Following the trail of lipids: Signals initiated by PI3K function at multiple cellular membranes

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Phosphatidylinositol 3,4,5-trisphosphate [PtdIns(3,4,5)P3] is the signaling currency of the phosphoinositde 3-kinase (PI3K)/AKT pathway; transduction through this axis depends on this signaling lipid. Formation of PtdIns(3,4,5)P3 is dictated not only by PI3K activation but also by the localization and access of PI3K to its substrate PtdIns(4,5)P2 (phosphatidylinositol 4,5-bisphosphate). PI3K/AKT-mediated signaling is antagonized by PtdIns(3,4,5)P3 dephosphorylation. Although previously typically considered an event associated with the plasma membrane, it is now appreciated that the formation and metabolism of PtdIns(3,4,5)P3 occur on multiple membranes with distinct kinetics. Modulated activity of phosphatidylinositol lipid kinases and phosphatases contributes to intricately orchestrated lipid gradients that define the signaling status of the pathway at multiple sites within the cell.

Introducing the PI3K/AKT Pathway

The response to extracellular mitogenic stimuli requires the transduction of signals from the cell periphery to the interior of the cell. Extracellular mitogenic signals bind and activate receptor tyrosine kinases at the plasma membrane, thereby recruiting and subsequently activating phosphoinositide 3-kinases (PI3Ks). Activated PI3K catalyzes the formation of phosphatidylinositol 3,4,5-trisphosphate [PtdIns(3,4,5)P3]. AKT and the kinase PDK1, which phosphorylates and activates AKT, both bind PtdIns(3,4,5)P3 and are brought into proximity at sites where PtdIns(3,4,5)P3 is abundant, thereby instigating their signal transduction properties. The PI3K/AKT signaling axis transduces signals generated by a plethora of extracellular stimuli and modulates multiple cellular processes, including metabolism, proliferation, survival, and protein synthesis (1, 2). Components of the PI3K pathway contribute to one of two main functions: the synthesis of PtdIns(3,4,5)P3 or the interpretation of or response to the lipid signals generated.

Enzymatic control of the synthesis and metabolism of PtdIns(3,4,5)P3 is regulated by mitogens. Class I PI3K enzymes produce PtdIns(3,4,5)P3 (3) by catalyzing the addition of phosphate to the substrate PtdIns(4,5)P2 (phosphatidylinositol 4,5-bisphosphate) (4). Hydrolysis of PtdIns(3,4,5)P3 is mostly, although not exclusively, achieved by two classes of enzymes. One is known as phosphatase and tensin homolog, deleted on chromosome 10 (PTEN), which dephosphorylates PtdIns(3,4,5)P3 at the 3 phosphate position of PtdIns(3,4,5)P3, the substrate of phosphatidylinositol 4-kinase PIP5K1 (5). The other is the SH2 (Src homology 2)-containing inositol phosphatase (SHIP) enzymes, which hydrolyze the 5' position of PtdIns(3,4,5)P3, thus generating PtdIns(3,4)P2 (6). Consequently, the locale of active class I PI3K, the accessibility of PI3K to their PtdIns(4,5)P2 substrate, and the subcellular distributions of PTEN, SHIP, and other PtdIns(3,4,5)P3-dephosphorylating enzymes define the spatiotemporal activity of the PI3K/AKT axis.

In addition to AKT-mediated mitogen-induced signaling, PI3K-dependent formation of PtdIns(3,4,5)P3 contributes to cellular and tissue organization. Distinct pools of PtdIns(3,4,5)P3 in the plasma membrane control such processes as the formation of the basolateral membrane in epithelial cells (7), orientation of the mitotic spindle in adherent cells (8), and formation of the axonal growth cone in neurons (9). Furthermore, processes specific to the plasma membrane environment, such as phagocytosis, depend on acute and controlled enrichment of, among other phosphoinositides, PtdIns(3,4,5)P3 (10).

Beginning with PtdIns(4,5)P2 at the Plasma Membrane

The PtdIns(3,4,5)P3 precursor lipid PtdIns(4,5)P2 is paramount for mitogen-induced signal transduction by the PI3K/AKT pathway. As the lipid substrate from which PtdIns(3,4,5)P3 is formed, PtdIns(4,5)P2 must be present for signal transduction to occur. Thus, the availability and localization of PtdIns(4,5)P2 is key when considering PI3K/AKT pathway signal propagation and intensity.

