Published in final edited form as:

Mol Cancer Res. 2021 April ; 19(4): 543–548. doi:10.1158/1541-7786.MCR-20-0818.

Phosphorylation and Driver Mutations in PI3K α and PTEN Autoinhibition

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Abstract

PI3K and PTEN are the second and third most highly mutated proteins in cancer following only p53. Their actions oppose each other. PI3K phosphorylates signaling lipid PIP₂ to PIP₃. PTEN dephosphorylates it back. Driver mutations in both proteins accrue PIP₃. PIP₃ recruits Akt and PDK1 to the membrane, promoting cell cycle progression. Here we review phosphorylation events and mutations in autoinhibition in PI3K and PTEN from the structural standpoint. Our purpose is to clarify how they control the autoinhibited states. In autoinhibition a segment or a subunit of the protein occludes its functional site. Protein-protein interfaces are often only marginally stable, making them sensitive to changes in conditions in living cells. Phosphorylation can stabilize or destabilize the interfaces. Driver mutations commonly destabilize them. In analogy to ‘passenger mutations’, we coin ‘passenger phosphorylation’ to emphasize that the presence of a phosphorylation recognition sequence logo does not necessarily imply function. Rather, it may simply reflect a statistical occurrence. In both PI3K and PTEN autoinhibiting phosphorylation events are observed in the occluding ‘piece’. In PI3K α the ‘piece’ is the p85 α subunit. In PTEN it is the C-terminal segment. In both enzymes the stabilized interface covers the domain that attaches to the membrane. Driver mutations that trigger rotation of the occluding piece or its deletion prompt activation. To date, both enzymes lack specific, potent drugs. We discuss the implications of detailed structural and mechanistic insight into oncogenic activation and how it can advance allosteric precision oncology.

Keywords

K-Ras; KRAS; passenger mutations; kinase recognition sequences; kinase consensus sequences; phosphosites; PI3K

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The authors declare no potential conflicts of interest.

Introduction

Phosphatidylinositol 3-kinase α (PI3K α) lipid kinase and phosphatase and tensin homologue (PTEN) phosphatase are essential enzymes in the cell. Activated PI3K α phosphorylates signaling lipid phosphatidylinositol 4,5-bisphosphate (PIP₂) to phosphatidylinositol 3,4,5-trisphosphate (PIP₃) (1). PTEN dephosphorylates PIP₃ back to PIP₂. The critical importance of the enzymes that regulate PIP₃ levels is seen from the prevalence of their mutations (2). The frequencies of oncogenic mutations in PI3K α (*PIK3CA*) and PTEN rank second and third in cancer, next only to p53. Both are components of the K-Ras signaling system. Unlike Raf and PI3K α , PTEN is not activated directly by Ras. However, because it modulates the outcome of PI3K α activation, it is a critical component of the Ras circuitry. PIP₃ recruits Akt and phosphoinositide-dependent protein kinase 1 (PDK1) to the plasma membrane (1). Through a kinase signaling cascade involving the mammalian target of rapamycin complex (mTORC), the outcome promotes cell cycle progression. The other major effector of Ras, Raf protein kinase, acts through the mitogen-activated protein kinase (MAPK) cascading kinases. At the ‘bottom’ of the pathway, phosphorylated extracellular signal-regulated kinase (ERK) (3) also acts to promote progression of the cell cycle. Its role in cell division complements PI3K cell growth action.

The PI3K structural studies of Roger Williams and his co-workers embody remarkable mechanistic insight that still stands out today (4–7). Mario Amzel and Sandra Gabelli have also carried out seminal structural work (8). PI3K α activation mechanism has only been published in 2019 (9) and 2020 (10), as well as the role of Ras in it (11). As to PTEN, there are structural data, crystal structure (12) and more recently hydrogen-deuterium exchange mass-spectrometry (HDX-MS) data as well (13,14). However, detailed mechanistic insight into PTEN phosphatase also still lags, and to date no specific effective drugs in the clinics for either. This may partly reflect their catalytic reactions on small lipids at the membrane. For PTEN it is particularly challenging since the drugs would need to activate – not deactivate – it. For PI3K the difficulty lies in the high homology/identity of the active site among isoforms and the shared features of the ATP binding cavities.

