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Phosphoinositide Kinases†

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Polyphosphoinositides constitute less than 0.1% of total cellular lipids yet are among the most intriguing and most studied of lipids because of their roles in intracellular signaling. Several years ago the phosphoinositide pathway was thought to be simple: phosphatidylinositol 4-kinase phosphorylates phosphatidylinositol (PI) and phosphatidylinositol-4-phosphate 5-kinase phosphorylates phosphatidylinositol 4-phosphate (PI-4-P) to produce phosphatidylinositol 4,5-bisphosphate (PI-4,5-P_2). Phospholipase C hydrolyzes PI-4,5-P_2 to produce the signaling molecules diacylglycerol and inositol 1,4,5-trisphosphate (Ins-1,4,5-P_3) (see pathway with boldface letters in Figure 1A). The enzymes and products in this pathway have been found in virtually every eukaryotic organism and tissue studied. Recently, the complexity of phosphoinositide metabolism has increased due to the discovery of phosphatidylinositol 3-kinase and its products, phosphoinositides phosphorylated at the D-3 position of the inositol ring (for the structure of PI and the numbering system of the inositol ring see Figure 1B). Two monophosphorylated phosphoinositides are now known: PI-4-P and PI-3-P (Whitman et al., 1988). In addition to PI-4,5-P_2, two new diphosphoinositides have been described, PI-3,4-P_2 and putative PI-3,5-P_2 (Auger et al., 1989a). PIP_3 (probably PI-3,4,5-P_3) has also been identified in several cell types (Auger et al., 1989a; Kucera & Rittenhouse, 1990; Nolar & Lapetina, 1990; Traynor-Kaplan et al., 1988, 1989). The discovery of these novel D-3-phosphorylated lipids has uncovered a new group of potential intracellular signals. As shown in Figure 1A there are several possible pathways by which the polyphosphoinositides could be synthesized. In addition, phosphatases with both lipid and site specificity degrade these lipids (not shown). This review will focus on the phosphoinositides kinases and their products, with particular emphasis on the novel compounds.

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Identification of Phosphoinositides. Since the discovery of the D-3-phosphorylated phosphoinositides, it is clear that thin-layer chromatography (TLC) alone cannot be relied on to separate and identify phosphoinositides. Although PI-3-P has a migration position slightly different from that of PI-4-P in some TLC systems (Whitman et al., 1988), no system has yet been developed that reproducibly resolves these two isomers. A TLC system has been described that separates PI-4,5-P_2 from PI-3,4-P_2 (Pignataro & Ascoli, 1990), but the value of this system is limited at present since the migration position of the putative PI-3,5-P_2 in this system has not been described. PIP_3 can be reliably separated from the lower phosphorylated phosphoinositides by several TLC systems (Auger et al., 1989a); however, without a standard, lyso-PI-4,5-P_2 could be misidentified as PIP_3. Confirmation of all these lipids requires HPLC analysis of the deacylated product.

Rigorous proof of the structure of any novel phosphoinositide is, of course, necessary. To date, this has been done only for PI-3-P produced in vitro by middle T antigen associated PI 3-kinase (Whitman, et al., 1988) and PI-3-P produced in vivo in astrocytoma cells (Stephens et al., 1989). Proof of the structure of PI-3-P involved deacylation and deglyceration to produce the inositol bisphosphate followed by periodate oxidation, reduction, and dephosphorylation to produce a polyol. The structure of the polyol depended on the phosphorylation sites of the inositol bisphosphate. The polyol was identified by comigration with standards on HPLC, from which the structure of PI-3-P was deduced.

The structures of the other D-3-phosphorylated compounds have been inferred on the basis of several lines of evidence. Most often, the deacylated and deglycerated product has been compared to inositol polyphosphate standards (Auger et al., 1989a; Kucera & Rittenhouse, 1990; Nolan & Lapetina, 1990; Pignataro & Ascoli, 1990; Stephens et al., 1989; Traynor-Kaplan et al., 1988, 1989). Analyses of phosphoinositides produced in vivo are most convincing when the cells are labeled with [^{3}H]inositol as opposed to $^{32}PO_4^{3-}$, since with $^{32}PO_4^{3-}$ one cannot be certain the product contains *myo*-inositol. Verifi-

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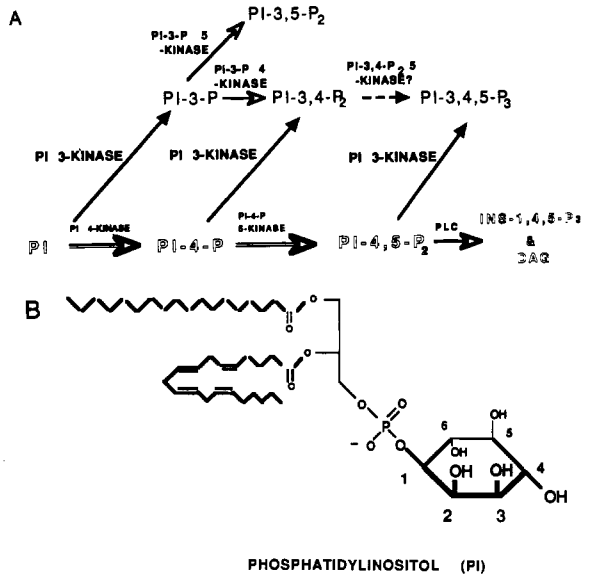

FIGURE 1: Phosphoinositide pathways. (A) The canonical PI pathway is shown in outline. PI 3-kinase products are shown in boldface. Also shown is the PI-3-P 4-kinase described in platelets and the PI-3-P 5-kinase described in platelets and sf9 cells. The existence of a PI-3,4-P_2 5-kinase has not been shown. (B) Structure of phosphatidylinositol and numbering of the ring.

cation that phospholipids produced in vivo contain *myo*-inositol attached to diacylglycerol by a phosphodiester bond at the D-1 position of the inositol ring is essential for conclusive identification of these lipids (Figure 1A). These analyses have not yet been published for the putative PI-3,4-P_2, PI-3,5-P_2, and PI-3,4,5-P_3 found in vivo.

The structures of the polyphosphoinositides produced in vitro have been more reliably determined. PI-3,4-P_2 has been produced by adding ATP and PI-4-P to PDGF receptor associated (Auger et al., 1989a) and polyoma virus middle T associated (Serunian et al., 1990) PI 3-kinase and to PI 3-kinase purified to homogeneity from rat liver (Carpenter et al., 1990). Since a significant fraction of the added PI-4-P is converted to this lipid, the PIP_2 produced must contain both *myo*-inositol attached to diacylglycerol through a phosphodiester bond at the D-1 position and a phosphate at the D-4 position. The deacylated and deglycerated product comigrates with a [^{3}H]inositol 1,3,4-trisphosphate standard on HPLC (Auger et al., 1989a). Finally, this structure has been confirmed by periodate oxidation of the inositol trisphosphate to produce altritol (unpublished results of L. Stephens, K. Auger, and L. C. Cantley) and by dephosphorylation to produce a compound that comigrates with *myo*-inositol 3-phosphate (although *myo*-inositol 1-phosphate and *myo*-inositol 3-phosphate are enantiomers and comigrate, exchange of ^{32}P into the phosphodiester bond at the 1-position can be excluded in this case).