PtdIns(4,5)P2 is the most abundant polyphosphoinositide in mammalian cells with its concentration highest at the inner leaflet of plasma membrane where it comprises ~1 to 2% of total phospholipids in the systems where it has been measured (11, 12). Subsequent to mitogen stimulation, minimal changes in total cellular amounts of PtdIns(4,5)P2 occur (13). Despite the high abundance of PtdIns(4,5)P2 in the plasma membrane, passive localized enrichment is unlikely because lateral diffusion of newly synthesized PtdIns(4,5)P2 occurs more rapidly than its synthesis (12). Inherent in these observations are several key concepts. Primarily, resting cells have the necessary concentration of precursor lipid such that in times of growth factor-–induced stimulation, sufficient class I PI3K substrate is available for PtdIns(4,5)P2 synthesis. The accumulation of PtdIns(4,5)P2 in a localized vicinity requires processes in addition to PtdIns(4,5)P2 synthesis itself. Mechanisms for localized enrichment of PtdIns(4,5)P2 include the binding of PtdIns(4,5)P2 to anchor proteins, “corralling” of PtdIns(4,5)P2 in lipid substructures called lipid rafts, and the recruitment of PtdIns(4,5)P2–producing enzymes into specific regions (12–15).

PtdIns Flux in Membrane Ruffles

Plasma-membrane enrichment of PtdIns(4,5)P2 is common, occurring at such sites as membrane ruffles and the phagosomal cup (12, 16). Proline-rich inositol polyphosphate 5-phosphatase (PIPP), which has 5-phosphatase activity for PtdIns(3,4,5)P3, exhibits a predominant membrane ruffle distribution, both in the absence and presence of growth factors (17, 18). Skeletal muscle– and kidney-enriched inositol phosphatase (SKIP), which also has 5-phosphatase activity for PtdIns(3,4,5)P3, is predominantly enriched at the endoplasmic reticulum (17). Upon stimulation with epidermal growth factor (EGF), SKIP redistributes to membrane ruffles in a transient manner (17). Membrane ruffles are structures involved in cell motility, and the role of PtdIns lipids in that process is a well-characterized process (19). The remainder of this Review will focus on PtdIns lipid flux in the clathrin-mediated endocytosis pathway and how these lipids are synthesized and metabolized upon internal membranes.
PtdIns Flux in Clathrin-Coated Membranes

Forming a pool of class I PI3K substrate in clathrin-coated pits

Of particular relevance to growth factor–induced PI3K/AKT-mediated signaling is the sequestration of PtdIns(4,5)P$_2$ into the membrane of clathrin-coated pits. Clathrin-coated pits form at the plasma membrane and are precursor structures to the vehicles of clathrin-mediated endocytosis, the clathrin-coated vesicle (20). Clathrin-coated vesicles internalize a wide range of molecules, including nutrients, growth factors, and receptors (14). With the exception of clathrin itself, most of the protein components required for clathrin-coated pit nucleation, maturation, and fission directly bind PtdIns(4,5)P$_2$ (11, 21). The recruitment efficiency of clathrin-coated pit nucleation factors, including the lipid PtdIns(4,5)P$_2$ and the adaptor protein AP-2, is enhanced at “hotspots” in the plasma membrane, which are determined by the presence of cortical actin (22). The cortical actin network concentrates plasma membrane PtdIns(4,5)P$_2$ in specific regions of the membrane to increase clathrin-coated pit-forming potential (Fig. 1).