Among the aspects not fully understood, especially in PI3K α , is phosphorylation. PI3K α and PTEN have multiple phosphorylation sites (15). Here we focus on phosphorylation sites and driver mutations playing a role in autoinhibition. We suggest that the reason for multiple identified phosphorylation sites and the failure to assign clear functional roles to some of them could be that there simply may not be any. Computationally, phosphorylation sites are largely identified based on short sequence logos, although contextual information is also considered. Statistically, with multiple kinases in the cell and across tissues, non-functional occurrences of such ‘passenger phosphorylation’ logos can be expected although they may not stand out in sequence alignments. The ultimate identification of phosphorylated sites is by experiment. However, observations can be controversial, probably due to different protocols, including cell lines.

Below, we discuss the questions of (i) why phosphorylation and driver mutations evolved at autoinhibition interfaces and (ii) how detailed structural knowledge of regulation and activation mechanisms can suggest venues in allosteric drug development.

Phosphorylation in Autoinhibition

Phosphorylation is common in autoinhibition and the reason for this can be straightforwardly understood (16). In autoinhibition a segment of the protein, as in PTEN, or a subunit, as in PI3K α , covers the active site thereby shielding the protein from degradation (17,18). The kinetic barriers between the active and autoinhibited states are low and surmountable under physiological conditions. The interfaces between the regulatory segment (or subunit) and the kinase or the catalytic domain are generally weak, facilitating the tipping of the stability scales toward exposure of the active site and activation upon receipt of a signaling cue. Under physiological conditions, PI3K α and PTEN are mostly in the autoinhibited state. However, with small differences in energy, a certain fraction of the population can switch to the active state even in the absence of an external cue.

The role of phosphorylation is to stabilize or destabilize interactions. From the standpoint of evolution, exploiting phosphate groups is a pragmatic solution. It allows the interfaces to be weak and easy to modulate. At the same time, phosphorylation events are straightforward to manipulate via a panoply of kinases and phosphatases, and they provide an extra layer of control. In both PI3K α and PTEN, some phosphates strengthen the autoinhibited states by locking favorable interactions. As we show below for PI3K α , the consensus logo of the kinase recognition sequences around the phosphates may also help stabilizing the interaction (Fig. 1 and Fig. 2). However, the presence of a phosphosite does not necessarily imply that it has a functional role. It may simply reflect a statistical ‘passenger phosphorylation’ probability. With multiple kinases in the cell, a ‘passenger phosphosite’ may be phosphorylated and observed experimentally, which may explain the unsuccessful efforts to determine its function. One such possible example is phosphorylation sites pTyr368, pTyr508, and pTyr580 in the p85 α regulatory subunit of PI3K α . Early on, they were identified as major Tyr phosphorylation sites following overexpression of p85 α and insulin receptor (IR) in Cos7 cells (19,20). However, they were not observed to change the PI3K α activity and the biological relevance of the sites has been controversial. Further attempts by Barbara Geering, whose comprehensive 2006 dissertation focused on PI3K class IA phosphorylation and expression levels, failed to assign specific functional role for these sites (20). Our structural modeling of pTyr368, pTyr508, and pTyr580 indicates that the corresponding phosphates would be exposed to the solvent with no contacts with any residue in the p110 α catalytic subunit, thus unlikely to act in autoinhibition.