Similar experiments indicate that the PIP_3 produced by phosphorylation of PI-4,5-P_2 with PDGF receptor associated PI 3-kinase (Auger et al., 1989a) and purified rat liver PI 3-kinase (Carpenter et al., 1990) is almost certainly PI-3,4,5-P_3. The deacylated and deglycerated product comigrates with [^{3}H]inositol 1,3,4,5-tetrakisphosphate standard and is resistant to periodate oxidation. Since the starting material was PI-4,5-P_2, the resistance to periodate oxidation (an indication that no unmodified adjacent alcohol groups exist) could only be explained by the structure PI-3,4,5-P_3 or PI-2,4,5-P_3. The latter structure is unlikely since the deacylated and deglycerated product comigrate with inositol 1,3,4,5-tetrakisphosphate and since the enzyme that synthesizes this lipid adds a phosphate to the D-3 position of PI and PI-4-P (Carpenter et al., 1990).

The least well characterized of the lipids shown in Figure 1A is the putative PI-3,5-P_2. This lipid has been synthesized in this laboratory by adding [3-^{32}P]PI-3-P and unlabeled ATP to purified PI-4-P 5-kinase (unpublished results of M. Whitman, L. Ling, and L. Cantley). The deacylated product of this lipid migrates approximately 1.5 min ahead of PI-3,4-P_2 on HPLC, and a [^{3}H]inositol-labeled lipid with a similar migration position has been detected in vivo (Auger et al., 1989a). Further work is needed to confirm the structure of the putative PI-3,5-P_2, but the in vitro synthesis from PI-3-P suggests that in vivo PI-4-P 5-kinase may phosphorylate PI-3-P at the D-5 position.

Phosphatidylinositol 4-Kinase. It has long been suspected that more than one species of PI kinase exists. Over 20 years ago Harwood and Hawthorne (1969) found two types of PI kinase in liver, on the basis of differential activation of the PI kinases in plasma membranes and endoplasmic reticulum by a nonionic detergent. Recently, Whitman et al. (1987) identified two types of PI kinase in fibroblasts on the basis of differences in sensitivity to adenosine and nonionic detergent. Type 1 PI kinase eluted from an anion exchange column earlier than type 2 PI kinase, was inhibited by detergent, and was not inhibited by adenosine. Type 2 PI kinase was activated by detergent and inhibited by adenosine. It was then shown that while type 2 PI kinase phosphorylates the D-4 position of the inositol ring as expected, type 1 PI kinase phosphorylates the D-3 position (Whitman et al., 1988). This was the first report of a PI 3-kinase or the existence of PI-3-P in vivo. Thus, type 1 and type 2 PI kinases are not isozymes but carry out distinct enzymatic reactions (Table I). PI 3-kinase is discussed further below.

Endemann et al. (1987) separated two types of PI 4-kinase from bovine brain by sucrose gradient centrifugation. These two enzymes have apparent sizes by sedimentation velocity of 55 and 230 kDa. The 55-kDa PI kinase is similar to the type 2 PI 4-kinase of fibroblasts in all properties investigated. Enzymes with properties similar to those of the fibroblast and brain type 2 PI 4-kinases have also been characterized from human red cell plasma membranes (Endemann et al., 1987; A. Graziani, G. Endemann, and L. Cantley, in preparation), porcine liver (Hou et al., 1988), bovine uterus (Porter et al., 1988), and A431 cells (Walker et al., 1988). This enzyme requires detergent for extraction from the membrane and is not significantly present in the soluble fraction of homogenized cells. The subunit molecular mass of the purified enzyme, 55 kDa (Hou et al., 1988; Porter et al., 1988; Walker et al., 1988; A. Graziani, G. Endemann, and L. Cantley, in preparation), is similar to that estimated by sucrose density gradient in cholate (Endemann et al., 1987), indicating that the protein is a monomer and binds a relatively small amount of cholate. Walker et al. (1988) did find significant Triton X-100 binding to the PI 4-kinase they purified. The apparent size of the enzyme was 120 kDa by glycerol gradient. The increase in the apparent size over the apparent size of the monomer by SDS–PAGE seemed to be due to detergent binding. Identification of the catalytic subunit for the type 2 enzyme has been facilitated by the ease with which enzymatic activity can be renatured from the 55-kDa protein following SDS–PAGE (Porter et al., 1988; Walker et al., 1988). By use of human red cell type 2 PI 4-kinase, as much as 25% of the total PI 4-kinase activity originally present in a sample can be recovered by renaturing the SDS–PAGE-purified 55-kDa peptide (A. Graziani, G. Endemann, and L. Cantley, in preparation). The

Table I: Phosphoinositide Kinases

source (ref)	size by SDS–PAGE (kDa)	native size (kDa)	substrate	location	K_M(ATP) (μM)	detergent effects	adenosine inhibition	comments
Phosphatidylinositol 4-Kinases								
type 2								
fibroblasts (Whitman, 1987)			PI		34	activates	yes	
bovine uterus (Porter et al., 1988)	55		PI		18			able to renature
rat liver (Hou et al., 1988)	55		PI	membranes	60		yes	
A431 cells (Walker et al., 1988)	55	120	PI	membranes	74		yes	able to renature
bovine brain (Endemann, 1987)		55	PI	membranes	54	activates	yes	
type 3								
bovine brain (Endemann et al., 1987)		230	PI	membranes	742	active in cholate	no	
bovine uterus (Li et al., 1988)		200	PI		250	inhibited by Triton X-100 at >0.2%		
rat brain (Yamakawa et al., 1988)	76	80	PI	membranes	150		no	
others								
bovine brain myelin (Saltiel, 1987)	45		PI, PI-4-P	myelin	150			able to renature PI kinase
S. cerevisiae (Belunis et al., 1989)	35		PI	membranes	300	activates	no	able to renature
Phosphoinositide 3-Kinase								
fibroblasts (Whitman et al., 1987)			PI		10	inhibits	no	
rat liver (Carpenter et al., 1990)	85 and 110	190	PI, PI-4-P, PI-4,5-P_2	cytosol	30–60	inhibits	no	
S. cerevisiae (Auger et al., 1990)			PI	membranes		inhibits	no	
PI-4-P 5-Kinases								
human erythrocytes (Ling et al., 1988)	53	150	PI-4-P, PI-3-P	membranes and cytosol	2	activates		
rat brain (Cochet & Chamboz, 1986)	45 (?)	100–110	PI-4-P		25			
rat brain (Van Dongen et al., 1988)	45		PI-4-P					
bovine brain (Moritz et al., 1990)	110	100–110	PI-4-P	membranes				
human erythrocytes (Anderson, 1990)								
type I			PI-4-P	membranes	25			
type II	53		PI-4-P	membranes and cytosol	<5			
PI-3-P 4-Kinase								
human platelets (Yamamoto et al., 1990)			PI-3-P	membranes and cytosol		inhibits		

type 2 enzymes from various sources were all found to have a relatively low K_M(ATP) (20–60 μM). Where investigated, adenosine inhibited these enzymes by competing with ATP (K_I = 10–70 μM). In all cases, the type 2 enzyme utilized substrate best when presented in a detergent micelle (Whitman et al., 1987).