PtdIns(4,5)P$_2$ regulates clathrin-coated pit assembly, with localized turnover of PtdIns(4,5)P$_2$ controlling several of the stages required for clathrin-coated vesicle formation (23). All three variants (α, β, and γ) of the main PtdIns(4,5)P$_2$-producing enzymes, phosphatidylinositol phosphate 5 kinases (PIPK5) (13), are detectable at the plasma membrane but not in clathrin-coated pits (23). Biochemical depletion of cellular PtdIns(4,5)P$_2$ results in fewer clathrin-coated pits (24). Conversely, synaptojanins, which are 5-phosphatidylinositol phosphatases that can use PtdIns(4,5)P$_2$ as a substrate (25), and oculocerebrorenal syndrome of Lowe (OCRL), another 5-phosphatidylinositol phosphatase with a preferred substrate of PtdIns(4,5)P$_2$ (26), are present in late-stage clathrin-coated pits (23, 27, 28). Consequently, these two lipid phosphatases can work in conjunction or independently, 

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**Fig. 1.** Phosphoinositide flux during mitogen-stimulated growth factor receptor signaling. Receptors bind ligands at the plasma membrane and accumulate at sites of clathrin-coated pit (CCP) nucleation (1). As the coated pit matures, the PtdIns profile changes, resulting in a peak of PtdIns(3,4,5)P$_3$ and AKT activation (2) due to PI3K associated with the activated receptor becoming activated within the maturing clathrin-coated pit. SHIP2 activity before vesicle fission terminates this signal. Synaptojanin and OCRL (3), which hydrolyzes PtdIns(4,5)P$_2$ producing PtdIns(4)P, are active in clathrin-coated vesicles (CCVs), enabling recruitment of uncoating proteins and removal of clathrin (4). Uncoated vesicles, now absent OCRL and synaptojanin, employ residual PtdIns(4,5)P$_2$ as a substrate for receptor-associated PI3K to generate a second burst of AKT signaling (5). Fusion with the early endosome (EE) compartment, which is enriched in PtdIns(3)P, recruits PTEN, thereby diminishing this second peak of PtdIns(3,4,5)P$_3$ accumulation. The upper graph represents AKT activity and the activity of the lipid phosphatases during the various stages of clathrin-mediated endocytosis. The lower graph represents the relative abundances of the various phosphoinositides during clathrin-mediated endocytosis. Note: The phosphoinositide abundances are relative for each individual lipid type. For example, the “high” concentration of PtdIns(3)P is orders of magnitude greater than the “high” concentration of PtdIns(3,4,5)P$_3$. The events that occur at the cell surface are shown at the top of the figure, and the intracellular events are shown between the two graphs.
depending on the cellular context, to reduce PtdIns(4,5)P$_2$ concentrations at this spatiotemporal junction. Synaptojanins are also associated with clathrin-coated vesicles. Animals with synaptojanin 1 knockout exhibit an accumulation of clathrin-coated vesicles, indicating reduced clathrin coat removal (29), a phenotype that also occurs in human patients lacking OCRL (30).

A model based on these data is that PtdIns(4,5)P$_2$ in the plasma membrane above a certain threshold is required for clathrin-coated pit formation and that a localized reduction in PtdIns(4,5)P$_2$ is necessary for maturation of the clathrin-coated pits into clathrin-coated vesicles and for clathrin uncoating. The observed reduction of PtdIns(4,5)P$_2$ within clathrin-coated pits correlates temporally with their growth and maturation (21). The mechanism by which PtdIns(4,5)P$_2$ hydrolysis contributes to clathrin uncoating involves heat shock protein 70 (HSP70), the protein that mediates clathrin release. HSP70 is guided to clathrin-coated vesicles by its J domain–containing partner auxilin, which interacts with clathrin-coated vesicles by binding to PtdIns(4)P (30), the product of synaptojanin- or OCRL-mediated hydrolysis of PtdIns(4,5)P$_2$.

Clathrin-mediated endocytosis shuttles PtdIns(4,5)P$_2$, and lipids derived from this precursor, from the plasma membrane into internal membranes. During clathrin-mediated endocytosis, PtdIns(4,5)P$_2$ concentrations are highest at the site of clathrin-coated pit formation, when free diffusion of lipids between clathrin-coated pits and the cellular “sink” of PtdIns(4,5)P$_2$ (that is, the plasma membrane) can occur. The concentration diminishes thereafter as PtdIns(4,5)P$_2$-hydrolyzing enzymes are recruited and lipid diffusion between clathrin-coated membranes and the plasma membrane is halted after the process of fission. The uncoated vesicles represent a localized but limited pool of PtdIns(4,5)P$_2$ that is distinct from the high concentrations present in the plasma membrane. Generation of PtdIns(4,5)P$_2$, which occurs predominantly at the plasma membrane, has not been documented on clathrin-coated vesicles. The reduced concentration of PtdIns(4,5)P$_2$ in internal membranes, occurring as part of the clathrin-coated vesicle maturation and clathrin-uncoating process, acts as a limiter on subsequent signaling potential by generating a local and internal, but controlled and restricted, pool of lipid that can be converted into PtdIns(3,4,5)P$_3$.

