Review

Lipid kinases play crucial and multiple roles in membrane trafficking and signaling

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Summary. Phosphotidylinositols (PIs) are known to play an essential role in membrane trafficking and signaling transduction. PIs serve multiple functions, such as recruitment of cytosolic proteins with PI phosphate (PIP) binding domains and modification of the physical properties of the membranes in which they reside. As substrates for phosphoinositide-specific lipases they function as a switch point in phosphoinositide metabolism. Recent work with epidermal growth factor receptor (EGFR) and colony stimulating factor-1 receptor (CSFR) has identified a possible connection between endocytosis of activated receptors and type-1 phosphatidylinositol-4-phosphate-5-kinase. Furthermore, serine/tyrosine phosphorylation of phosphatidylinositol-4-phosphate-5-kinase seems to be essential for its activities. Indeed, one of the products of the phosphatidylinositol-4-phosphate-5-kinases, PIP_2, has been shown to be involved in multiple steps of endocytosis, including the assembly of the clathrin coat, regulation of adaptor proteins, and production of endocytic vesicles via the regulation of dynamin. The discussion in this review focuses primarily on receptors with intrinsic enzymatic activity, specifically on receptor tyrosine kinases (RTKs). We will discuss their structure; mechanism of action and potential role in membrane trafficking and/or signaling through the regulation of phosphatidylinositol phosphate kinases.

Key words: Phosphotidylinositols (PI), Lipid kinases, Endocytosis, Signaling, PI4P5 kinase

Introduction

No cell lives in isolation. They are in fact highly responsive to stimuli from their environment. In multicellular organisms, cell death, survival and proliferation depend on an elaborate extra- and intracellular communication network. Signal transduction cascades mediate the sensing and processing of stimuli. These molecular switches in cells are constructed from receptors, enzymes, channels, and regulatory proteins, which detect, amplify and integrate diverse external signals.

Receptors are key elements in several intracellular processes (e. g., endocytosis, phagocytosis and secretion-exocytosis). For convenience, receptors may be sorted into four different types or classes: (1) G-protein linked receptors (e.g., receptors for glucagon and serotonin); (2) ion channels receptors (e.g., the acetylcholine receptor); (3) receptors with intrinsic enzymatic activity (e. g., epidermal growth factor receptor (EGFR) and insulin receptor (IR)) and, (4) receptors lacking intrinsic enzymatic activity (e.g. receptor for cytokines and interferon). The discussion in this review focuses primarily on receptors with intrinsic enzymatic activity, specifically on receptor tyrosine kinases (RTKs). We will discuss their structure, mechanism of action and potential role in membrane trafficking and/or signaling through the regulation of phosphatidylinositol phosphate kinases.

RTKs play an essential role in the regulation of protein phosphorylation, which in turn, will modulate an elaborate intracellular communication network that coordinates the growth, differentiation and metabolism of cells in several tissues and organs. RTKs form an important class of cell-surface receptors whose ligands are soluble or membrane-bound peptide/hormones, such as insulin and EGF. Binding of a ligand to this type of receptor stimulates the receptor's tyrosine activity, which subsequently activates a signal-transduction cascade leading to changes in cellular physiology and/or function, including cell proliferation and differentiation, cell death and survival, and membrane trafficking.

Some RTKs were identified in studies of human cancers associated with mutant forms of growth-factor receptors, which send a proliferative signal to cells even in the absence of growth factor (Zwick et al., 2002). All RTKs comprise an extracellular domain containing a ligand-binding site, a transmembrane α-helix, and a cytosolic domain that includes a region with protein

tyrosine kinase activity. Binding of ligand causes most RTKs to dimerize; the protein kinase of each receptor monomer then phosphorylates a distinct set of tyrosine residues in the cytoplasmic domain of its partner, a process termed autophosphorylation. Although these receptors exist as a dimer even in the absence of ligand, binding of ligand is required for autophosphorylation to occur.

The phosphotyrosine residues of the activated RTKs play an essential role in transducing hormone/growth factor signals to intracellular signaling molecules. Two different classes of proteins associate with the cytosolic domain of activated RTKs: (1) adapter proteins (e.g., growth factor receptor-bound proteins 2, 7 and 10 (Grb2,7 and 10)) that couple the activated receptors to other signaling molecules but have no intrinsic signaling properties; and (2) enzymes involved in signaling pathways, including p120 Guanosine Activated Protein (GAP)-Ras, Son of Sevenless (SOS), Phosphatidy-linositol (PI) 3-kinase, Phospholipase C (PLC)-γ-1, and PIP-kinase types I and II. These proteins recognize and interact with specific phosphotyrosine residues directly on the RTKs or through adapter proteins.

