Stereo-specific Substrate Recognition by Phosphatidylinositol Phosphate Kinases Is Swapped by Changing a Single Amino Acid Residue*

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Type I and type II phosphatidylinositol phosphate (PIP) kinases generate the lipid second messenger phosphatidylinositol (PtdIns) 4,5-bisphosphate and thus play fundamental roles in the regulation of many cellular processes. Although the two kinase families are highly homologous, they phosphorylate distinct substrates and are functionally non-redundant. Type I PIP kinases phosphorylate PtdIns 4-phosphate at the D-5 hydroxyl group and are consequently PtdIns 4-phosphate 5-kinases. By contrast, type II PIP kinases are PtdIns 5-phosphate 4-kinases that phosphorylate PtdIns 5-phosphate at the D-4 position. Type I PIP kinases, in addition, also phosphorylate other phosphoinositides in vitro and in vivo and thus have the potential to generate multiple lipid second messengers. To understand how these enzymes differentiate between stereoisomeric substrates, we used a site-directed mutagenesis approach. We show that a single amino acid substitution in the activationloop, A381E in Iβ and the corresponding mutation E362A in Iβ, is sufficient to swap substrate specificity between these PIP kinases. In addition to its role in substrate specificity, the type I activation loop is also key in subcellular targeting. The Iβ(E362A) mutant and other mutants with reduced PtdIns 4-phosphate binding affinity were largely cytosolic when expressed in mammalian cells in contrast to wild-type Iβ which targets to the plasma membrane. These results clearly establish the role of the activation loop in determining both signaling specificity and plasma membrane targeting of type I PIP kinases.

The lipid second messenger PtdIns(4,5)P₂, plays critical roles in the regulation of membrane trafficking, cytoskeletal organization, channel activities, nuclear functions, apoptosis, and signal transduction (1–3). Growing evidence suggests that the synthesis of PtdIns(4,5)P₂ at specific intracellular locations is dynamically regulated and signals the recruitment or the activation of specific proteins involved in mediating the distinct downstream effects of PtdIns(4,5)P₂ (4–6). PtdIns(4,5)P₂ also serves as the precursor to the second messengers inositol 1,4,5-trisphosphate (IP₃), diacylglycerol, and PtdIns(3,4,5)P₃ (3, 7). Consequently, the generation and degradation of PtdIns(4,5)P₂ is also required for initiation of these additional signals. Thus, a better understanding of the mechanisms that control the production of PtdIns(4,5)P₂ is key to understanding many cellular processes regulated by PI signaling. However, until recently, little was known regarding the temporal and spatial regulation of PtdIns(4,5)P₂ synthesis. The cloning and characterization of the PIP kinases that synthesize PtdIns(4,5)P₂, together with the solution of the first three-dimensional structure of one of the PIP kinases, serve as an entry point to understand the regulation of PI signaling processes at the molecular level (8–11).

PIP kinases are a novel family of lipid kinases that are structurally related to protein and PI 3-kinases despite an almost complete lack of primary sequence identity (9, 12, 13). To date three distinct subfamilies have been identified that share significant sequence homology in their catalytic domains but differ in their substrate usage, subcellular localization, and function (8, 10). Two of these subfamilies, the type I and type II PIP kinases, are responsible for the production of PtdIns(4,5)P₂, albeit by different pathways. The type I PIP kinases phosphorylate PtdIns(4)P at the D-5 hydroxyl group and are, thus, PtdIns(4)P 5-kinases (14, 15). The type II PIP kinases, in contrast, are PtdIns(5)P 4-kinases that phosphorylate PtdIns(5)P at the D-4 position to generate PtdIns(4,5)P₂ (16). The third class, III PIP kinases, of which yeast Fab1 is the prototype, does not generate PtdIns(4,5)P₂ but rather phosphorylates PtdIns(3)P to PtdIns(3,5)P₂ (16–18).

The distinct substrate specificities of type I and type II PIP kinases are likely physiologically significant because members of the two subfamilies are functionally not redundant and primarily localize to different cellular compartments (19, 20). Type I PIP kinases have been implicated in the regulation of actin reorganization, secretion, endocytosis, apoptosis, and ion channel activity (21–26). In yeast and mammalian cells type I PIP kinases have been predominantly detected at the plasma membrane (20, 24, 25, 27) but are also present in the nucleus and at the Golgi (28, 29). Type II PIP kinases are only found in metazoan and localize to the cytosol, the nucleus, the endoplasmic reticulum, and the actin cytoskeleton (28, 30–33). Recent reports suggest a role for the type II PIP kinases in Ca²⁺-induced granule secretion in platelets (34) and in aspects of tumor necrosis factor α-mediated signaling (35), but the precise function of type II PIP kinases in the regulation of these processes has not yet been identified.