Generating PtdIns(3,4,5)P$_3$ in clathrin-coated pits

Multiple growth factor receptors are internalized through clathrin-mediated endocytosis. For example, the activated EGF receptor (EGFR) is internalized through clathrin-mediated endocytosis (31). The activated receptor also interacts with various accessory proteins resident in clathrin-coated pits (32, 33). Platelet-derived GFR (PDGFR) is also localized to clathrin-coated pits in an active-phosphorylated state, and this accumulation in clathrin-coated pits coincides with a localized increase in both PI3K concentration and activity (34). Thus, clathrin-mediated endocytosis provides a mechanism by which both activated receptors, PI3K, and PtdIns(4,5)P$_2$ are sequestered in close proximity on a limited membrane.

Clathrin-mediated endocytosis is a dynamic process requiring specific biophysical changes to the membrane at various steps (Fig. 1). When evaluating the role of PtdIns flux in this process, I define the following stages: (i) clathrin-coated pit formation, (ii) clathrin-coated pit maturation into an invaginated structure, (iii) fission to release the mature pit into a clathrin-coated vesicle, (iv) and finally, removal of the clathrin coat to produce a mature uncoated vesicle ready to fuse with endosomes. Subsequent to clathrin removal, the vesicles eventually transition into the endosomal system by fusing with early endosomes.

Hydrolyzing PtdIns(3,4,5)P$_3$ in clathrin-coated pits

The production of PtdIns(3,4,5)P$_3$ is opposed by the molecule’s turnover. The hydrolysis of PtdIns(3,4,5)P$_3$, and therefore termination of PI3K/AKT-mediated signaling, starts immediately upon receptor activation and clathrin-coated pit formation. The PtdIns(3,4,5)P$_3$ phosphatase, SHIP2, is a ubiquitously expressed enzyme [in contrast, SHIP1 is primarily hematopoietic (35)] and is tethered to clathrin-coated pits by direct interaction with intersectin 1 (36), a modular scaffold that promotes the formation of clathrin-coated vesicles (37). SHIP2 accumulates in clathrin-coated pits but dissociates before vesicle fission and loss of clathrin (36). Consequently, SHIP2 is present to hydrolyze PtdIns(3,4,5)P$_3$ at the earliest stages of clathrin-coated pit formation. Because clathrin-coated pits also harbor active receptor-ligand complexes and exhibit PI3K activity (32–34), SHIP2 and the SHIP2 substrate PtdIns(3,4,5)P$_3$ are in close proximity at these sites. In SHIP2 knockdown cells, clathrin-coated pits exhibit a shorter lifetime than clathrin-coated pits in control cells. This shorter lifetime results from an increased accumulation of clathrin and more rapid maturation (36), suggesting that SHIP2, and presumably a reduction in PtdIns(3,4,5)P$_3$ or an increase in PtdIns(3,4)P$_2$ or both, controls the kinetics of clathrin-mediated endocytosis.

In addition to a role in clathrin-coated pit dynamics, SHIP-dependent PtdIns(3,4,5)P$_3$ dephosphorylation reduces AKT activation. Overexpression of SHIP2 reduces growth factor–stimulated AKT phosphorylation and activation, which are PtdIns(3,4,5)P$_3$-dependent (38). Unlike PTEN-null cells, which exhibit basal increases in PtdIns(3,4,5)P$_3$ and AKT phosphorylation in the absence of SHIP2, the increase in abundance of PtdIns(3,4,5)P$_3$ is limited to the period immediately after cell stimulation, and PtdIns(3,4,5)P$_3$ concentrations return to basal shortly after growth factor–induced signaling has been initiated (39, 40). These data suggest that SHIP2 antagonizes PI3K/AKT signaling at early stages of receptor activation, which may relate to the association of SHIP2 activity with clathrin-coated pits. Furthermore, these data outline the spatiotemporal layer of control of PtdIns(3,4,5)P$_3$ hydrolysis. One reason for the early association of SHIP2 with activated receptors in clathrin-coated pits might be to prevent PtdIns(3,4,5)P$_3$ diffusion out of pits into the adjacent plasma membrane. Such unchecked lipid diffusion might provide a pool of PtdIns(3,4,5)P$_3$ that escapes from the regulatory mechanisms that control PI3/AKT signaling.