Phosphorylation in PI3K α

Information relating to PI3K α phosphorylation sites, the roles they play and how they play them is still incomplete. It is especially meager for p110 α . For p85 α , observations point to cell-specific kinases’ involvement. Early on, p85 α serine phosphorylation was documented, with scant data on tyrosine phosphorylation (Fig. 1A–B). p110 α and to a lesser extent p110 β phosphorylate p85 α on Ser608 in the iSH2–cSH2 linker, decreasing catalytic activity

in vitro (21). Mutation of Ser608 to Ala or Glu decreased p110 α binding (21). Ser608 phosphorylation may regulate activity by stabilizing the autoinhibited state. The C-lobe of the kinase domain contains basic residues, and the phosphorylated Ser608 and its flanking acidic residues may interact with those basic residues. Contrasting the earlier work, a more recent study observed that autophosphorylation of Ser608 does not affect its activity (22). These opposing observations can be explained by the different experimental protocols. Our modeling supports the earlier studies and provides interaction details (Fig. 1B–C) and can explain the frequent truncation of the C-terminal in cancer. It suggests that the adjoining highly negatively charged residues (⁶⁰¹ENTED⁶⁰⁵ and ⁶¹¹EDDED⁶¹⁵) in p85 α can interact with the basic residues in the activation loop of the C-lobe, stabilizing the autoinhibition. The similarities among class IA PI3Ks (PI3K α , PI3K β and PI3K δ) in sequence, structure, and autoinhibition/activation mechanism, suggest that the phosphorylated Ser608 and Tyr607 and their nearby acidic residues can stabilize the isoforms autoinhibition as well (Fig. 1D). Direct data on dephosphorylation is largely lacking. In 3T3-L1 adipocytes, protein phosphatase 2C (PP2C) dephosphorylates p85 α and promotes PI3K α activity, and it was proposed that it dephosphorylates Ser608 (23). p110 β and p110 δ autophosphorylate Ser residues in their C-termini (Ser1070 in p110 β ; Ser1039 in p110 δ) (20), also downregulating catalytic activity. Protein kinase A (PKA) phosphorylates p85 α on Ser83 (23). p85 α Tyr phosphorylation by Tyr kinase Fyn, but not Lck, was also observed in T cells. The phosphorylation enhanced PI3K α activity. Lck overexpression in T cells, and Bcr-Abl in lymphoblast cells phosphorylate p85 α Tyr688 in the cSH2. The reduction in PI3K α activity suggests that Fyn, Lck and Abl do not phosphorylate the same tyrosine (20). Subsequent studies did not observe a change in activity following Tyr phosphorylation by Bcr-Abl. However, interactions with other proteins were changed. Further detailed data have been compiled and tested (20). Overall, observations are sometimes conflicting, and a coherent mechanistic picture is still unavailable possibly due to the presence of both functional and statistical passenger phosphosites. The lack of clear separation between these categories renders the data challenging to understand. This is further mired by the expectation that the function of some sites can be cell specific whereas only a couple of cell lines were probed.

The scarcity of data on phosphorylation of p110 α could reflect their rarity. A bulky negatively charged group in the catalytic domain could negatively impact its interaction with the negatively charged phospholipids. Instead, the phosphates reside on the interaction surface of p85 α , which rotates away in activation (9).

Phosphorylation in PTEN

The PTEN protein includes the PIP₂-binding motif (PBM, residues 1-15, whose basic residues bind anionic membrane lipids), phosphatase domain (PD, residues 16-185), and the C2 domain (residues 190-350), followed by the carboxy-terminal tail (CTT) and the PDZ binding motif (Fig. 2A). The disordered CTT is key to PTEN regulation (14). Phosphorylation of Ser362 (15), Thr366, Ser370, and the 380–385 cluster (Ser380, Thr382, Thr383, and Ser385) in the CTT are crucial for maintaining PTEN in a stable cytosolic state (14). Casein kinase 2 (CK2) and glycogen synthase kinase β (GSK β) can phosphorylate these sites *in vitro* (24), and other kinases such as polo-like kinase 3 (PLK3) may too (25). The phosphorylated tail mimics the phospholipid membrane (26). It interacts with the C2

and phosphatase domains, with the 380–385 cluster interacting with the Ca²⁺ loop (residues 321–342) and CBR3 loop (residues 258–268) of the C2 and the arginine loop (residues 35–49) of the phosphatase domain. These interactions, verified by hydrogen-deuterium exchange mass-spectrometry (HDX-MS) data (13), result in the ‘closed’ autoinhibited state (Fig. 2B). The roles of Thr366 and Ser370 phosphorylation sites are not entirely clear, although they can interact with the TI loop (13). PTEN is also phosphorylated by Src tyrosine kinases (27–29) in the C2 domain at Tyr240 and Tyr315 (15). The detailed mechanisms and conformational changes are also still unclear.