Insulin receptor

Insulin, insulin-like growth factors (IGFs) and their receptors play essential roles in growth and development, and in the maintenance of normal metabolic homeostasis. These receptors have been extensively reviewed elsewhere and are briefly discussed below (Roth et al., 1994; Liu and Roth, 1998; Pessin and Okada, 1999; Pessin and Saltiel, 2000; Whitehead et al., 2000, Watson and Pessin, 2001, Wiley and Burke, 2001, Saltiel and Pessin, 2002). While insulin is produced uniquely by the ß-cells of the pancreas, the IGFs are expressed ubiquitously, as are the IR and IGF-I receptor (IGF-IR). The IR and IGF-IR belong to a family of transmembrane receptor tyrosine kinases, each produced by expression of a distinct gene and expressed on the cell surface as a dimer of two alpha-beta chains joined by disulfite bridges to form a heterotetrameric receptor complex. Each ligand binds to its cognate receptor with high affinity. In addition, the expression of various receptors are often tissue specific. High levels of IR are found in liver and adipose tissue, whereas IGF-IRs are almost absent in liver and adipose tissue. This should partially explain some of the preferential effects of insulin on metabolic homeostasis.

Ligand binding to the receptor activates its intrinsic tyrosine kinase activity, resulting in autophosphorylation on tyrosine residues. These phosphotyrosine residues are docking sites for Src homology 2 (SH2)-containing molecules. The primary substrates that bind to both IRs and IGF-IRs include the insulin receptor substrates (IRS1-4) and Src homolog and Collagene homolog (Shc). IRS and Shc molecules interact with IR and IGF-IR at the juxtamembrane domains of each receptor and will become tyrosine-phosphorylated by the kinase

activity of each receptor. These phosphotyrosines exist in specific motifs, which in turn, are new docking sites for other proteins, such as PI3-kinase, Grb2, SH2 domain-containing inositol-5-phosphatase (SHIP2), and protein-tyrosine phosphatases1ß (PTP1-ß). PI3-kinase is a complex of a binding regulatory subunit and a catalytic subunit (see below). The regulatory subunits contain a SH2 domain, which will allow interaction with IRS molecules. Grb2 and other SH2-adapter molecules bind Shc and SOS, which is a key protein in the activation of Ras. These reactions lead to the activation of the PI3-kinase pathways and the Ras/Raf/mitogenic-activated protein (MAP) kinase pathways. The activation of the PI3-kinase pathways will produce a significant increase in PI(3,4,5)P$_3$ lipids. These lipids, in turn, will lead to activation of protein kinase B (PKB) or Akt. PKB/Akt is an essential element in the regulation of several intracellular processes, including endocytosis, apoptosis, cellular proliferation, glycogen synthesis and glucose transport. Function of the PI3-kinase/Akt pathway is also determined by phosphatidylinositol-3-phosphatases such as tensin homologue and SHIP2. Overexpression of these enzymes leads to decreased levels of PI(3,4,5)P$_3$. This might terminate signal transduction and/or change the nature of the phosphoinositides, altering the binding specificity to Pleckstrin (PH) or phox (PX) homology domains. Distribution of these genes or reduced expression of these messenger RNAs yields mice with increased insulin sensitivity. These are both essential for the biological responses of the IR and IGF-IR. EGFR and IR belong to the same family; share a structural similarity and are involved in similar intracellular signaling events. However, the IR and EGFR regulate different processes in metabolism, cell proliferation, apoptosis and differentiation.

Epidermal growth factor receptor

A considerable amount of work has been performed using EGFR and IR, and EGFR is one of the best-understood receptor trafficking systems. As a result there are several excellent reviews, which are succinctly summarized below (Hackel et al., 1999; Schlessinger, 2000, 2002; Sorkin, 2001). In the absence of ligand, these receptors are diffusely distributed at the cell surface and on stimulation are clustered in coated pits and rapidly internalized. Internalization requires intrinsic receptor kinase activity and specific motifs in the carboxy terminus domain of the receptor (Wells, 1999; Ceresa and Schmid, 2000; Yarden, 2001).

The EGFR family comprises four distinct receptors, ErbB1-4. These transmembrane receptors are composed of an extracellular ligand-binding domain and a cytoplasmic region with enzymatic activity. This structure enables signals to be transmitted across the plasma membrane, where they activate gene expression and ultimately induce cellular responses such as proliferation. The signal-transducing tyrosine kinase in the EGFR and related receptors does not phosphorylate