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1 The abbreviations used are: PtdIns(4,5)P₂, phosphatidylinositol 4,5-bisphosphate; DMEM, Dulbecco’s modified Eagle’s medium; PtdIns, phosphatidylinositol; PtdIns(3)P, phosphatidylinositol 3-phosphate; PtdIns(3,4)P₂, phosphatidylinositol 3,4-bisphosphate; PtdIns(3,4,5)P₃, phosphatidylinositol 3,4,5-trisphosphate; PtdIns(4)P, phosphatidylinositol 4-phosphate; PtdIns(5)P, phosphatidylinositol 5-phosphate; PI 3-kinase, phosphoinositide 3-kinase; PIP, phosphatidylinositol phosphate.
Although it was generally thought that the physiological function of type I and type II PIP kinases involves the generation of PtdIns(4,5)P₂ only, recent findings suggest that their cellular roles may be more complex. Two reports demonstrated that type I and type II PIP kinases also use other phosphoinositide substrates in vitro, although typically with lower efficiency (15, 36). Type I PIP kinases are the most promiscuous enzymes and phosphorylate PtdIns(3)P on both the 4- and 5-hydroxyls forming PtdIns(3,4)P₂ and PtdIns(3,5)P₂. Moreover, they can generate PtdIns(3,4,5)P₃ by the successive phosphorylation of PtdIns(3)P on the D-4 and D-5 positions of the inositol head group (15). At least some of the three mammalian type I isoforms can also generate PtdIns(5)P from phosphatidylinositol and may thus be responsible for the synthesis of this novel lipid (36). Type II PIP kinases also use PtdIns(5)P as a substrate; however, the product of this reaction is the activation of PtdIns(4,5)P₂ only, recent findings suggest that their molecular mechanisms by which these kinases specifically recognize and bind the closely related substrates.

To reveal these mechanisms, we focused our study on two specific PIP kinase isoforms, human type Iβ and type IIβ. By using chimeras of Iβ and IIβ, we have previously shown that the substrate specificity of type I and type II PIP kinases is determined by an ~25-amino acid region, termed the activation loop (19). This small domain is diagnostic of the different PIP kinase subfamilies and is topologically equivalent to the activation loop in protein kinases (9). Because the loop region was disordered in the three-dimensional structure of Iβ, the first PIP kinase to be crystallized (9), it is unclear how substrate is recognized and bound by any given PIP kinase subfamily. However, activation loop sequences contain a limited number of residues that are conserved in a type-specific manner, suggesting that the differences in substrate preference might be controlled by few amino acid residues. To identify these specificity determinants, we generated an array of mutants within the Iβ and IIβ activation loops by site-directed mutagenesis, and we characterized their activity toward phosphoinositide substrates. We report that mutation of a single residue within the activation loop is sufficient to switch substrate preference of IIβ to that of Iβ and vice versa. Moreover, in agreement with earlier findings (19), we find that mutations in Iβ that decrease the affinity for PtdIns(4)P impair the ability of the kinase to localize to the plasma membrane. These results establish a dual role of the activation loop in defining substrate specificity and promoting membrane association and indicate that PtdIns(4)P binding is critical for the localization of Iβ to the plasma membrane.

**EXPERIMENTAL PROCEDURES**

**Materials, Strains, and Plasmids—**Synthetic PtdIns(3)P, PtdIns(4)P, and PtdIns(5)P dipalmitoyl esters were purchased from Echelon (Salt Lake City, UT). LipofectAMINE Plus, Opti-MEM, and fetal bovine serum were obtained from Invitrogen. Ni²⁺, LipofectAMINE Plus, Opti-MEM, and fetal bovine serum were obtained from Invitrogen. Ni²⁺, LipofectAMINE Plus, Opti-MEM, and fetal bovine serum were obtained from Invitrogen. Ni²⁺, LipofectAMINE Plus, Opti-MEM, and fetal bovine serum were obtained from Invitrogen. Ni²⁺, LipofectAMINE Plus, Opti-MEM, and fetal bovine serum were obtained from Invitrogen.

**Lipid Kinase Assays—**Lipid kinase activity assays were carried out for 4 min at room temperature in 50-μl reactions containing 50 mM Tris, pH 7.6, 10 mM MgCl₂, 0.5 mM EGTA, 20 μM substrate (0.25–100 μM for kinetic studies) presented in micelles, and 50 μM ATP (10⁻² M/ATP, 5 μCi/reaction). Reactions were terminated and lipid products extracted and analyzed as described before (15). The data of kinetic studies are representative of two separate experiments done in duplicate. Km values were obtained from the slopes and y axis intercepts of the primary double-reciprocal plots. Units for kinetic values are μM for Km and pmol/min per mg of purified protein were based on a Bradford assay for Vmax.