SHIP2 activity cleaves PtdIns(3,4,5)P$_3$ to produce PtdIns(3,4)P$_2$. Yet, studies with the pleckstrin homology (PH) lipid-binding domain of AKT (PHAKT), which is routinely used as a probe to detect the presence of PtdIns(3,4,5)P$_3$ and which also binds PtdIns(3,4,5)P$_3$ (41), indicate PtdIns(3,4,5)P$_2$ does not accumulate in clathrin-coated membranes. Experiments with PTEN-null cells that are expressing PHAKT or PHBTK1 [the PH domain of BTK1, which is a highly specific PtdIns(3,4,5)P$_3$ probe (42)] show that endocytosed membranes are enriched for probe, but this signal is lost just before clathrin-coated vesicle fission (42), suggesting that clathrin-coated pit maturation and fission are preceded by a reduction in PtdIns(3,4,5)P$_3$ and PtdIns(3,4)P$_2$. These data illustrate several important points. First, PTEN is not responsible for this loss of PtdIns(3,4,5)P$_3$ in prefusion clathrin-coated pits. Second, the loss of PHAKT probe in a manner similar to that of PHBTK1 indicates that PtdIns(3,4)P$_2$ does not become enriched in clathrin-coated pits, at least to levels detectable with PH-based protein probes.

However, assessment of class II PI3K activity, which mediates the phosphorylation of PtdIns(4)P to generate PtdIns(3,4)P$_2$, has identified the role of these enzymes in the late stages of clathrin-coated pit maturation. Abrogation of class II PI3Kα activity by RNA interference or depletion of PtdIns(3,4)P$_2$, by INPP4B (an inositol 4-phosphatase) overexpression impedes clathrin-coated vesicle maturation, preventing fission (43). The necessity of a specific PtdIns(3,4)P$_2$-producing enzyme in late-stage clathrin-coated structures, which is either recruited or activated after SHIP2 dissociation, suggests that the reduction in PtdIns(3,4,5)P$_3$ and the increase in PtdIns(3,4)P$_2$ are temporally and functionally distinct events with different roles in clathrin-mediated endocytosis.
In addition to metabolizing PtdIns(3,4,5)P$_3$, the action of inositol 5-phosphatases in concert with inositol 4-phosphatases combines as a method of PtdIns(3)P production (44). Consequently, 5'-dephosphorylation of PtdIns(3,4,5)P$_3$ can initiate an enzymatic sequence that increases PtdIns(3)P abundance in clathrin-mediated endocytosis-derived membranes before fission. This process would predispose such membranes to fuse with the early endosome compartment, a compartment in part defined by high PtdIns(3)P concentrations.

**PtdIns Flux in the Endosomal Compartment**

**Generating PtdIns(3,4,5)P$_3$ on early endosomes**

Vesicles containing the EGFR or PDGFR internalized through clathrin-mediated endocytosis are positive for the guanosine triphosphatase (GTPase) Rab5a, which mediates fission with the early endosomes (45). These Rab5a-positive structures in the endosomal compartment contain active receptors (46–48) and recruit multiple signal-transducing effector proteins, including p85α (46, 49), a regulatory protein partner of class I PI3K heterodimer. Because p85α binds to the catalytic subunit of class I PI3K, this indicates that the Rab5a-positive endosomes also contain the catalytic subunit of class I PI3K. Chemical inhibition of the autophosphorylation properties of EGFR or PDGFR at the plasma membrane in the presence of growth factors and subsequent activation of the receptors by removal of the inhibitor after their transition into the endosomal compartment has dissected these signaling axes in detail. Recruitment of p85α, presumably as part of an active PI3K dimer, to endosomal membranes occurs in the absence of active plasma membrane receptor, and the endosome-originating signals elicit apoptotic and progrowth responses. Furthermore, PI3K inhibitors block the endosome-generated progrowth responses (46, 49). Thus, internal membranes that receive lipids and proteins through clathrin-mediated endocytosis have the requisite PI3K signaling substrate and catalytic activity to produce PI3K-mediated signals.

