization of YB-1 and on the ability of YB-1 to bind to RNA (Bader and Vogt 2005). The interaction of YB-1 with RNA is not sequence-specific, and YB-1 can bind to multiple sites on the mRNA. However, for the inhibition of protein synthesis, binding at or near the cap structure of mRNA is essential. According to a recent model, YB-1 then interferes with binding of eIF4G (eukaryotic initiation factor 4G) to mRNA and thus competes with the assembly of the eIF4F initiation complex on the mRNA (Svitkin et al. 2009).

The specific sensitivity of PI3K-induced transformation to rapamycin and to expressed YB-1 supports the conclusion that the oncogenic effects of PI3K are mediated by TORC1 and that they involve stimulation of protein synthesis. This activity of TOR appears necessary for transformation, but it is probably not sufficient. A gain of function in TOR alone has so far not been found to transform cells. Cells lacking TSC1 or TSC2 show constitutive activation of TOR, but are not transformed (Kwiatkowski et al. 2002; Zhang et al. 2003a). Patients with heritable loss of function in the TSC complex develop hamartomas, but aggressive cancers are rare (Al-Saleem et al. 1998; Kwiatkowski and Manning 2005; Marcotte and Crino 2006). However, in rodent model systems, inactivating mutations of either TSC1 or TSC2 increases cancer incidence, possibly due to secondary mutations (Everitt et al. 1992; Kobayashi et al. 1999; Kwiatkowski et al. 2002).

If gain of function in TOR is necessary but not sufficient for transformation, what then are the other necessary, complementing oncogenic activities that originate with PI3K signaling? There likely will be several. A possible candidate is one of the multiple targets of AKT: NFκB. The transcriptional activity of NFκB is upregulated in AKT-transformed cells. This increased function is dependent on AKT and is abolished by small molecule inhibitors of AKT and by a dominant negative mutant of AKT (Bai et al. 2009). In AKT-transformed cells, the total amount of IκB inhibitor protein is dramatically decreased. Blocking NFκB activity with the super-repressor of NFκB (IκBSR) induces a cellular resistance that is selective for PI3K- and

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AKT-induced transformation. Thus, NFκB activity is essential for the oncogenicity of PI3K and AKT. Although there is no general agreement on how AKT communicates with NFκB, the balance of the evidence supports the idea of a phosphorylation cascade that connects the two proteins. AKT can phosphorylate IKKα (IκB kinase) *in vivo* (Ozes et al. 1999), and the activated IKK complex then phosphorylates the p65 subunit of NFκB (Sakurai et al. 1999), enhancing its transcriptional activity. It is, however, possible that the essential requirement for NFκB in PI3K-induced transformation can be satisfied with a basal level of activity and that the AKT-mediated gain of function represents a secondary consequence of transformation.

The identification of essential components in the oncogenic pathway is important for therapeutic considerations and helps define suitable drug targets. In this regard, the catalytic subunit p110 of PI3K remains a strong candidate, but more understanding of isoform-specific functions in various cancers and cell types and on different contributing genetic backgrounds (e.g. gain of function in receptor tyrosine kinases, loss of function in PTEN) is needed (Garcia-Echeverria and Sellers 2008; Kong and Yamori 2008; Maira et al. 2008; Wymann and Schneiter 2008; Yap et al. 2008). TOR emerges as another promising drug target (Guertin and Sabatini 2009). Small molecule inhibitors of TOR would also block the effects of AKT-independent signaling to TOR. The situation with AKT is more complex, because not all PI3K-driven tumors show AKT dependence (Vasudevan et al. 2009). Our understanding of the oncogenic signals emanating from PI3K is still evolving. New pathways and feedbacks are being characterized and novel interacting proteins discovered. Tissue- and cell-type specific differences in PI3K signaling are being defined. All these advances will eventually result in the recognition of new drug targets.