Driver Mutations in Autoinhibition

Mutations that relieve the autoinhibition of proteins involved in cell proliferation are drivers of cancer (16,30,31). Mutations can be at the interface in which case they are ‘orthosteric’ drivers. However, mostly they are allosteric drivers. They act indirectly, by conformational changes that weaken the interface between the autoinhibiting segment and the catalytic domain. These mutations can drive cancer since the difference in stabilities between the autoinhibited and the active states is small.

Driver mutations in PI3K

Mutations in class I PI3K catalytic subunit p110 α can confer a strong gain of function (32,33). They act through two main mechanisms (9): (i) relieving the autoinhibition by shifting the equilibrium toward an ‘open’ state with the exposed active site at the membrane and (ii) promoting the interaction with the membrane. For (i), examples include the E542K and E545K charge reversal mutations in the helical domain (Fig. 1B). These mutations mimic the interaction of the receptor tyrosine kinase (RTK, such as PDGFR) pYxxM motif with the nSH2 in the p85 α subunit. This interaction triggers conformational changes that disrupt the iSH2–C2 interface, swivel the adaptor binding domain (ABD), and culminate with exposure of the active site at the membrane. The rotation of the iSH2 domain of p85 α is coupled with the interacting ABD of p110 α . Notably, the ABD has drivers as well, either at the interface or in the linker (Fig. 1B). None of the regions has direct contacts with the catalytic site. However, the outcome is active site exposure at the membrane, activation and catalysis. By breaking and/or forming interactions, allosteric events shift the ensemble toward the stabilized (here mutated) state (34–36). (ii) H1047R is an example for the second mechanism. It provides positive charges at the kinase domain surface promoting the interaction (11,37) (Fig. 1B).

Oncogenic mutations in the iSH2 and nSH2 domains promote proliferation with varied potency. They weaken the autoinhibitory interaction between p85 α and p110 α while retaining the stabilizing interaction between p85 α iSH2 and the ABD of p110 α (32). Apart from G376R in the nSH2 (32), the mutations cluster in the iSH2 domain and involve residues that interact with the p110 α C2 domain (38,39). Similar to mutations in the helical domain (9,40), mutations in the inhibitory iSH2–C2 interaction weaken it, shifting the equilibrium toward the open, exposed state (8,38,39,41). p85 α deletion mutants, KS459delN and DKRMNS560del induced high transforming activity, R574fs and T576del intermediate activity and the effects of the others were an order of magnitude lower (32).

Notably, a single strong driver cannot fully activate PI3K. Two weak drivers may or may not; and two, one weak and one strong (42) or multiple strong (43) drivers on the same allele, can promote phosphorylation of downstream proteins and tumor development (44). Under such circumstances, one (e.g. H1047R) can promote membrane attachment, substituting for Ras (11). The other reduces the transition state barrier (e.g. E542K or E545K) (45–47). Occurrence of two (or more) strong drivers is rare, since Vasan *et al.* (42) did not observe them. However, in a large landscape study, Saito *et al.* (43) did. In principle, two strong hotspot mutations may generate ~1,000-fold higher downstream Akt phosphorylation than the single mutations (42,45).

Mutations in PTEN also relieve the autoinhibition

PTEN deletion mutations are common, and modest alterations can promote cancer without mutations or loss of even one allele (15). With PIP₃ being the substrate, membrane binding can control PTEN (14). Different from PI3K α , PTEN mutations reduce the affinity to the membrane. Oncogenic mutations abolishing the C2–phosphatase domains interface (48) result in inactivation. Interface mutations S170N/G/I/R and R173C/H/L are common in cancer (Fig. 2C) (12).