The type 3 PI 4-kinase, like the type 2, is a membrane-associated enzyme and is maximally active when substrates are presented in detergent micelles (Endemann et al., 1987). The enzymes are distinct however: they have different apparent molecular weights, type 3 has a higher K_M(ATP) and is relatively resistant to inhibition by adenosine (Table I), and a monoclonal antibody that causes greater than 90% inhibition of the type 2 PI 4-kinase has no effect on the type 3 enzyme (Endemann et al., 1990). An enzyme purified from bovine uterus (Li et al., 1989) has properties similar to those of the brain type 3 enzymes. This protein has an apparent size by gel filtration of 200 kDa and like the brain type 3 enzyme is relatively resistant to inhibition by adenosine. Yamakawa and Takenawa (1989) purified an enzyme from rat brain that is similar to type 3 in that it is relatively resistant to inhibition by adenosine and is activated by detergents. The K_M(ATP) is intermediate between type 2 and the other type 3 enzymes, but this is a much purer enzyme. However, its apparent size by gel filtration (80 kDa) appears to be smaller than sizes previously reported for the type 3 enzyme. The specificity for phosphorylation of the D-3 versus D-4 position of the inositol ring was not investigated for this enzyme, but the activation by detergent suggests that it is a PI 4-kinase. A 76-kDa peptide copurified with this PI kinase, but no renaturation of activity from SDS–PAGE was reported.

Kinase activities that phosphorylate PI and PI-4-P were shown to copurify from bovine brain myelin (Saltiel et al., 1987). Phosphatidylinositol kinase activity was renatured from a 45-kDa band of SDS–PAGE, but the renaturation of the PI-4-P kinase activity was not reported. Whether the same

protein has two activities or whether the PIP kinase activity is part of a complex or a contaminant is not known. The phosphorylation sites on the inositol ring were not determined. The other PI 4-kinases discussed above have no associated PI-4-P kinase activity.

A PI kinase was also purified from the membrane fraction of the yeast *Saccharomyces cerevisiae* (Belunis et al., 1988). This enzyme was activated by nonionic detergents and appeared to be a PI 4-kinase. A 35-kDa peptide copurified with the enzyme. A soluble PI 4-kinase was also observed in *S. cerevisiae* (Auger et al., 1989b).

Studies of the intracellular distribution of PI 4-kinase activity in mammalian tissues have found that it is almost exclusively membrane associated. Hepatocytes have been most often studied to determine with which membrane fraction(s) PI kinase is associated, and although HPLC analysis of the PIP produced has not been done in these cell fractionation studies, PI-3-P makes up less than 10% of the total PIP in most mammalian cells. The majority of [^{32}P]PI-4-P in $^{32}PO_4^{3-}$-labeled hepatocytes migrates with the plasma membrane fraction, but a significant amount comigrates with lysosomes (Seyfred & Wells, 1984). Studies in which membrane fractions have been assayed for PI kinase activity have found the majority of the activity in intracellular membranes, either endoplasmic reticulum or Golgi apparatus (Jergil & Sundler, 1983; Lundberg & Jergil, 1988). The findings of these two types of studies can be reconciled if the activity of PI 4-kinase is differentially regulated in different membranes or if PI-4-P is transported to other membranes, especially the plasma membrane, shortly after synthesis. PI kinase has been found in nuclear membranes from rat liver cells (Smith & Wells, 1983) and in nuclei from Friend erythroleukemia cells stripped of their envelopes (Couo et al., 1987). In platelets, 30% of the PI kinase was found in the detergent-insoluble portion or "cytoskeletal" fraction (Nahas et al., 1989). Careful analysis of type 2 versus type 3 PI 4-kinase activities in subcellular fractions has not been done; however, bovine brain coated vesicles have both types of activity while red cell plasma membranes have only type 2 PI 4-kinase (Endemann et al., 1987; unpublished results).

Regulation of Phosphatidylinositol 4-Kinase. Regulation of PI 4-kinase activity is poorly understood. Most experiments suggesting that this enzyme is regulated have been conducted in whole cells, permeabilized cells, or cell lysates. In these systems it is difficult to determine the contributions of phosphatases, phospholipases, and PI-4-P 5-kinase and the availability of ATP and/or PI to the observed changes in PI-4-P [for an evaluation of the complexity of this analysis, see Chahwala et al. (1987)]. Such analyses are also complicated by the presence of multiple PI 4-kinases at multiple intracellular locations. Despite these complexities there is some evidence that PI 4-kinase may be regulated by cAMP (Holland et al., 1988; Kaibuchi et al., 1986; Kato et al., 1989), phorbol esters, diacylglycerol, concanavalin A, and calcium ionophore (Boon et al., 1985; de Chaffoy de Courcelles et al., 1984; Halenda & Feinstein, 1984), and GTPγS (Chahwala et al., 1987) in certain systems. Walker and Pike (1987) found an increase in the PI kinase activity (apparently PI 4-kinase) of membranes isolated from A431 cells after treatment of the cells with EGF. Such results suggest that this enzyme may be regulated by protein kinases or GTP binding proteins. Thus far, we know of no published evidence that a PI 4-kinase is phosphorylated or that a purified PI 4-kinase is regulated by a protein kinase or a G protein.

Peptides and other small molecules may regulate PI 4-kinase activity by direct interaction with the enzyme. Polyamines, histones, and polylysines activate PI 4-kinase up to 8-fold in crude preparations from A431 cells (Vogel & Hoppe, 1986). The type of PI 4-kinase activated and whether the activation is by direct binding to the protein remain to be determined. The physiological relevance of this activation is also unknown. As discussed above, adenosine specifically inhibits the purified type 2 PI 4-kinase under conditions where type 3 and PI 3-kinase are not affected. This inhibition may be physiologically relevant under conditions of ATP depletion in the cell (Buckley, 1977; Doctrow & Lowenstein, 1985; Whitman et al., 1987). *S*-Adenosylhomocysteine inhibits PIP production in neutrophils in vivo and is a competitive inhibitor of PI kinase in neutrophil membranes in vitro (Pike & DeMeester, (1988). Quercetin inhibits PI 4-kinase, presumably through competing with ATP for binding to the active site s does the isoflavone orobol (Nishioka et al., 1989). 2,3-Dihydroxybenzoic acid and 2,3-dihydroxybenzaldehyde inhibit PI 4-kinase with IC_{50} of 0.7 and 0.45 μg/mL, respectively (Nishioka et al., 1989). A synthetic wasp venom causes a 2–3-fold increase in the activity of PI and PIP kinases in MDCK cell membranes (Eng & Lo, 1990). The mechanism of this activation is unknown.