**Cell Culture, Transfection, Indirect Immunofluorescence, and Confocal Microscopy—**Human osteosarcoma MG-63 cells were grown in Dulbecco’s modified Eagles medium containing 10% fetal bovine serum and antibiotics. Semi-confluent cultures grown on glass coverslips were transiently transfected and processed for indirect immunofluorescence as described previously (19). Cells were examined using a 60× plan oil immersion lens (NA 1.4) on a Bio-Rad MR 1000 laser-scanning confocal microscope mounted transversely to an inverted Nikon Diaphot 200. The microscope was controlled by the Lasersharp 3.2 program (BioRad). Z series were created by sequentially scanning green and red channels at 0.4-μm steps. Single sections or projections built with the Lasersharp 3.2 program were exported to Adobe Photoshop 6.0 for final image processing.
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RESULTS

The N-terminal Portion of the Activation Loop Determines Substrate Specificity—To identify the substrate specificity determinants in the activation loops, we first bisected the loop and constructed four chimeras, termed \( \beta \)-II\( \alpha \)N, \( \beta \)-II\( \alpha \)C, \( \beta \)-II\( \beta \)N, and \( \beta \)-II\( \beta \)C, in which either the N- or the C-terminal portions of the \( \beta \) and II\( \beta \) activation loops were reciprocally exchanged (Fig. 1B). An invariant lysine residue positioned in the middle of both loops (Lys-366 in \( \beta \) and the corresponding Lys-385 in II\( \beta \)) was used as an anchor point for the construction of the chimeras. The four chimeric kinases were expressed in E. coli, purified to homogeneity, and tested for their ability to phosphorylate synthetic phospholipid substrates. We found that the N-terminal portion of the activation loop was both necessary and sufficient to confer the substrate specificity of the donor enzyme. As could be expected, the \( \beta \)-II\( \beta \)C chimera, in which the N-terminal portion of the \( \beta \) loop was replaced with the corresponding region from II\( \beta \), gained the ability to phosphorylate PtdIns(4,5)P but could no longer phosphorylate PtdIns(5)P (Fig. 2, lanes 3 and 9). By contrast, the \( \beta \)-II\( \alpha \)C chimera, which contained the C-terminal portion of the \( \beta \) activation loop, behaved like wild-type \( \beta \) and efficiently phosphorylated PtdIns(5)P but did not utilize PtdIns(4,5)P as a substrate (Fig. 2, lanes 4 and 10). Thus, it appears that replacement of the N-terminal portion of the II\( \beta \) activation loop with the corresponding region of \( \beta \) is sufficient to swap substrate specificity suggesting that this region contains the residues defining substrate specificity.

These results were corroborated by the characterization of the substrate usage of the \( \beta \)-II\( \alpha \)N and \( \beta \)-II\( \beta \)C chimeras. Accordingly, the \( \beta \)-II\( \alpha \)N chimera retained the substrate specificity of wild-type \( \beta \) and exclusively phosphorylated PtdIns(4,5)P but not PtdIns(5)P (Fig. 2, lanes 6 and 12). By contrast, the \( \beta \)-II\( \beta \)C chimera, which contained the N-terminal activation loop region of II\( \beta \), gained the ability to phosphorylate PtdIns(5)P, in contrast to wild-type \( \beta \) that did not efficiently utilize this substrate (Fig. 2, lanes 5 and 11). Interestingly, whereas the \( \beta \)-II\( \beta \)C chimera strongly preferred PtdIns(4,5)P as a substrate, it did not completely lose its ability to phosphorylate PtdIns(4,5)P (Fig. 2, lanes 3). These findings indicate that additional residues that are possibly present in the C-terminal portion of the activation loop are required for more complete exclusion of PtdIns(4,5)P. At present, we cannot distinguish whether residues in the \( \beta \) loop are required for active exclusion of PtdIns(4,5)P or whether the PtdIns(4,5)P-binding site extends further into the C-terminal portion of the \( \beta \) activation loop. Nevertheless, these combined results suggest that the amino acid residue(s) necessary for discrimination between PtdIns(4,5)P and PtdIns(5)P are mainly located within the first 12 amino acids of the activation loop of type I and type II PIP kinases.