when the receptors are in isolation. A number of different ligands, including EGF-like molecules, transforming growth factor (TGF-α) and neuregulins, activate the receptor by binding to the extracellular domain and inducing the formation of receptor homodimers or heterodimers. Tyrosine residues on one receptor are presumably cross-phosphorylated by the other member of the receptor pair and these in turn form docking sites for signaling complexes composed of cytoplasmic enzymes and adapter proteins. The subsequent dissociation of these signaling complexes releases activated effectors and adapter proteins into the cytoplasm, where they stimulate many different signal transduction cascades, such as the MAPK pathway, PI3-kinase, the anti-apoptotic PKB/Akt and several transcriptional regulators. The EGFR family of receptor tyrosine kinases lies at the head of a complex signal transduction cascade that modulates cell proliferation, survival, adhesion, migration and differentiation. While growth-factor-induced EGFR signaling is essential for many normal morphogenic processes and is involved in numerous additional cellular responses, the aberrant activity of members of this receptor family has been shown to play a key role in the development and growth of tumor cells. Finally, the EGFR signal is inactivated primarily through endocytosis of the receptor-ligand complex. The contents of the resulting endosomes are then either degraded or recycled to the cell surface.

PLCγ-1

One of the prominent signaling proteins activated by EGFR is the gamma 1 isoform of phospholipase C (PLCγ1). This enzyme, which has two SH2 domains, catalyzes the hydrolysis of phosphatidylinositol(4,5)-biphosphate (PIP_2), generating the second messengers diacylglycerol and inositol triphosphate and liberating PIP_2-bound proteins. PLC-γ1 activity is positively modulated in vivo by association with EGFR. Its function (i.e. PIP_2 hydrolysis) is probably spatially limited to the EGFR surface because PIP_2 hydrolysis does not occur in the endosome compartment due to the limited access of EGFR-bound PLC-γ1 to its substrate in endocytic trafficking organelles (Wells et al., 1999; Carpenter, 2000).

PI3-kinase

PI3-kinases were the first mammalian lipid kinases discovered and since then multiple mammalian PI3-kinases have been identified (Carpenter et al., 1990). These are divided into three classes, are regulated by different mechanisms and produce different 3-phosphoinositde products. Below is a brief description of the three classes of PI3Ks and for more detailed reviews the reader is referred to the following excellent reviews (Carpenter and Cantley, 1990; Fruman et al., 1998; Hinchliffe et al., 1998a; Wymann and Pirola, 1998; Backer, 2000).

Class I

The class I PI3-kinases are heterodimers composed of a 110-120 kDa catalytic subunit, of which there are 3 mammalian isoforms (p110α, p110ß, p110δ) (Hiles et al., 1992; Hu et al., 1993; Vanhaesebroeck et al., 1997), and a regulatory subunit. There are currently two isoforms of the regulatory subunits (p85α and p85ß) (Carpenter et al., 1990, Escobedo et al., 1991, Otsu et al., 1991, Skolnik et al., 1991); however there are also two splice variants of p85α (p50α and p55ß) (Antonetti et al., 1996; Fruman et al., 1996; Inukai et al., 1996, 1997), and a third gene encodes p55γ (Pons et al., 1995), which has a similar structure to p55α. The catalytic subunits consist of a p85 interacting domain at its extreme N-terminus, a RBD (ras-binding domain), an internal C2 domain, a PI kinase homology domain and a kinase domain. The p85 subunits are composed of an SH3 domain at its N-terminus, followed by a Rho-GAP domain sandwiched between 2 proline-rich (PR) regions, then two SH2 domains separated by the "inter-SH2 domain" that interacts with the catalytic subunits (Fruman et al., 1998; Wymann and Pirola, 1998). These catalytic and regulatory subunits make up the class IA PI3-kinases. The class IA kinases are preferentially activated by RTKs when the p85 subunits associate with the intrinsic receptor kinase or its substrate, through their SH2 domains. The class IB group consists of a single catalytic protein termed p110γ (Stephens et al., 1994b, 1997; Stoyanov et al., 1995). It has 35% identity to p110α and contains a PI kinase homology domain, a kinase domain and a RBD, but differs in the extreme N-terminus and does not interact with the p85 regulatory subunits. p110γ subunit associates with a different regulatory subunit, p101, which has no apparent homology to any other known proteins. The class IB kinases interact with trimeric G-proteins. All of the class I PI3-kinases are able to phosphorylate the free 3-position of PI, PI(4)P, or $PI(4,5)P_2$ in vitro (Fruman et al., 1998). The class IA kinases can also phosphorylate PI(5)P (Rameh et al., 1997). Interestingly, stimulation of these kinases in vivo only results in increased production of $PI(3,4)P_2$ and $PI(3,4,5)P_3$, and the bulk of $PI(3,4)P_2$ appears to be the product of a 5-phosphatase on $PI(3,4,5)P_3$ (Carpenter and Cantley, 1990). This suggests that the major substrate for the class I PI3-kinases in vivo is $PI(4,5)P_2$.