**Generating PtdIns(3,4,5)P$_3$ on APPL1-positive vesicles**

Not all internal membranes that shuttle into or comprise the early endosome compartment are equivalent; a myriad of endosomal vesicle type exist. One such population of internal membranes is that of the adaptor protein, phosphotyrosine interaction, PH domain, and leucine zipper containing 1 (APPL1)–positive endosome. APPL1 interacts with EGFR (50), exhibits a prosurvival function that requires endosomal localization, and mediates this antiapoptotic effect through AKT (51). Although APPL1 interacts with the EGFR (50), the interaction of APPL1 with the guanosine 5'-triphosphate (GTP)-bound (active) form of Rab5a (52, 53) is necessary for its association with vesicles derived from clathrin-mediated endocytosis (42). The tethering of APPL1 to Rab5a-positive structures is prevented in the absence of GTP-bound Rab5a or subsequent to GTP hydrolysis. Thus, the APPL1 association with the vesicles is dictated by the GTPase activity of Rab5a (52), and its interaction with EGFR may ensure that APPL1 associates with signaling-active vesicles.

APPL1-positive vesicles are distinct from “canonical” early endosomes, which are defined by the presence of early endosome antigen 1 (EEA1) and PtdIns(3)P; minimal colocalization of EEA1 and APPL1 is observed (42, 52). Instead, most APPL1-positive vesicles progress through other intermediate compartments, for example, WDF2-positive vesicles (42), before incorporation into the endosomal system.

Several data characterize APPL1-positive vesicles as a hub for PI3K/AKT signaling. APPL1 directly binds AKT with a higher affinity for AKT2 than AKT isoforms 1 and 3 (54). Stimulation of cells with insulin reduces the interaction between APPL1 and AKT2, and the active-phosphorylated form of AKT2 does not interact with APPL1 (54). These observations indicate that APPL1 is a binding partner for unphosphorylated (inactive) AKT2, from which AKT2 is released and activated. Thus, it might be predicted that APPL1 recruits inactive AKT2, and to a lesser degree the other AKT isoforms, to vesicles containing the active receptor and associated PI3K, where AKT becomes activated. A study in zebrafish also found colocalization of Appl1, Akt, and the Akt substrate glycogen synthase kinase 3β (Gsk-3β) in the same vesicular structures. Furthermore, knockdown of Appl1 reduced Akt and Gsk-3β phosphorylation (51). Diminished AKT phosphorylation and activation as well as the resulting abrogation of GSK-3β phosphorylation subsequent to Appl1 knockdown has been reproduced in vitro studies in human cell lines (42, 55).

APPL1 also interacts with p110α (54), the catalytic subunit of class I PI3K dimers, suggesting that APPL1 may also enhance PI3K activity associated with clathrin-coated vesicle-derived compartments. This function of APPL1 leads to a model in which two stages of PtdIns(3,4,5)P$_3$ production occur during endocytosis. The first burst of PtdIns(3,4,5)P$_3$ production occurs during clathrin-coated pit formation and maturation through the recruitment of PI3K to the activated receptors located in these structures, and this PtdIns(3,4,5)P$_3$ signal is quenched mostly by the activity of SHIP2. The second burst occurs after fission and loss of the clathrin coat, and is mediated by PI3K associated with the APPL1-positive vesicles.

During clathrin-mediated endocytosis of activated receptors, such as EGFR, only a portion of the activated receptors enter the APPL1-positive compartment; only a subset of EGFR and APPL1 co-localize (42, 52). However, tracking of the maturation of EGFR-positive structures in live cells shows that about two-thirds of total active EGFR (42) transits through APPL1-positive structures. Additionally, the residence time of activated receptors and PI3K in the APPL1-positive compartment correlates with the duration of the PI3K/AKT-mediated signal.