Determinants of Type II Substrate Specificity—Sequence alignment of the PIP kinases indicates a series of amino acid residues that are highly conserved within type I or type II PIP
kinase activation loops but are divergent between them (Fig. 1A). Interestingly, the majority of these type-specific residues are clustered within the N-terminal portion of the activation loop that we identified above as controlling substrate preference. Because these residues are good candidates for specificity determinants, they were targeted by site-directed mutagenesis. Thus, a panel of mutants was created in which type-specific residues were flanking the N-terminal activation loop region were mutated (summarized in Fig. 1C). In addition, a number of invariant residues within and flanking the N-terminal activation loop region were mutated (Fig. 1C). The resulting mutant variants were expressed in E. coli and purified, and their lipid kinase activity toward PtdIns(4)P and PtdIns(5)P was determined. Of all the mutants analyzed, only the IIβ(A381E) substitution resulted in a switch in specificity (Fig. 2B). Both substitutions did, however, cause a moderate (A380E) to severe (H382E) decrease in activity toward PtdIns(5)P indicating that both of these invariant residues are required for aspects of PtdIns(5)P binding or catalysis (Fig. 1C and Fig. 2B). Thus, the positioning of the glutamate residue within the activation loop is critical for stereo-specific recognition of substrate, a finding that is consistent with the conservation of this residue in all type I PIP kinases.

Next, we wanted to assess whether the presence of negative charge rather than the specific chemical nature of the amino acid is critical for switching substrate preference. Accordingly, we created a mutant in which Ala-381 was substituted with asparagine, and we tested its ability to phosphorylate PtdIns(4)P and PtdIns(5)P. Interestingly, the introduction of an asparagine residue at 381 only partially changed the specificity of the IIβ enzyme. The IIβ(A381D) mutant exhibited dual substrate specificity and was able to utilize PtdIns(4)P and PtdIns(5)P with equal efficiency (Fig. 2B). This finding suggests that the switch in substrate specificity in IIβ(A381E) cannot be solely attributed to the introduction of a negative charge at position 381 but may require a specific and likely direct interaction of the longer side chain in glutamate with either substrate or other residue(s) in the kinase.

**Determinants of Type I Substrate Specificity**—By having established that substitution of Ala-381 with glutamate in IIβ is sufficient to switch substrate specificity, we wanted to investigate the effect of the reciprocal mutation in IIβ. Therefore, a mutant, IIβ(E362A), was constructed in which Glu-362, a residue that is conserved among type I PIP kinases, is substituted with alanine. This mutation was sufficient to switch substrate preference toward PtdIns(5)P because this substrate, but not PtdIns(4)P, was readily phosphorylated by the mutant kinase (Fig. 3, lanes 4 and 8). Although the IIβ(E362A) mutant protein was able to phosphorylate PtdIns(4)P at higher substrate concentrations (>50 μM), PtdIns(5)P was still the preferred substrate under these conditions (data not shown). Taken together, it appears that the substrate preference of type I and type II PIP kinases is critically determined by the presence or the absence of the glutamate residue in the activation loop.

**Phosphorylation of PtdIns(3)P by Mutant Activation Loop Variants**—In vitro, type I and type II PIP kinases also phosphorylate PtdIns(3)P, although with lower efficiency than their primary substrates PtdIns(4)P and PtdIns(5)P, respectively. Although type I PIP kinases produce a mixture of PtdIns(3,4)P₂ and PtdIns(3,5)P₂, type II PIP kinases largely generate PtdIns(3,4)P₂ (15, 36). To investigate whether the differences in phosphorylation site choice between type I and type II PIP kinases are influenced by the presence or absence of the glutamate residue in the activation loop, we compared the activity of the IIβ(E362A) and IIβ(A381E) mutant kinases toward synthetic PtdIns(3)P. Typically PtdIns(3,4)P₂ and PtdIns(3,5)P₂, the two expected products of the kinase reactions, can be separated from each other by the TLC running system (40). By this method, PtdIns(3,4)P₂ can be distinguished from PtdIns(3,5)P₂ by its slightly different RF value (see Fig. 4, lanes 1 and 2). We thus used the difference in migration rate between the two 3-phosphorylated species to define the products generated by the IIβ(E362A) and IIβ(A381E) mutant kinases.