Class II

The class II PI3Ks (PI3KC2) are large (170-210kDa), monomeric enzymes, containing the internal C2 domain, PI kinase homology domain and kinase domain, which are highly homologous to those in Class IB (MacDougall et al., 1995). In addition, the PI3KC2 enzymes have another C2 domain and a PX domain at their C-terminus (Fruman et al., 1998). PI3KC2s preferentially phosphorylate PI and PI(4)P *in vitro*, and in the presence of phosphatidylserine human PI3KC2α

also phosphorylates PI(4,5)P$_2$ (Domin et al., 1997).

Class III

The first class III PI3-kinase was identified by Emr and coworkers in a screen for yeast vacuolar sorting mutants (Schu et al., 1993). This archetype of the class III is VPS34 and orthologues have been described in plants (Welters et al., 1994), Drosophila (Linassier et al., 1997), *C. elegans* (Roggo et al., 2002), algae (Molendijk and Irvine, 1998) and mammals (Stephens et al., 1994a; Volinia et al., 1995). They contain the PI kinase homology domain and a kinase domain and are restricted to a single enzymatic product; PI(3)P. FYVE domains are specific to PI(3)P lipids, showing negligible affinity for PI(4)P. FYVE domains-containing proteins localize to endosomal membranes containing PI(3)P lipids (Table 1). PI3-kinases have been shown to be involved in many cellular processes including mitogenesis, apoptosis, cytoskeletal regulation and vesicular trafficking. Here we will briefly review their role in vesicular trafficking. A direct role for PI3-kinases in membrane trafficking was first described when the bovine p110 cDNA was cloned and found to have high sequence homology with VPS34 (Hiles et al., 1992). VPS34 was shown to be involved in the receptor-mediated movement of soluble hydrolases from the Golgi to vacuoles in *Saccharomyces cerevisiae* (Herman and Emr, 1990; Schu et al., 1993, Stack et al., 1993; Stack and Emr, 1994). Using selective PI3-kinase inhibitors, wortmannin and LY294002, several groups have shown that mammalian lysosomal hydrolases were secreted rather than targeted correctly, providing strong evidence of a role for PI3-kinases in trafficking between the TGN and endosomes/lysosomes (Brown et al., 1995, Davidson, 1995, Martys et al., 1996). Yeast screens and PI3-kinase inhibition studies were also used to show a function for PI3-kinases in endocytic trafficking. In yeast, end12 (an allele of VPS34) was found to be important for targeting α-factor for degradation, as end12 mutants produced an inhibition in the endocytic pathway (Shepherd et al., 1996). Receptors with mutations in their PI3-kinase binding domains and specific inhibitors of PI3-kinases were used to show that PI3-kinases were not required for receptor

Table 1. A summary of proteins and their specific protein domains. The table shows a list of proteins, that were referred to in the text, along with their PI binding domain, the PI they associate with and a general function of the protein. Below is a brief description of the respective domains. *PH domain*: Pleckstrin-homology (PH) domains are found in a wide variety of signaling proteins. Some PH domains bind with high affinity (low μM or nM Kd) to specific phosphoinositides such as PI(4,5)P$_2$, PI(3,4)P$_2$ or PI(3,4,5)P$_3$. Binding to phosphoinositides may allow PH proteins to respond to lipid messengers, for example, by relocation to membranes. *PX domain:* The Phox homology (PX) domain is the most recently identified member of the family of phospholipid-binding domains. Consisting of ~120 amino acids, the PX domain is found in more than 100 proteins, including the p40phox and p47phox components of the NADPH oxidase complex, sorting nexins, phospholipases D1 and D2 and CISK. Cell biology studies have established that PX domains function predominantly as D3-phosphorylated phosphoinositide (PI(3)P) binding modules, targeting the PX domain-containing proteins to the membranes. FYVE domain: The FYVE (Hrs and EEA1) domain is a small, cysteine-rich Zn^{2+} binding domain of approximately 60 amino acids. The FYVE domain has been shown to specifically bind PI(3)P. This observation has implicated FYVE domain–containing proteins in a signaling role downstream of PI3 kinase. ENTH domain: First identified in the endocytic protein epsin 1, the epsin NH2-terminal homology (ENTH) domain is a membrane-binding motif of approximately 150 amino acids. Proteins containing this domain have been shown to bind to phospholipids including PI(4,5)P$_2$ and PI(1,4,5)P$_3$. Consistent with these findings, the primary function suggested for the ENTH-domain-containing proteins is to act as clathrin adaptors in endocytosis. The ENTH domain proteins associate with the phospholipid bilayer allowing recruitment of clathrin components and clathrin accessory factors to the cell membrane.