The half-life of APPL1-positive endosomes is dictated by the abundance of PtdIns(3)P at these structures. Inducible enzymatic reduction of PtdIns(3)P on endosomal membranes results in enlarged APPL1-positive structures with enhanced activated receptor residence time and correlates with an increase in the active form of signal transduction proteins (42). Recruitment of PtdIns(3)P-binding proteins, such as EEA1 and WDF2, correlates with loss of APPL1, which is consistent with PtdIns(3)P abundance as a terminating factor in the lifetime of APPL1-positive endosomes (42).

**Hydrolyzing PtdIns(3,4,5)P$_3$ on PtdIns(3)P-positive vesicles**

The APPL1 compartment functions as a site of PtdIns(3,4,5)P$_3$ production; however, PtdIns(3,4,5)P$_3$ generation does not go unchecked. OCRL is an APPL1 binding partner (27). Therefore, PtdIns(4,5)P$_2$ hydrolysis occurs at the APPL1-positive endosomes, which would prevent excessive PtdIns(3,4,5)P$_3$ production by limiting the availability of the PI3K substrate. Additionally, because structures derived from APPL1-positive endosomes enter into the PtdIns(3)P-rich early endosome compartment, the PI3K/AKT-mediated signaling period ends because PtdIns(3,4,5)P$_3$ is metabolized. These two processes—limiting PI3K substrate availability and increasing PtdIns(3,4,5)P$_3$ turnover—are not equivalent: the former limits the maximal signal that can be produced [that is, PtdIns(3,4,5)P$_3$] and thereby the signaling potential, whereas the latter terminates the signal.

PtdIns(3)P-enriched membranes form a second zone within which a high probability of PtdIns(3,4,5)P$_3$ hydrolysis exists. PTEN maintains low amounts of cellular PtdIns(3,4,5)P$_3$ (56) by dephosphorylating this lipid at the 3 position (5). PTEN is enriched on membranes with high concentrations of PtdIns(3)P. The interaction of the CBRR3 loop of PTEN with PtdIns(3)P tethers PTEN to these membranes (57). CBRR of PTEN is also required for maximal PtdIns(3,4,5)P$_3$ dephosphorylation; when overexpressed in
PTEN-null cells, CBR3-mutant PTEN fails to constitutively diminish AKT phosphorylation, unlike the wild-type protein (57). Thus, PTEN associated with PtdIns(3)P-rich membranes forms a roadblock—a region of PtdIns(3,4,5)P3 dephosphorylating activity—in the path of endocytosed, receptor-enriched, and PI3K-enriched membranes. Such a system in which PTEN quenches the signal on internal membranes provides a flexible and adjustable mechanism for regulation of growth factor–derived signaling in cells. Modulation of the localized generation of PtdIns(3)P might be useful for fine-tune PTEN activity. Multiple processes, including redistribution away from its substrate through altered production of PtdIns(3)P and phosphorylation-mediated inhibition of PTEN activity, reduce PTEN activity in cells exposed to various types of stress (58).

Exosomes originate from multivesicular bodies from the late endosomal system, which in turn matures from early endosomes (59). Some exosomes serve as an intercellular transport vehicle for PTEN (60). PtdIns(3)P-mediated tethering of PTEN provides a potential mechanism by which PTEN in the early endosomal pathway might become incorporated into vesicles destined to be released as exosomes.

**PtdIns Flux at the Endoplasmic Reticulum, Golgi, and Mitochondria**

PtdIns-dependent signaling and regulators of these signals have also been observed at various other internal membranes, including the endoplasmic reticulum, Golgi, and mitochondria. For example, the accumulation and hydrolysis of PtdIns(3,4,5)P3 at the Golgi occur after growth factor receptor stimulation (61), and PTEN associates with mitochondria and the endoplasmic reticulum (62), in addition to endosomal structures enriched for PtdIns(3)P. There are extensive interactions between and flux of molecules through the endosomal, endoplasmic reticulum, Golgi, and mitochondrial membranes. Evidence suggests that clathrin-mediated endocytosis of activated growth factor receptors may be a mechanism for delivering PTEN to these internal compartments, as well as suggesting a potential function for PTEN at these sites. For example, clathrin-dependent localization of EGFR to mitochondria occurs in murine fibroblasts (63), suggesting that PTEN may antagonize PI3K-dependent signaling by EGFR that reaches the mitochondria. EGFR-containing Rab5a-positive endosomes can also colocalize, as measured by microscopy, with endoplasmic reticulum. This observed colocalization is to a degree comparable to the colocalization of these endosomes with microtubules, the cytoskeletal structures that mediate endosomal movement in cells (64, 65). The exact nature of PTEN tethering to the endoplasmic reticulum remains to be elucidated, but localization of this protein at these membrane sites is consistent with Rab5a-positive (and therefore APPL1-positive) membranes intersecting a PTEN-positive compartment during endocytosis. It is possible that Rab5a-positive and also APPL1-positive membranes accumulate PTEN during the endocytic process and deliver PTEN to the endoplasmic reticulum.