Conclusion

PI3Ks have oncogenic potential. The requisite gain of function can be achieved by mutation or by differential expression. Oncogenicity is mainly associated with class I PI3Ks and correlated with the ability to produce PIP_3. PIP_3 is the critical PI3K product that links lipid kinase activity to a network of downstream signals originating in AKT. In sensitive experimental systems, gain of function in a single PI3K isoform is sufficient to induce oncogenic transformation. These systems can be used for quantitative determination of oncogenicity and serve as models for investigations of transformation-associated changes in the cellular phenotype. Transformed focus assays in cell culture remain the gold standard for measuring and comparing oncogenic activity; such assays include all four isoforms of class I PI3K and can yield valuable data on the anti-oncogenic potency of drug candidates. The experimental systems of PI3K-induced oncogenic transformation also allow determination of specific changes in signaling pathways, cell behavior and metabolism.

The PI3K pathway is deregulated in the majority of human cancers. In sporadic tumors and in cancer cell lines, there are numerous other genetic and epigenetic changes that have been extensively documented by the human cancer genome project (Cancer Genome Atlas Research Network 2008; He et al. 2008; Jones et al. 2008; Parsons et al. 2008; Wood et al. 2007). In these situations, PI3K signaling can be expected to make a contribution to the oncogenic phenotype of the cell, but rarely will it function as the sole or dominant transforming event. The oncogenic phenotype of human cancer is the composite of all genetic and epigenetic changes. Experience with inhibitors of PI3K reflects this complexity. The growth of cells experimentally transformed by PI3K is generally highly sensitive to PI3K inhibitors. Few human cancer cell lines show such sensitivity. However, combinations of inhibitors that target critical nodes in cellular signaling or single inhibitors that target a critical combination of oncoproteins can be very effective (Cheng et al. 2009; Fan et al. 2009; Fan et al. 2007; Fan et al. 2006; Fan and Weiss 2006; Jaiswal et al. 2009; Nelander et al. 2008). These observations are detailed in other chapters of this book.

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The greatest challenge in the area of PI3K oncogenicity remains the identification of mutant-specific inhibitors that have drug-like properties. The highly targeted therapeutic potential of such inhibitors justifies intense efforts by industry and in academic laboratories.

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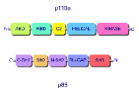

Figure 1.
Domain structure of catalytic and regulatory subunits of PI3K.
ABD, adaptor-binding domain; RBD, RAS-binding domain; C2, C2 domain; HELICAL, helical domain; KINASE, kinase domain; C-SH2, C-terminal SH2 domain; iSH2, inter-SH2 domain; N-SH2, N-terminal SH2 domain; RhoGAP, Rho GTPase-activating protein homology domain; SH3, SH3 domain.

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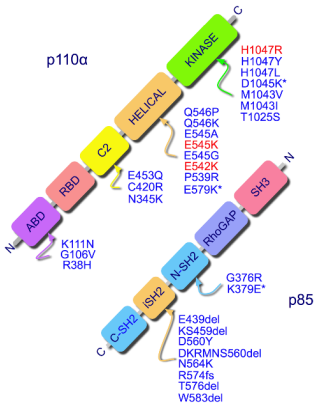

Figure 2.

Cancer derived and engineered gain-of-function mutations in p110α and in p85.

p110α: The hot-spot mutations are in red, rare mutations in blue; engineered gain-of-function mutations are marked by an asterisk.

p85: The engineered gain-of-function mutation is marked by an asterisk.

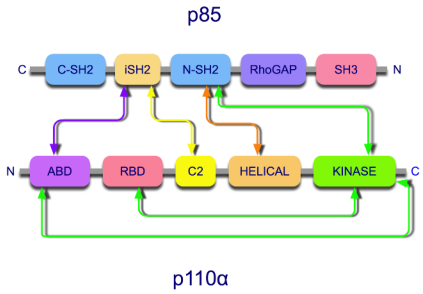

Figure 3.

p110α-p85 domain interactions of importance for gain-of-function mutations. Green arrows mark interactions with the kinase domain that could explain why the H1047R mutant is RAS-independent, but p85-dependent. The orange arrow signifies the inhibitory interaction between the N-terminal SH2 domain of p85 and the helical domain of p110α that is released by the helical domain mutation in p110α and the N-terminal SH2 domain mutation in p85. The orange and yellow arrows mark similar, probably inhibitory interactions between the inter-SH2 domain of p85 and the C2 or adaptor-binding domain of p110α, respectively. These interactions may be modulated by mutations in the respective domains of p110α.

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