PROTEIN	DOMAIN	TARGET	FUNCTION
Grb2	PH	PI(4,5)P$_2$	Endocytic adaptor protein
p120 GAP-Ras	PH	PI(4,5)P$_2$	GAP for Ras protein
mSOS	PH	PI(3,4,5)P$_3$	GEF for Ras
Dynamin	PH	PI(4,5)P$_2$	Endocytosis: CCV formation
PKB/Akt	PH	PI(3,5)P$_2$ & PI(3,4,5)P$_3$	Cell survival: apoptosis
PLC-γ-1	PH	PI(4,5)P$_2$	Cell signaling
PLC-δ-1	PH	PI(4,5)P$_2$	Cell signaling
IRS-1	PH	PI(4,5)P$_2$	Adaptor protein
ARNO	PH	PI(3,4,5)P$_3$	GEF for Arf Proteins
EFA6	PH	PI(3,4,5)P3	GEF for Arf Proteins
ARAP1/3	PH	PI(3,4)P$_2$ & PI(3,4,5)P$_3$	GAP for Arf Proteins
ASAP1	PH	PI(4,5)P$_2$	GAP for Arf Proteins
Centaurin-α	PH	PI(3,4,5)P$_3$	GAP for Arf Proteins
CISK	PX	PI(3,5)P$_2$ & PI(3,4,5)P$_3$	Cell survival: proliferation
PI3KC2-γ	PX	PI(4,5)P$_2$	Cell signaling
PLD	PH & PX	PI(4,5)P$_2$	Cell signaling
EEA1	FYVE	PI(3)P	Endocytosis: fusion
HRS	FYVE	PI(3)P	Endocytosis: sorting
EPSIN1	ENTH	PI(4,5)P$_2$	Endocytic adaptor protein
AP180	ENTH	PI(4,5)P$_2$	Endocytic adaptor protein
CALM	ENTH	PI(4,5)P$_2$	Endocytic adaptor protein
AP-2	ENTH	PI(4,5)P$_2$	Endocytic adaptor protein

internalization, but were important in targeting of internalized receptors to lysosomes. Further evidence of a role for PI3-kinases in the endocytic pathway came from *in vitro* endosomal fusion assays, in which addition of wortmannin or LY294002 inhibited endosomal fusion, as did a catalytically inactive p110, whereas a constitutively active p110 subunit enhanced fusion (Jones and Clague, 1995; Li et al., 1995). Using mutants of Rab5, the small GTP binding protein involved in endosomal fusion, researchers were able to show that PI3-kinases acted upstream of Rab5 (Li et al., 1995; Clague and Urbe, 2001; Zerial and McBride, 2001). Recent research has revealed a close link between the Arf-GTPases and PI3-kinases and provides further evidence for their role in vesicular trafficking (Venkateswarlu, et al., 1998; Cullen and Venkateswarlu, 1999; Venkateswarlu and Cullen, 2000). The products of PI3-kinases, $PI(3,4,5)P_3$ and the subsequent $PI(3,4)P_2$; have been shown to be important as second messengers for membrane localization and allosteric modulation of their targets. Many of these proteins involved in vesicular trafficking have been shown to associate with the products of PI3-kinases through specific protein domains, such as PH, PX and FYVE (Simonsen et al., 2001; Wishart, et al., 2001; Itoh and Takenawa, 2002) (Table 1). Examples of these molecules include several Arf-GEFs (guanine nucleotide exchange factors), such as ARNO and EFA6 (Turner and Brown, 2001), which interact through their PH domains. Simply, $PI(3,4,5)P_3$ formation recruits the Arf-GEFS to the membrane, where they activate the respective Arf. These in turn stimulate downstream effectors, such as PLD and vesicle coat proteins, and finally the Arf-GAPS (Centaurin-α, ASAP1 and ARAP1/3) are recruited to inactivate the Arfs (Santy and Casanova, 2002).

PI4-kinase

The PI4-kinases are responsible for the addition of a phosphate group at the D4 position of PI, which is thought to be the sole substrate of the PI4-kinases. Currently, the PI4-kinases are assigned to two classes, class II and III, according to different biochemical properties (Endemann et al., 1987; Whitman et al., 1987). The original class I kinases were discovered to be PI3-kinases. The type II kinases are 45-55kDa membrane-associated proteins that can be renatured from SDS-gels. The characteristics of these types II kinases are that they are inhibited by adenosine (K_i <50μM) and Ca^{2+}, they exhibit lower Km values for ATP and PI, and they are insensitive to wortmannin and LY294002 (Balla, 1998; Fruman, et al., 1998). The first type II kinase, type IIα, was only recently cloned and was shown to have the expected enzymatic characteristics and determined to be membrane-associated due to a palmitoylation site in the center of the protein (Barylko et al., 2001). In 2002, type IIβ was identified and shown to have a high degree of similarity to type IIα in its C-terminal catalytic domain, but low

similarity in its N-terminus (Balla et al., 2002). Both enzymes exhibit PI4-kinase activity that is connected with microsomal fractions, and epitope tagging showed that both enzymes localized to the endosomal vesicular compartments (Balla, et al., 2002).