Cancer-associated mutations are distributed throughout PTEN. The highest frequency mutations (R130Q/G) are in the lipid phosphatase catalytic region (49). Some reduce membrane association, like K13E which also impacts activation, as do S10N, G20E, L42R, and F90S, around the PIP₂-binding pocket (14,49) (Fig. 2C). Some at the C-terminal, including CTT deletions (14,50) cause tumor development (51,52). The mutations abolish the CTT interaction, expose the C2/DUSP surface enabling membrane interaction, PIP₃ binding and dephosphorylation to PIP₂. Mechanistic details of activation at the membrane, the role of some phosphorylations and deactivation by some mutations, are still unavailable. Also unclear is why some mutations are oncogenic, whereas others can promote other disorders, such as autism (53). With its dearth of drugs, PTEN presents a weighty and tantalizing target to decipher.

Detailed Mechanistic Insight Can Guide Innovative Pharmacology

Analysis of all class IA PI3K crystal structures resulted in a set of conformational changes including those in the activation loop that are associated with the presence *versus* the absence of the nSH2. The changes are also associated with oncogenic mutations (Fig. 3A–B) (54). Together, they suggest that an acidic patch on the nSH2 domain interacts with basic residues in the activation loop. When the nSH2 is displaced by the binding of a phosphorylated RTK, the activation loop is no longer constrained, and its basic residues can assume a conformation appropriate for recognizing the phosphates of the lipid substrate (Fig. 3C). The detailed structural analysis provides novel insight into the potential mechanisms that mediate activation in the absence of the nSH2 of p85 α (54). It identifies a specific conformation of the activation loop, a shift in the position of the α 11 helix and the structural basis for the nSH2 regulation of PI3K α (Fig. 3C). The extended activation loop accommodates the basic residues for substrate binding and phosphorylation (Fig. 3D). The importance of the nSH2 is borne out by cancer-derived mutations (32). The activation

mechanism suggests that iSH2 movement is vital not only to the active conformation but also to membrane interactions and can be considered in innovative allosteric drug discovery. C2 and ABD, both away from the catalytic site, are responsible for iSH2 movement. An allosteric drug could interfere with this movement by stabilizing the C2 and ABD conformations. Targeting pockets in the large ABD interface with the kinase domain (55), or in the C2/helical domain interface may be one way. Sterically impeding iSH2 movement could be another.

Detailed mechanistic insight may also consider combined orthosteric and allosteric drugs, and allosteric drugs at positions of allosteric rescue mutations (55). For the first, such drug combination has been shown in metastatic anaplastic lymphoma kinase (ALK), where a steric mutation (C1156Y) interfered with crizotinib binding. Subsequent treatment with lorlatinib, another ATP-competitive drug, led to steric interference by L1198F allosteric mutation. However, the mutation resensitized ALK to crizotinib (56). Allosteric compound GNF-5 successfully mimicked this allosteric mutation (57). G2032R mutation similarly resurrected crizotinib in ROS1-related cancer (58). Treatment of Bcr-Abl kinase in myeloid leukemia (CML) by ATP-competitive imatinib and nilotinib, led to steric clashes by T315. Emerging allosteric mutations re-sensitized Bcr-Abl (57,59). Like GNF drugs, allosteric asciminib in the myristoyl pocket of Abl1, can combine with orthosteric ponatinib (60), overcoming Y253H and E255V mutations (61). Additional examples of allosteric/orthosteric drug combinations are available (62,63). For the second strategy examples also exist, however to date they are untested (55). Allosteric drugs can mimic the conformational changes promoted by the mutations (64). Our discussion above suggests that rather than obtaining them by large-scale screening as in the case of GNF5, detailed mechanistic structural insight can also powerfully guide the drug search.