A PI kinase in the yeast *S. cerevisiae* seems to be regulated by cAMP-dependent protein kinase, although it is not clear whether it is a PI 4-kinase or a PI 3-kinase. In one study, yeast mutants of the RAS genes (ras1, ras2) or adenylate cyclase gene (cry1), which have low cAMP-dependent protein kinase activity, were shown to have diminished PI and PIP kinase activities (Kato et al., 1989). The enzymes were activated by the addition of cAMP or in a mutant (bcy1) that reverses the cry1 phenotype by eliminating the cAMP dependence of the cAMP protein kinase. cAMP was concluded to inhibit PI kinase activity in yeast in two other studies (Holland et al., 1988; Kaiuchi et al., 1986). The activities in all of these studies were assumed to be PI 4-kinase and PI-4-P 5-kinase, but we recently showed that the major PIP produced in *S. cerevisiae* is PI-3-P rather than PI-4-P (Auger et al., 1989b), raising the possibility that PI 3-kinase was being measured in the above studies (see further discussion under Phosphatidylinositol 3-Kinase). Ergosterol has been found to increase PI kinase activity in sterol-starved yeast, and PI kinase activity has also been found to increase in G1 in sterol auxotrophic yeast (Dahl et al., 1987). These studies did not distinguish between PI 3-kinase and PI 4-kinase.

Phosphatidylinositol 3-Kinase. PI 3-kinase was originally described as a PI kinase (thought at the time to be a PI 4-kinase) that copurified with the protein-tyrosine kinases, pp60$^{v\text{-}src}$, polyoma middle T antigen/pp60$^{c\text{-}src}$ complex, and the PDGF receptor (Kaplan et al., 1987, 1986; Sugimoto et al., 1984; Whitman et al., 1985). It was distinguished from the major PI kinase in fibroblasts because it was inhibited by nonionic detergents but resistant to inhibition by adenosine. The protein-tyrosine kinase associated PI kinase was named type 1 and the adenosine-sensitive kinase named type 2 (Whitman et al., 1987). Because the products of the type 1 and type 2 PI kinases exhibited slightly different migration rates on TLC, they were further analyzed. The products of the two enzymes were compared for their abilities to serve as substrates of the purified human red cell PI-4-P 5-kinase. The product of the type 1 enzyme was a poor substrate compared to that of the type 2 PI kinase, indicating a structural difference between the two lipids (unpublished results of Whitman, Ling, and Cantley). Reverse-phase HPLC analysis of the two lipids produced from type 1 versus type 2 PI kinase

acting on the same commercial PI showed no differences in the fatty acid composition, indicating that the structural difference was in the head group. Chemical analysis of the head group by the procedure discussed above revealed that the type 1 enzyme produces PI-3-P while type 2 produces PI-4-P (Whitman et al., 1988). PI 3-kinase is present in all eukaryotic organisms tested but has not been found in bacteria. It is present in all tissues, but the highest specific activity is in liver and spleen (Carpenter et al., 1990).

We have recently purified PI 3-kinase 27 000-fold to homogeneity from rat liver (Carpenter et al., 1990). The majority of PI 3-kinase activity is found in the soluble fraction of homogenized cells, although a significant fraction is associated with the membrane in growth factor stimulated and/or oncogene-transformed fibroblasts (Cohen et al., 1990a,b). The apparent size of the soluble enzyme is 190 kDa by gel filtration and sucrose density gradient centrifugation. The purified enzyme is a dimer of one 110-kDa peptide and one 85-kDa peptide. Two different forms of the enzyme with distinct 110-kDa subunits can be separated by ion exchange chromatography and SDS–PAGE. The 110-kDa subunits of the two forms are related peptides with similar but apparently distinct sequences. No differences in enzymatic activities of the two forms of the enzyme have been detected thus far. The purified PI 3-kinase phosphorylates PI, PI-4-P, and PI-4,5-P_2 at the D-3 position of the inositol ring. The V_{max} of the enzyme and the relative utilization of these three substrates is quite sensitive to the composition of the lipid vesicle (lipids presented in detergent micelles are not substrates). When phosphatidylserine is added as a carrier, PI-4,5-P_2 is the preferred substrate. Interestingly, in crude liver cytosol PI 3-kinase activity is readily detectable, but PI-4-P and PI-4,5-P_2 3-kinase activities are not detectable, suggesting the presence of an inhibitor in the cytosol that preferentially inhibits the PIP and PIP_2 kinase activities of PI 3-kinase. This inhibitor is apparently removed during purification.

An 85-kDa phosphoprotein was originally implicated as the PI 3-kinase because its presence correlated with PI kinase activity in immunoprecipitates of polyoma middle T and in anti-phosphotyrosine immunoprecipitates of the PDGF receptor (Courtneidge & Heber, 1987; Kaplan et al., 1987, 1986). Recently, Cohen et al. showed that the middle T antigen of polyoma virus has a remarkable affinity and specificity for this 85-kDa protein (Cohen et al., 1990a,b). When total cell proteins are separated by SDS–PAGE and then transferred to nitrocellulose and blotted with ^{32}P-labeled middle T, the only detectable band is the 85-kDa peptide! For these experiments, the middle T protein is phosphorylated on tyrosine by pp60$^{c\text{-}src}$, and this phosphorylation appears to be necessary for optimal association. Evidence that the protein blotted is the 85-kDa subunit of the PI 3-kinase was provided by the ability of middle T to blot the 85-kDa subunit (but not the 110-kDa subunit) of the purified enzyme (Carpenter et al., 1990). The middle T associated 85-kDa protein is phosphorylated on both serine and tyrosine residues in intact cells (Kaplan et al., 1987). This protein is also phosphorylated on both tyrosine and serine residues in response to stimulation of cells by PDGF (Kaplan et al., 1987). Both the 110-kDa subunit and 85-kDa subunit of the purified PI 3-kinase associate with and are phosphorylated on tyrosine by purified middle T/pp60$^{c\text{-}src}$ complex in vitro (unpublished results of Carpenter and Auger). Preliminary results indicate that both subunits of the PI 3-kinase are recruited to the PDGF receptor in response to stimulation of cells by PDGF. The 110- and 85-kDa peptides are tightly associated in vitro; attempts to separate them and renature activity have failed, making assignment of the catalytic subunit uncertain.