**Implications for PtdIns Signaling from Multiple Membranes**

The production and accumulation of PtdIns(3,4,5)P3 resulting from PI3K activation are dynamic. Distinct membranes, containing both activated PI3Ks and PtdIns(4,5)P2 substrate, accumulate PtdIns(3,4,5)P3 at different rates, which are determined by multiple factors, including the presence of lipid phosphatases and lipid kinases, phosphoinositide concentrations, and the abundance of other proteins that regulate the activity of the lipid-metabolizing enzymes or mediate their recruitment. The PI3K-dependent accumulation of PtdIns(3,4,5)P3 enables AKT-mediated signaling. The location, timing, and degree to which this lipid accumulates at specific sites define how the signal propagates and the cellular response.

The PtdIns lipid profile of membranes during growth factor receptor endocytosis controls the intensity and the sites at which the receptor-mediated signaling will activate AKT (Fig. 1). At clathrin-coated pits, when activated P3Ks are tethered in close proximity to a high abundance of their substrate, the concentration of PtdIns(3,4,5)P3 rises sharply. Enrichment of SHIP phosphatases during clathrin-coated pit maturation diminishes the concentration of PtdIns(3,4,5)P3 before fission, acting to acutely limit signaling potential in this location at this time. The PtdIns(3,4,5)P3 equilibrium then shifts toward accumulation in membrane locations with high APPL1 content, leading to signal transduction before signaling membranes enter the PtdIns(3)P-rich early endosomal compartment, where PTEN serves to quench signaling. Signaling is not constant but tidal. A myriad of enzymatic and lipid components are choreographed to adjust the PtdIns(3,4,5)P3 equilibrium at different stages after receptor activation.

The mechanisms and processes described herein in the context of mitogen stimulation reveal that the PI3K/AKT signal occurs beyond the plasma membrane and that clathrin-mediated endocytosis of activated growth factor receptors provides a means to deliver mediators of the signal to the interior of the cell. Furthermore, as PtdIns(3,4,5)P3 formation by PI3K occurs at multiple intracellular membrane locations, similar to the events that occur during clathrin-mediated endocytosis, a finely tuned and orchestrated lipid flux also occurs, which defines the output of the pathway therein.

Thinking of the dynamics of the PI3K/AKT pathway in terms of PtdIns status has impact for cancer therapeutics targeting this pathway. Understanding that the signal through this axis is tidal, membrane-specific, and dependent on a myriad of protein and lipid components necessitates a more refined assessment of PI3K activity, which has been identified as a prime therapeutic target in various cancers. The understanding that a multitude of protein and lipid elements are essential for the correct quenching of PI3K/AKT signaling presents new therapeutic options. For example, promotion of VPS34 [the dominant the PtdIns(3)P-producing enzyme in mammalian cells] function in PTEN-positive cancer with oncogenic mutations in class I PI3K may be a viable strategy. In this same context, inhibition of MT1M or MTMR2, phosphatases that use PtdIns(3)P as a substrate to generate PtdIns(4,5)P2 (66, 67), may also prove a viable therapeutic strategy. Knowledge regarding the dynamics of the lipid signature associated with the growth factor–mediated PI3K/AKT pathway should be integrated into the rationale of therapeutic strategies targeting this pathway in cancer, as well as for the further dissection of how the cell responds to mitogen stimulation.

**REFERENCES AND NOTES**


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Following the trail of lipids: Signals initiated by PI3K function at multiple cellular membranes

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