Several type III kinases have been cloned and are found on both membranes and in soluble fractions with molecular weights of either ~110 or ~210kDa. The biochemical characteristics used to classify these PI4-kinases are their sensitivity to wortmannin (IC_{50}~50-100 nM) and LY294002 (IC_{50}~50-100 μM), higher Kms for ATP and PI, and insensitivity to adenosine and $Ca2^+$. Examination of the sequences of the cloned class III PI4-kinase has led to further classification (class IIIα or β), depending on differing homologies in their kinase domains (Balla, 1998; Fruman, et al., 1998; Gehrmann and Heilmeyer, 1998).

The PI4-kinases are important enzymes because they are the only phosphatidylinositol kinases responsible for the addition of the D4 position phosphate, which is an essential precursor to the highly important secondary messengers $PI(4,5)P_2$ and $PI(3,4,5)P_3$ (see below). In many of the processes in which $PI(4,5)P_2$ and $PI(3,4,5)P_3$ are involved, it is not known whether the PI(4)-kinases are key regulatory elements. Below are some examples in which the production of PI(4)P has been shown to be specifically regulated. Using yeast genetics, the specific functions of the PI4-kinases, Pik1 and Stt4 (staurosporine sensitive mutant 4), were examined. Several groups have shown that Pik1 mutants demonstrate important defects in the secretion of newly synthesized proteins, suggesting a crucial role for Pik1 synthesized PI(4)P in secretory pathway trafficking (Balla, 1998; Gehrmann and Heilmeyer, 1998). In addition, Walch-Solimena and Novick localized a pool of Pik1 to the *trans*-Golgi and therefore Pik1 has been proposed to regulate Golgi to plasma membrane transport (Walch-Solimena and Novick, 1999). On the other hand, Stt4 mutants do not affect the secretory pathway, but disrupt actin remodeling. Specific upstream regulators of Pik1 and PI4-kinase β have also been described, and interestingly Arf has been shown to stimulate both PiK1 and PI4-kinase-β activity (Balla, 1998).

PIP-kinases

The final group of lipid kinases in this review is the phosphatidylinositol phosphate kinases (PIP-kinases). Currently, three families of PIP-kinases are known and are grouped by the similarity of their conserved kinase domains. Initially, these kinases were thought to phosphorylate the 5 hydroxyl position of PI(4)P to form the important secondary messenger $PI(4,5)P_2$; however recent studies of the substrate specificity of the PIP-kinases have demonstrated that although they share homology in their catalytic domains, they have different substrate specificity, subcellular localization and functions. An interesting feature of the PIP-kinases is

that they have no statistical sequence similarity to other known lipid kinases, such as PI3-kinases or PI4-kinases that do share sequence homology (Ishihara, et al., 1996, Loijens and Anderson, 1996, Loijens, et al., 1996, Fruman, et al., 1998, Hinchliffe, et al., 1998a, Ishihara, et al., 1998). Crystallization of type IIß PIP-kinase and mutation analysis of type I and II PIP-kinases have shown that the PIP-kinases share structural features with other kinases, such as activation loops and putative nucleotide binding sites (see below) (Ishihara, et al., 1998, Rao, et al., 1998, Kunz, et al., 2000, 2002).