Regulation of Phosphatidylinositol 3-Kinase. There is compelling evidence that PI 3-kinase is regulated by a variety of pathways that involve activation of protein-tyrosine kinases. PI 3-kinase activity has been found tightly associated with virtually every protein-tyrosine kinase that has been investigated for this activity. In addition to the tyrosine kinases discussed above, PI 3-kinase activity associates with pp62$^{v\text{-}yes}$ (Fukui et al., 1989), v-*abl*, and BCR-*abl* oncoproteins (L. Varticovski, G. Daley, P., Jackson, D. Baltimore, and L. Cantley, in preparation), insulin receptor from insulin-stimulated cells (Ruderman et al., 1990), CSF-1 receptor from CSF-1-stimulated cells (Varticovski, 1989), and the v-*fms* oncoprotein (Varticovski, 1989). Gutkind et al. (1990) have recently found that PI 3-kinase will immunoprecipitate with pp60$^{c\text{-}src}$ and p59$^{c\text{-}fyn}$ in thrombin-stimulated platelets. Anti-phosphotyrosine antibody immunoprecipitates from IGF-1 stimulated cells also have PI 3-kinase activity (Kapeller et al., 1990). Most of the subfamilies of the protein-tyrosine kinase superfamily (Hanks et al., 1988) are represented, suggesting that the primordial protein-tyrosine kinase associated with PI 3-kinase and that this association has been retained through evolution of the subfamilies. A possible exception is the EGF receptor, which in our hands does not appear to associate with the PI 3-kinase. However, Bjorge et al. (1990) do report association of PI 3-kinase with the EGF receptor. In any event, it seems likely that PI 3-kinase provides a signal used in common by most protein-tyrosine kinases (see below for further discussion of physiological role).

The evidence that protein-tyrosine kinases regulate PI 3-kinase is thus far indirect. ^{3}H-Labeled quiescent fibroblasts or smooth muscle cells have detectable levels of PI-3-P but no detectable PI-3,4-P_2 or PI-3,4,5-P_3. Within 5 min of stimulation by PDGF, [^{3}H]inositol-labeled lipids, with migration positions by HPLC identical with those of the latter two structures, appear in the cell (Auger et al., 1989a). The appearance of these lipids is temporally consistent with appearance of enzymatic activities that convert PI-4-P to PI-3,4-P_2 and PI-4,5-P_2 to PI-3,4,5-P_3 in PDGF receptor immunoprecipitates. Also, in agreement with these results, there is an increase in the amount of membrane-associated PI 3-kinase activity in the PDGF-stimulated cells, indicating recruitment from the cytosol (unpublished results of Auger and Cantley).

The model proposed on the basis of these results is that activation of the PDGF receptor results in recruitment of the PI 3-kinase from the cytosol to the cytoplasmic domain of the receptor. This recruitment would bring the enzyme to the membrane where PI-4-P and PI-4,5-P_2 are available as substrates. Although the 85-kDa subunit (and probably the 110-kDa subunit) of the PI 3-kinase become phosphorylated on tyrosine and serine during this recruitment (see above), the phosphorylation need not play a role in activation. The PI 3-kinase purified from rat liver cytosol has a fairly high specific activity (2–3 μmol mg^{-1} min^{-1}) and does not appear to be significantly phosphorylated on tyrosine (Carpenter and Cantley, unpublished results). In preliminary studies, phosphorylation of the pure enzyme with middle T/pp60$^{c\text{-}src}$ (>1 mol/mol on the 85-kDa subunit) does not appear to affect V_{max} or substrate specificity. However, as discussed above, there is some evidence that the crude cytosolic enzyme has an associated inhibitor that affects substrate specificity. Thus, it is possible that tyrosine phosphorylation of the recruited PI 3-kinase causes dissociation of the inhibitor, thereby allowing

phosphorylation of PI-4-P and PI-4,5-P_2. Further work is needed to test this possibility.

The mechanism by which activated protein-tyrosine kinases cause recruitment of PI 3-kinase has been investigated by the study of mutants of these proteins. In the case of the middle T/pp60$^{c\text{-}src}$ complex, mutations of either pp60$^{c\text{-}src}$ or middle T affect the association with PI 3-kinase. A kinase-defective mutant of pp60$^{c\text{-}src}$ complexes with middle T when the two proteins are expressed in the baculovirus system. However, this complex, unlike wild-type middle T/pp60$^{c\text{-}src}$, will not associate with purified PI 3-kinase in vitro (unpublished results of Auger, Carpenter, and Cantley). These results indicate that the tyrosine kinase activity of pp60$^{c\text{-}src}$ is essential for the complex formation. Mutation of tryosine-315 of middle T, the major site for phosphorylation by pp60$^{c\text{-}src}$, to a phenylalanine does not affect the ability of middle T to complex with pp60$^{c\text{-}src}$ in vivo or the ability of middle T to activate the protein-tyrosine kinase activity of pp60$^{c\text{-}src}$, but it does significantly reduce the association with PI 3-kinase (Talmadge et al., 1989; Whitman et al. 1985). These and other results indicate that phosphorylation of middle T at tyrosine-315 creates the binding site for association with the 85-kDa subunit of PI 3-kinase. Recent results from Hanafusa's laboratory (Mayer & Hanafusa, 1990) have suggested a novel mechanism for association of proteins that are phosphorylated on tyrosine residues. Many protein-tyrosine kinases and substrates of protein-tyrosine kinases contain a highly conserved ~70 amino acid domain that has been labeled the SH-2 domain (for *src* homology). This domain appears to provide a binding site for sequences that contain phosphotyrosine. Thus, a likely possibility is that phosphotyrosine–315 of middle T complexes with an SH-2 domain on the 85-kDa subunit of PI 3-kinase.

Studies of mutants of the PDGF receptor have led to a similar conclusion. Association of PI 3-kinase with this receptor requires the presence of a region of the cytosolic domain called the KI insert domain (a sequence in the kinase domain unique to the PDGF receptor) (Coughlin et al., 1989). Mutation of tyrosine-751 in this domain, a major autophosphorylation site, to phenylalanine or glycine dramatically reduces the association with PI 3-kinase (Kazlauskas & Cooper, 1989). Sequence similarity between this region of the PDGF receptor and the region around tyrosine-315 of middle T [consensus sequence: Glu/Asp-Tyr(P)-X-Pro-Met-Y-Asp-X, where X is hydrophobic and Y is Glu in middle T and Leu in the PDGF receptor] suggests that this domain is the binding site for the 85-kDa subunit of PI 3-kinase.

There is also a report in which phorbol ester was found to increase PI 3-kinase activity in middle T immunoprecipitates from middle T transformed fibroblasts (Raptis et al., 1988). In neutrophils the appearance of PIP_3 is activated by GTPγS and inhibited by pertussis toxin (Traynor-Kaplan et al., 1988, 1989), which suggests that a G protein is involved in the response.

Phosphatidylinositol-4-phosphate 5-Kinase. PI-4-P 5 kinase catalyzes the phosphorylation of PI-4-P at the D-5 position of the inositol ring to form PI-4,5-P_2. There is no evidence for an alternative route of synthesis of PI-4,5-P_2. The purified red cell plasma membrane enzyme will not significantly phosphorylate PI. PI-4-P 5-kinase is found in both soluble and membrane-bound forms. The proportion of the enzyme reported to be in the cytosolic fraction in various cells varies from 30 to 80% (Imai et al., 1986; Van Dongen et al., 1984; Ling, 1989).