Cell signaling needs to be sensitive to the environment. This is possible only if the difference in the stabilities between the closed and open states is small (16,17). This principle clarifies autoinhibition and its release. In cancer, allosteric events can modify the expression pattern of the protein, or as we discuss here act via a covalent change involving e.g. phosphorylation or oncogenic mutations. Allosteric oncogenic mutations often act by relieving it. Phosphorylation events can act by stabilizing or by relieving it. Here we discussed autoinhibition in two critical enzymes in cell signaling whose functions oppose each other. Together, they strike an essential balance of a key signaling lipid. Both are sought-after drug targets. PTEN is particularly hard to target. Mutants often involve large (or entire) deletions, making them difficult to modulate. Catalytically, drugging would involve restoring function, which is hard to achieve. A network approach could be advantageous; but would require a comprehensive list of its related and alternative pathways. Nevertheless, progress is being made (65).

Acknowledgements

This project has been funded in whole or in part with federal funds from the National Cancer Institute, National Institutes of Health, under contract HHSN26120080001E. The content of this publication does not necessarily reflect the views or policies of the Department of Health and Human Services, nor does mention of trade names, commercial products, or organizations imply endorsement by the U.S. Government. This Research was supported [in part] by the Intramural Research Program of the NIH, National Cancer Institute, Center for Cancer Research.

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Figure 1.

Phosphorylation and diver mutations in PI3K autoinhibitions. (A) PI3Kα is an obligate dimer with the catalytic p110α subunit and the regulatory p85α subunit with the phosphorylation sites. (B) The phosphorylation sites stabilize, and the driver mutations destabilize the autoinhibition. The hotspot driver mutations (E542K, E545K in helical domain and H1047R in kinase domain) promote the nSH2 release and membrane interactions in PI3K activation. The weak driver mutations in ABD and C2 domain destabilize the ABD/KD and C2/iSH2 interfaces. (C) Modeling suggests that phosphorylation sites in p85α subunit (pS608 and the adjacent pY607) may enhance the autoinhibition by interacting with the basic residues in kinase domain (based on PDBID: 4OVV). (D) Sequence comparison indicates a general role of p85α phosphorylation in enhancing the autoinhibitions of the class IA PI3Ks (PI3Kα, PI3Kβ and PI3Kδ).

Figure 2.

Phosphorylation and diver mutations in PTEN autoinhibitions. (A) PTEN consists of the PIP₂-binding motif (PBM, 1-15), the phosphatase domain (PD, 16-185), the C2 domain (190-350), the carboxy-terminal tail (CTT) and PDZ binding motif. (B) The phosphorylation sites in CTT (pS380, pT382, pT383, pS385) interact with the arginine loop (35-49) in PD, and CBR3 (258-268) and Ca2 loop (321-342) in C2, resulting in the “closed” conformation for enhancing the autoinhibition. (C) The driver mutations reducing the membrane interactions (S10N, K13E, G20E, L42R and F90S), interfering with the catalysis (R130Q/G) and breaking the PD/C2 interface (S170N/G/I/R and R173C/H/L) in PTEN decrease the PTEN activities.

Figure 3.

Structural insights into PI3K α activation by nSH2 release. (A) nSH2 domain in p85 α subunit interacts with the p110 α subunit, maintaining the "closed" autoinhibited conformation (PDBID: 4OVV). (B) nSH2 release by phosphorylated tyrosine motifs in RTK results in the "open" activated conformations (PDB ID: 5DXH). The iSH2 domain moves and exposes the kinase domain surface for catalysis. (C) The acidic patch of nSH2 domain (D337, E341, E342, and E345) in "closed" autoinhibited conformation interacts with the basic boxes and confine a "collapsed" activation loop. Upon nSH2 release, the activation loop becomes extended. (D) The extended activation loop gathers the basic residues (⁹⁴¹KKKK⁹⁴⁴ in activation loop and K776 in P-loop) for substrate binding in phosphorylation (modeled based on PDB ID: 5DXH, 2Y3A and 1E8X).