The type I PIP-kinases are also classified as PI(4)P5-kinases because they have been shown to predominately synthesize the secondary messenger $PI(4,5)P_2$, by the phosphorylation of the 5 hydroxyl position of PI(4)P in vivo. In addition, in vitro the type I PIP-kinases can phosphorylate the 4 and 5 positions of PI(3)P to form $PI(3,4)P_2$ and $PI(3,5)P_2$. This reaction can be carried out sequentially by the type I PIP-kinases to produce $PI(3,4,5)P_3$ (Loijens, et al., 1996, Fruman, et al., 1998, Hinchliffe, et al., 1998a). Recently, the type I PIP-kinases have been shown to phosphorylate the 5-position of $PI(3,4)P_2$ to produce $PI(3,4,5)P_3$ in cells exposed to oxidative stress (Halstead, et al., 2001). Several reports have shown that the activities of the type I PIP-kinases are stimulated by phosphatidic acid from 8- to 50-fold under some assay conditions (Moritz, et al., 1992, Jenkins, et al., 1994). The type I PIP-kinases that are found throughout the animal and plant kingdom and in yeast and mammalian cells are localized predominately at the plasma membrane but are also present at the nucleus and Golgi. Currently, there are 3 three known mammalian type I PIP-kinases. Both the human and mouse alpha and beta forms were cloned independently by two groups in 1996 (Ishihara, et al., 1996, Loijens and Anderson, 1996), however the alpha/beta terminology was reversed. The mammalian alpha and beta PIP5-kinases are 61kDa proteins, which migrate at 68kDa, have 67% identity overall and have 75% identity in their catalytic domains. In 1998, Ishihara et al cloned the third isoform of the PIP5-kinases from the murine ß-cell line MIN6 (Ishihara, et al., 1998). This novel isoform, termed PIP-kinase Iγ, has two splice variants of 87 and 90kDa, exhibits approximately 80% homology in its kinase core domain and shares 40% homology in its N-terminus with type Iß. In addition to cloning the third mammalian type I PIP-kinase, Ishihara et al carried out mutation studies on the cDNAs of all three isoforms in an attempt to map the functional features of the PIP5-kinases (Ishihara, et al., 1998). They showed that the core kinase domain is a central 380-amino acid section that has 70-80% identities between the three isoforms and less than 40% identity with type II PIP-kinase-α. PI(4)P-kinase assays demonstrated that type Iß had the highest Vmax for PI(4)P and type Iγ underwent the greatest stimulation with phosphatidic acid. A putative phosphate-binding domain was also described in the amino region of this conserved kinase domain of PIP5-kinase α. In 2000, Kunz et al further characterized the catalytic domain of both type I and II PIPKs by showing that substrate specificity was determined by a ~25 amino acid region, which they termed the "activation loop" (Kunz, et al., 2000). Recently, Kunz et al ascribed dual roles to the activation loop in substrate specificity and in promoting membrane association (Kunz, et al., 2002). Using site-directed mutagenesis studies, they demonstrated that a single amino acid change was capable of swapping the stereo-specific substrate recognition of the type I and II PIP-kinases (Kunz et al., 2002).

The type I PIP-kinases have been linked to many cellular processes, including actin reorganization, secretion, endocytosis, apoptosis and ion channel regulation; however in this review we will focus on their role in endocytosis. The product of the type I PIPKs, $PI(4,5)P_2$, acts early in endocytosis by regulating clathrin coat formation. Many proteins involved in clathrin-mediated endocytosis interact specifically with $PI(4,5)P_2$, such as epsin, CALM, AP180, AP2 and dynamin (Wakeham, et al., 2000, Cullen, et al., 2001, Martin, 2001, Osborne et al., 2001, Simonsen et al., 2001, McLaughlin, et al., 2002). A role for type I PIP-kinases in endocytosis was first suggested when an inactive N-terminal deletion mutant of the murine type Iß was shown to inhibit the ligand-induced downregulation of the colony stimulating factor-1 receptor (Davis, et al., 1997). More recently, the type I PIP-kinases have been shown to interact with the cytoplasmic tails of the EGFR, and truncated, kinase-dead mutants of the type Iß were able to inhibit EGFR endocytosis and recruitment of clathrin light chain and dynamin on the plasma membrane (Cochet, et al., 1991, Barbieri, et al., 2001). The published data suggest that type I PIP-kinases play an early role in the internalization of receptor-mediated endocytosis; however, Galiano, et al. (2002) showed that type I PIP-kinases can have a secondary function in endocytosis and demonstrated a role for these kinases in endosomal vesicle trafficking.

The type II PIP-kinases have previously been characterized as PI(5)P4-kinases because their primary function is the phosphorylation of the 4 position of phosphatidyl-5-phosphate to form $PI(4,5)P_2$. As with the type I PIP-kinases, the type II PIP-kinases are also able to phosphorylate PI(3)P in vitro, however the sole product is $PI(3,4)P_2$. The type II PIP-kinases are found only in metazoans and have been localized to the cytosol, nucleus, endoplasmic reticulum and actin cytoskeleton. There are currently three mammalian isoforms of the type II PIP-kinases (α, ß, γ), which share ~60% homology in their kinase domains, and these are 47kDa proteins that migrate at ~57kDa. The human type IIα was the first cloned in 1995 (Boronenkov and Anderson, 1995, Divecha, et al., 1995), whereas the ß-isoform was identified through a yeast 2-hybrid screen with the juxtamembrane region of p55 tumor necrosis factor-α receptor (Castellino, et al., 1997). The rat γ-isoform was cloned by Itoh et al. (1998). The three

isoforms of type II PIP-kinases exhibit different subcellular localization: the alpha is a cytosolic enzyme, as is the beta isoform (which also has a nuclear localization), and the gamma is located in the endoplasmic reticulum. Interestingly, the cytosolic type IIα has been shown to interact with all three isoforms of the type I PIP-kinases, causing its relocation to the plasma membrane (Hinchliffe et al., 2002).

As mentioned above, the typeIIβ kinase has been crystallized and its structure resolved to 3Å. The type IIβ kinase forms a disc-shaped homodimer, through N-terminal interactions, and its structure suggests that an electrostatic mechanism is involved in membrane targeting. Interestingly, the kinase has a protein kinase ATP-binding core and shows that structurally the phosphoinositide kinases all belong to a superfamily (Rao et al., 1998).