The PI-4-P 5-kinases of the human red cell have been studied in detail. A membrane-associated PI-4-P 5-kinase was purified to homogeneity (Ling et al., 1989). This enzyme can be extracted from the membrane by high salt in the absence of detergent. It has an apparent molecular mass of 150 kDa by gel filtration and 53 kDa by SDS–PAGE. The enzyme demonstrated product inhibition by PI-4,5-P_2 and activation by phosphatidylserine and, as mentioned above, will phosphorylate PI-3-P under certain conditions. Anderson and collaborators (Bazenet et al., 1990) have separated two distinct PI-4-P 5-kinase from the red cell membrane and named them type 1 and type 2. The type 2 enzyme is identical with the enzyme purified by Ling et al. (1989) and is also identical with the major soluble PI-4-P 5-kinase of the red cell cytosol. Curiously, the type 2 enzyme appears to be abundant only in cells of bone marrow origin. The tissue distribution of the type 1 enzyme is more ubiquitous. Studies with inhibitory antibodies indicate that it is the type 1 rather than type 2 enzyme that is responsible for most PI-4,5-P_2 synthesis in the red cell. The subunit composition of the type 1 enzyme is still uncertain.

A cytosolic PIP-4-P 5-kinase was purified from rat brain and shown to have a molecular mass by gel filtration of 100–110 kDa (Cochet & Chambaz, 1986). The major band on SDS–PAGE of this preparation was 45 kDa. Moritz et al. (1990) have also purified a PI-4-P kinase from bovine brain membranes that has a molecular mass of 110 kDa by SDS–PAGE. The product of this enzyme has not been analyzed.

Cell fractionation studies have shown that most of the membrane-associated PI-4-P 5-kinase activity partitions with the plasma membrane. Consistent with this finding, the plasma membrane is enriched in PI-4,5-P_2 compared to internal membranes (Augert et al., 1989; Lundberg & Jergil, 1988; Seyfred & Wells, 1984). However, PIP kinase and PIP_2 synthesis has also been found in isolated nuclei (Couo et al., 1987; Smith & Wells, 1983).

Regulation of Phosphatidylinositol-4-phosphate 5-Kinase. The regulation of PI-4-P 5-kinase is poorly understood. Chemoattractants and leukotriene B4 have been found to activate the PIP kinase (probably a PI-4-P 5-kinase) associated with neutrophil membranes (Pike et al., 1990). A 2-fold increase in the V_{max} was found. GTPγS has also been found to increase the activity of a PIP kinase in rat brain (Smith & Chang, 1989) and placental membranes (Urumow & Wieland, 1986). No effect of GTPγS was seen on the soluble rat brain PIP kinase, which again suggests these may be different enzymes.

Recently, Gill and collaborators (Gordon Gill, personal communication) have found evidence for regulation of PI-4-P 5-kinase by the EGF receptor. This enzyme was found to associate with the EGF receptor in an EGF-dependent manner. The mechanism by which this association occurs is not clear, but it appears to involve the intrinsic protein-tyrosine kinase activity of the receptor. It is tempting to speculate that a mechanism similar to that outlined above for the PI 3-kinase recruitment to the PDGF receptor occurs. Whether the PI-4-P 5-kinase is the type 1 or type 2 described by Anderson (see above) or a third type remains to be determined.

Small molecules and peptides have been shown to affect PIP kinase activity in crude systems. As with PI 4-kinase, polyamines activate a PIP kinase partially purified from rat brain: in the presence of polyamines the Mg^{2+} concentration required for optimum activity is lowered (Lundberg et al., 1986). The synthesis of PI-4,5-P_2 in intact cells has been reported to be increased by phorbol esters, DAG, concanavalin A, and A23187 (Boon et al., 1985; de Chaffoy de Courcelles et al.,

1984; Halenda & Feinstein, 1984).

Phosphatidylinositol-3-phosphate 4-Kinase. A new phosphoinositide kinase activity was recently discovered in human platelets (Yamamoto et al., 1990). This activity converts PI-3-P to PI-3,4-P_2. Attempts to phosphorylate PI-3-P with purified type 2 PI 4-kinase and partially purified type 3 PI 4-kinase have failed (A. Graziani, G. Endemann, and L. Cantley, in preparation), indicating that the PI-3-P 4-kinase is distinct from previously reported enzymes. The presence of this enzyme in platelets indicates that the PI-3,4-P_2 produced in response to stimulation by thrombin may result from phosphorylation of PI-3-P at the D-4 position rather than phosphorylation of PI-4-P at the D-3 position as proposed for PDGF-stimulated smooth muscle cells (Auger et al., 1989a).

Physiological Roles for PI-4-P and PI-4,5-P_2. The best known role of PI-4-P is as a substrate for PI-4-P 5-kinase to form PI-4,5-P_2, the precursor to the well-known second messengers diacylglycerol and inositol 1,4,5-trisphosphate. However, there is evidence that in some cells only a small subfraction of total cellular PI-4-P serves as a precursor to PI-4,5-P_2 formation. Inokuchi and Imboden (1990) double labeled Jurkat cells with [^{3}H]inositol and $^{32}PO_4^{3-}$ and then stimulated the cells with an antibody to CD3. Within the first 60 s the primary response was breakdown of PIP_2 to IP_3 and DAG. From 1 min to greater than 20 min there was continued hydrolysis of newly synthesized PIP_2 in these cells. During this later time there was evidence of increased flux through PI and PIP_2 but not PIP, suggesting that PIP_2 was formed from a small pool of PIP with very high turnover so that no increase in flux was detected in the total PIP pool. This was confirmed by determining the specific activity of the 1-phosphate of PIP_2 which was 8–10 times greater than that of the 1-phosphate of PIP. These data suggest that in lymphocytes most of the PIP in the cell is not immediately available for conversion to PIP_2. The PI 4-kinase and PI-4-P 5-kinase enzymes involved in synthesis of the PI-4,5-P_2 that undergoes rapid turnover appear to be colocalized in a subcompartment of the cell. There are conflicting data as to whether there is a distinct pool of PI-4,5-P_2 which is preferentially hydrolyzed by phospholipase C in response to a stimulus (Berridge, 1987).

The existence of multiple cellular locations for PI-4-P along with the evidence that much of this PI-4-P (and perhaps some of the PI-4,5-P_2) is not immediately involved in PI turnover raises the possibility that these lipids have other functions besides being intermediates in PI turnover. Several actin binding proteins have been found to bind to both PI-4-P and PI-4,5-P_2. The best studied is the actin filament breaking protein gelsolin (Jamney et al., 1987; Jamney & Stossel, 1987, 1989). Either lipid (but not PI or inositol polyphosphates) will release gelsolin from severed ends of actin filaments, raising the possibility that these lipids regulate new filament formation. Since gelsolin association with PI-4-P is weak compared to the association with PI-4,5-P_2, it is likely that PI-4-P does not play a role in gelsolin–actin interactions in vivo. PI-4,5-P_2 has also been found to bind to profilin and to cause disassembly of actin–profilin complexes (Lassing & Lindberg, 1985). It has recently been shown that PIP_2 bound to profilin is not susceptible to hydrolysis by phospholipases C (Goldschmidt-Clermont et al., 1990). In vitro each profilin molecule binds seven PI-4,5-P_2 molecules. In vivo there is enough profilin that the majority of PI-4,5-P_2 could be bound to it. This could explain the low rate of hydrolysis of PI-4,5-P_2 in unstimulated cells. Other actin-associated proteins known to interact with PI-4,5-P_2 include myosin type I from *Acanthamoeba* (Adams & Pollard, 1989; Miyata et al., 1989) and the glycophorin–band 4.1 complex of the red cell (Anderson & Marchesi, 1985). In addition, a casein kinase (type 1) from the human red cell tightly associates with the PI-4,5-P_2–glycophorin complex and may be regulated by this association (Bazenet et al., 1990). This enzyme is thought to regulate cytoskeletal associations. Thus, these lipids have been strongly implicated as regulators of the actin component of the cytoskeleton.

Membrane-associated ATPases have also been shown to be affected by PI-4-P and PI-4,5-P_2. The red cell plasma membrane Ca pump (Penniston, 1982) and a nuclear envelope RNA-dependent ATPase (Smith & Wells, 1984) have been shown to be activated by polyphosphoinositides. In the former case PI-4,5-P_2 was more potent than PI-4-P, but in the latter case PI-4-P was most effective.

A requirement for phosphoinositides in secretion by adrenal chromaffin cells has recently been suggested by Eberhard et al. (1990). It is possible that this effect is mediated through interactions with cytoskeletal proteins with which adrenal chromaffin granules and phosphoinositides both interact.

PI-4-P is a good substrate for most phospholipases C that have been investigated and its hydrolysis could contribute to hormone-stimulated diacylglycerol production. Recently, PI-4-P was reported to regulate DNA polymerase α activity, although the activating molecule appeared to be inositol 1,4-bisphosphate produced from a phospholipase C activity in the polymerase α preparation (Sylvia et al., 1988). Thus, there is suggestive evidence but no conclusive evidence that both PI-4-P and PI-4,5-P_2 have cellular functions besides being intermediates in PI turnover.

Physiological Role for D-3-Phosphorylated Phosphoinositides. The D-3-phosphorylated phosphoinositides are not intermediates in the canonical PI turnover pathway (Figure 1A). It is likely that the D-3 polyphosphoinositides themselves, rather than inositol phosphates derived from them, act as second messengers. The phospholipases that act on PI, PI-4-P, and PI-4,5-P_2 do not hydrolyze any of the D-3 polyphosphoinositides (Lips et al., 1989; Serunian et al., 1989), indicating that this pathway is regulated independently of the cannonical pathway. Attempts to find a phospholipase C that hydrolyzes these new lipids have failed. In addition, unlike PI-4-P and PI-4,5-P_2 which are present at significant concentrations in unstimulated cells and generally decline in response to hormones or growth factors, PI-3,4-P_2 and PI-3,4,5-P_3 are absent in quiescent cells and are produced rapidly upon stimulation (Auger et al., 1989a). Thus, these lipids are likely to be signals rather than precursors of signals.

The route of synthesis of PI-3,4-P_2 in vivo is not known. Purified PI 3-kinase will phosphorylate PI-4-P to form PI-3,4-P_2, but not as well as it phorphorylates PI or PI-4,5-P_2 (Carpenter et al., 1990). PI 4-kinase purified from human red cells or type 3 PI 4-kinase from bovine brain will not phosphorylate PI-3-P (A. Graziani, G. Endemann, and L. Cantley, in preparation). We have identified a PI-3-P 4-kinase in platelets that will phosphorylate PI-3-P to PI-3,4-P_2 (Yamamoto et al., 1990). Minimal amounts of PIP_3 are generated in platelets, relative to PI-3,4-P_2. In other systems, where PI-3,4-P_2 and PIP_3 are correlated with growth they are generated in approximately equal amounts. This argues that in platelets the PI-3,4-P_2 generated in response to stimulation is due primarily to activation of the PI-3-P 4-kinase and not PI 3-kinase. In cells where PI-3,4-P_2 and PIP_3 are generated in more equal amounts, their synthesis may be due to activation of PI 3-kinase. EGF causes a very large increase in PI-3,4-P_2 (comparable to the PI-4,5-P_2 level) in Leydig tumor cells under conditions where it acts as a differentiation signal rather than

a mitogenic signal (Pignataro & Ascoli, 1990). A PI-3-P 4-kinase may also account for the synthesis of PI-3,4-P_2 in the Leydig tumor cell line since PIP_3 is not found in this system. The stimulation of PI-3,4-P_2 production in platelets by GTPγS suggests involvement of a G protein, which could be acting on PI-3-P 4-kinase. Phorbol esters have also been reported to activate PI-3,4-P_2 synthesis in platelets by a mechanism which may be mediated through protein kinase C (Yamamoto & Lapetina, 1990). This activation may also be mediated through an effect on the PI-3-P 4-kinase.

As with PI-3,4-P_2, the route of synthesis of PI-3,4,5-P_3 in vivo is not known. Since PI-4,5-P_2 is a very good substrate for the PI 3-kinase, it is likely that phosphorylation of PI-4,5-P_2 at the 3-position is the major pathway of synthesis. However, one cannot exclude phosphorylation of PI-3,4-P_2 at the 5-position or phosphorylation of PI-3,5-P_2 at the 4-position.

A compound tentatively identified as PI-3,5-P_2 on the basis of its migration pattern on HPLC is seen in smooth muscle cells labeled in vivo with [^{3}H]inositol, and a small amount is seen in platelets given [^{32}P]PI-3-P as a substrate (Auger et al., 1989a; Yamamoto et al., 1990). The levels of this compound do not seem to change with stimulation by PDGF. As with the other D-3-phosphorylated phosphoinositides, no function has been identified for PI-3,5-P_2. The route of synthesis of this compound is not known. The two potential routes by which it could be synthesized are phosphorylation of PI-5-P by PI 3-kinase, but PI-5-P has never been shown to exist. We have been able to phosphorylate PI-3-P at the 5-position with a kinase from sf9 cells which associates with the middle T antigen expressed in the baculovirus system (K. Auger and L. Cantley, unpublished observations). The PIP kinase purified from human red cells (Ling et al., 1989) will phosphorylate PI-3-P at the D-5 position of the inositol ring to varying degrees, depending on the enzyme preparation (L. Ling, M. Whitman, A. Graziani, and L. Cantley, unpublished observations).