The regulation and function of type II PIP-kinases is poorly understood; however, unlike the type I kinases, they are not stimulated by phosphatidic acid and currently no small G-proteins have been found to exert an influence on type IIs (Loijens, et al., 1996; Fruman, et al., 1998; Hinchliffe, et al., 1998a). Recent data suggests that one mode of regulation may be through phosphorylation of the type II PIP-kinases (Hinchliffe et al., 1998b, Huang et al., 2001) and several PIP-kinase kinases have begun to be identified in platelets, including casein kinase II and two serine/threonine kinases (Hinchliffe et al., 1998b, 1999, 2002). Hinchliffe et al. also provide evidence for degradation of type II PIP-kinases by the calcium-stimulated protease calpain in thrombin-stimulated platelets (Hinchliffe, et al., 1999). The role for calpain in type II PIP-kinases regulation is currently not understood.

In a recent review, (Hinchliffe et al., 1999) suggested three possible functions for type II PIP-kinases. The first is that the type IIs are acting as phosphatidylinositol 3 phosphate 4-kinases to produce PI(3,4)P$_2$; the second is they are present to clear PI(5)P, by converting it to PI(4,5)P$_2$; the third is that the type IIs are involved in a minor pathway for the production of PI(4,5)P$_2$ and allow for control of a specific pool of PI(4,5)P$_2$ production.

Type III PIP-kinases have recently been reviewed and will therefore only be covered briefly here (Shisheva, 2001). In 1999, the first mammalian-type III PIP-kinase was cloned from the murine adipocyte cDNA library and revealed that, in addition to the PIP-kinase domain, the protein contained an N-terminal zinc-binding FYVE finger, a chaperonin-like region and a DEP domain (Shisheva et al., 1999). This protein has been known as p235, in reference to its molecular weight, and as PI-kinase-FYVE; however we shall refer to it as a class III PIP-kinase. Interestingly, the murine type III has extensive homology to the Saccharomyces cerevisiae type III PIP-kinase, Fab1p, which has been shown to be a PI(3)P5-kinase responsible for vacuole morphology (Cooke, et al., 1998). Complementation studies using Δfab1 yeast cells showed that transfected murine type III kinase could restore basal PI(3,5)P$_2$

synthesis; this was not seen with the mammalian type I PIP-kinases (McEwen et al., 1999). These data strongly suggest that the type III kinases are PI3P5-kinases and play a role in membrane trafficking. The mammalian type IIIs localize to vesicles of the late endocytic pathway, through interaction in their FYVE domain with PI(3)P, and their enzymatic activity was required to maintain late endocytic membrane integrity(Shisheva, et al., 1999, Ikonomov, et al., 2002, Sbrissa, et al., 2002). The mammalian type III PIP-kinase also contains an intrinsic protein kinase, capable of trans- and autophosphorylation; therefore, late endosomal localization and protein kinase activity could allow the type IIIs to be involved in signaling from endosomes.

Concluding remarks

The key to understanding the cell physiopathology mediated by cell surface receptors lies not only in the identification of downstream effectors but also in the temporal-spatial association of the interaction with these effectors. Clearly, membrane trafficking plays an important role both in controlling the location of signaling interactions and in regulating the cellular degradation-recycling of the activated receptor.

Recent data have indicated that there are even greater levels of complexity in PI-kinase endocytosis and signaling than we previously realized. In particular, biochemical analysis has revealed new factors that regulate PI-kinase activity, many of which are regulated by growth factors.

PI-kinase molecules are simple molecules and yet they play key regulatory roles in a large number of biological processes because of their location at the branch-point from the plasma membrane and endosome receptors to numerous signaling pathways. Probably one of principal functions of PI-kinase is to recruit proteins to the plasma membrane where they become activated. There are three types of mammalian PI-kinases that show different biochemical and biological properties. Localization in specialized microdomains of the plasma membrane and/or internal membranes may be an important aspect of PI-kinase signaling, while proteins such as phosphatases, kinases and small GTPases may facilitate or block PI-kinase incorporation into particular signaling complexes, or may permit access only to certain effector proteins. It is unclear how these factors regulate PI-kinase in tumor cells, but the assembly of specific PI-kinase isoforms into signaling complexes may be determined by their association with specific membrane sub-domains or assembly factors, or by their access to specific effectors.

By defining how each of these aspects of PI-kinase regulation affects tumor cell endocytosis and signaling, we will gain a greater understanding of the development and growth of tumor cells. From a biomedical prospective, the identification of these sites and knowledge of the kinetics of receptor activation of downstream effectors provides an opportunity to design

rational therapeutic strategies to manipulate a given signaling pathway.

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