Virtually every oncoprotein and growth factor receptor of the protein-tyrosine kinase superfamily that has been investigated has been found to associate with the PI 3-kinase (see Phosphatidylinositol 3-Kinase). In addition mutants of polyoma middle T (Cohen et al., 1990; Courtneidge & Heber, 1987; Kaplan et al., 1987, 1986; Talmadge et al., 1989; Whitman et al., 1985), pp60$^{v\text{-}src}$ (Fukui & Hanafusa, 1989), and the PDGF receptor (Coughlin et al., 1989; Kazlauskas, 1989) that fail to associate with PI 3-kinase were invariably defective in stimulating cell growth. The in vivo levels of PI-3,4-P_2 and PI-3,4,5-P_3 (but not PI-3-P) are elevated in transforming mutants of middle T but not in transformation-defective mutants (Serunian et al., 1990). These mutational studies have strongly implicated the PI 3-kinase in growth regulation and suggested that the products of this enzyme, PI-3,4-P_2 and/or PI-3,4,5-P_3, may be critical growth signals.

Although it is unlikely that activation of PI 3-kinase is the single critical event for cell transformation by these oncogenes, this possibility has not been adequately tested. There are examples of mutants in the middle T gene product (Drucker & Roberts), pp60$^{v\text{-}src}$ (Fukui & Hanafusa, 1989), and the *abl* oncoprotein (L. Varticovski, G. Daley, P. Jackson, D. Baltimore, and L. Cantley, in preparation) that are transformation defective but still associate with the PI 3-kinase, indicating that activation of PI 3-kinase alone is not sufficient for transformation. However, in those mutants that have been investigated to date, the in vivo levels of the products of this enzyme (PI-3,4-P_2 and PI-3,4,5-P_3) were depressed compared to those in transformed cells (Varticovski, 1990; Ling, Cantley, and Roberts, unpublished results), raising the possibility that the cellular location of the oncoprotein–PI 3-kinase complex may be important for transformation. For example, a mutant *abl* protein lacking an amino-terminal myristylation sequence has protein-tyrosine kinase activity and associates with the PI 3-kinase but does not have elevated levels of PI-3,4-P_2 or PI-3,4,5-P_3 and fails to transform fibroblasts (L. Varticovski, G. Daley, P. Jackson, D. Baltimore, and L. Cantley, in preparation). In this case the failure to elevate these lipids could be explained by the failure of the complex to associate with the membrane. In fact, one could argue that the general observation that oncoproteins of the protein-tyrosine kinase family must associate with the membrane to transform cells is because the targets with which they associate (PI 3-kinase, PI-specific phospholipase C-γ, and PI-4-P 5-kinase, for example) act on membrane lipids. Clearly, additional studies of the in vivo levels of the D-3 phosphoinositides in cells transfected with mutants of oncogenes will be needed to determine their necessity and/or sufficiency for transformation.

In some terminally differentiated cells the D-3-phosphorylated polyphosphoinositides appear to play a role unrelated to growth. Both PIP_3 and PI-3,4-P_2 are produced in neutrophils in response to stimulation by formyl peptide (Traynor-Kaplan et al., 1988, 1989). PI-3,4-P_2 and very small amounts of PIP_3 have been found in platelets in response to thrombin, GTPγS or a thromboxane A_2 analogue (Kucera & Rittenhouse, 1990; Nolan & Lapetina, 1990).

The inositol phospholipid pathway has become quite complex. During the next several years it is likely that additional phosphoinositide kinases will be purified and characterized. More importantly, the regulation of phosphoinositide kinases will be better understood and the intriguing links of inositol phospholipids to cellular functions, especially growth and transformation, may soon be clarified.

Registry No. Phosphoinositide kinase, 9077-69-4.

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Accelerated Publications

Mixed DNA/RNA Polymers Are Cleaved by the Hammerhead Ribozyme†

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ABSTRACT: A series of chemically synthesized oligodeoxyribonucleotides containing one or two ribonucleotides (DNA/RNA mixed polymers) at and/or adjacent to the cleavage site of the substrate can be cleaved by the "hammerhead" ribozyme. In comparison with the all-RNA substrate, the predominantly deoxyribonucleotide substrates have (1) lower optimal temperatures of cleavage, (2) approximately 6-fold higher K_m's and 7-fold lower k_{cat}'s at 30 °C, and (3) 15-fold higher K_m's and 8-fold lower k_{cat}'s at 37 °C. The extent to which the RNA substrate cleavage is inhibited in the presence of an all-DNA (K_I = 13 μM) and an RNA substrate analogue with a dC at the cleavage site (K_I = 0.96 μM) supports the contention that the formation of the ribozyme–substrate complex with the predominantly deoxyribonucleotide substrates (D substrates) is impaired. The weaker binding of D substrates was confirmed by thermal denaturation and determination of the T_m of the complex. Analysis of the kinetic data also suggests that the conformation of the catalytic core of the ribozyme–substrate complex differs from that of the all-RNA complex, a change that results from the presence of a DNA/RNA heteroduplex in the complex.

Self-cleavage of RNA has been demonstrated to play an important biological role in the processing of some RNAs, i.e., in plant virus satellite, viroid, virusoid, hepatitis δ virus, and newt satellite RNAs (Forster et al., 1988; Talbot & Bruening, 1988; Epstein & Gall, 1987). Unlike self-splicing and RNase P catalysis, RNA self-cleavage leads to 2′,3′-cyclic phosphate and 5′-hydroxyl products and, with the exception of the hepatitis virus in the above list, is associated with a consensus secondary structure and 13 consensus nucleotides called the hammerhead domain (Uhlenbeck, 1987; Gerlach & Haseloff, 1988; Koizumi et al., 1988; Forster et al., 1988; Jeffries & Symons, 1989). This structural domain can be separated into two RNA fragments: an enzymatic fragment (the ribozyme) and a substrate fragment as shown in Figure 1. The structural model consists of three RNA double helices which delimit the consensus nucleotides CUGAXGA (X can be any nucleotide) and GAAAC in the ribozyme and GUC in the substrate, many of which have been the object of nucleotide replacement studies in order to obtain information on their role in the cleavage mechanism (Koizumi et al., 1988; Sheldon & Symons, 1989a; Sampson et al., 1987).

We have recently introduced the use of chemically synthesized DNA/RNA mixed polymers to evaluate the significance of various 2′-hydroxyls in hammerhead catalysis (Perreault et al., 1990). These molecules may also give some clue to the reason why nucleic acid catalysis seems to be restricted to RNA. This work showed that 2′-OHs in the region of U^8GAUGA^{13} of the ribozyme and the 2′-hydroxyl adjacent to the scissile phosphate bond are important factors in cleavage. Now, we describe the application of the mixed polymer analogues of Figure 1 to analyze the structural requirements of the substrate in this reaction. Our results show that predominantly DNA substrates can be cleaved, demonstrating that the helical regions I and III of the hammerhead

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