Under these conditions, IIβ generated an equal mixture of PtdIns(3,4)P₂ and PtdIns(3,5)P₂ from PtdIns(3)P, whereas IIβ produced PtdIns(3,4)P₂ only (Fig. 4, lanes 1 and 2), in agreement with earlier reports (36, 19). Despite its ability to phosphorylate PtdIns(4)P at the D-5 position, IIβ(A381E) exclusively produced PtdIns(3,4)P₂ (Fig. 4, lane 5). Thus, although substrate preference is swapped from a type II to a type I PIP
FIG. 4. PtdIns(3)P phosphorylation by activation loop mutants. Lipid kinase activities toward synthetic PtdIns(3)P of Iβ (lane 1), IIβ (lane 2), and the Iβ-IIβloop chimera (lane 3) are compared with those of the Iβ(E362A) (lane 4) and IIβ(A381E) variants (lane 5). Phosphorylated phospholipids were separated by thin layer chromatography for 2.5 h. An autoradiogram of the kinase assay is shown. The positions of PtdIns(3,4)P₂ and PtdIns(3,5)P₂ as well as of the origin are indicated by arrows. A third, as yet unidentified, phosphorylated product (denoted by X) with faster migration speed than PtdIns(3,4)P₂ and PtdIns(3,5)P₂ was observed and is likely a contaminant present occasionally in the PtdIns(3)P preparation.

kinase in the Iβ(A381E) kinase, phosphorylation site choice is not modified accordingly. Similar results were previously observed with the Iβ-Iβloop chimera (19) (Fig. 4, lane 3) that contained the entire type I activation loop region inserted into the Iβ backbone. These results suggest that phosphorylation site choice and generation of PtdIns(3,5)P₂ are not solely determined by the activation loop sequence but may also involve kinase core residues.

Interestingly, phosphorylation site choice was affected by the Glu-362 to alanine substitution in Iβ. This mutant lost its ability to phosphorylate PtdIns(3)P at the D-5 position of the inositol head group and only generated PtdIns(3,4)P₂ (Fig. 4, lane 4). This finding is in agreement with our earlier observations that substitution of the Iβ activation loop with that of IIβ abrogates generation of PtdIns(3,5)P₂ (19). Therefore, although the activation loop does not exclusively determine phosphorylation site choice, activation loop residues, particularly Glu-362 in Iβ, likely affect the orientation of substrate and its presentation to active site residues involved in the phosphotransfer reaction.

Kinetic Properties of Mutant PIP Kinases—To investigate the substrate preference of the Iβ(A381E) mutant in more detail, we determined the apparent Kₘ, Vₘₐₓ, and Vₘₐₓ/Kₘ values for the various substrates. As expected, PtdIns(4)P was the preferred substrate of the Iβ(A381E) mutant and was bound and converted to PtdIns(4,5)P₂ with considerable affinity and efficiency (Vₘₐₓ/Kₘ ratio of 39, see Table I). In fact, the apparent Kₘ value for PtdIns(4)P binding (Kₘ of 19 μM, Table I) was comparable with that of the previously reported Iβ-Iβloop chimera (19) in which the entire loop was exchanged (Kₘ of 18 μM, see Table I). By contrast, no significant phosphorylation of PtdIns(5)P was detected within the substrate concentration range assayed (0.25–100 μM). Similar to wild-type Iβ, PtdIns(3)P was only a weak substrate for the Iβ(A381E) mutant (Vₘₐₓ/Kₘ ratio of 1.5, see Table I) and was bound and converted with relatively weak affinity and efficiency, respectively. Taken together, these results demonstrate that substitution of Ala-381 with glutamate is sufficient, both qualitatively and quantitatively, to convert the PtdIns(5)P 4-kinase into a PtdIns(4)P 5-kinase.

Our kinetic studies also confirmed that the Iβ(E362A) mutant preferentially used PtdIns(5)P over PtdIns(4)P and PtdIns(3)P. The Vₘₐₓ/Kₘ ratio for PtdIns(5)P was 6-fold greater than that for PtdIns(4)P and 18-fold greater than that for PtdIns(3)P (Table I). Interestingly, the Iβ(E362A) mutant was still able to bind PtdIns(4)P with relatively high affinity (Kₘ of 5.5 μM, see Table I). However, the Vₘₐₓ of the reaction was significantly reduced resulting in a dramatic decrease in overall activity toward PtdIns(4)P (Vₘₐₓ/Kₘ ratio of 28, see Table I). By contrast, the Iβ(E362A) mutant bound (Kₘ of 3.5 μM) and phosphorylated PtdIns(5)P with high affinity and efficiency (Vₘₐₓ/Kₘ ratio of 169, see Table I), making PtdIns(5)P the preferred substrate of the mutant. Taken together, these results confirm that substrate preference of type I and type II PIP kinases is critically determined by the presence or the absence of the glutamate residue in the activation loop. The Iβ(E362A) mutant kinase has a low Kₘ value for PtdIns(4)P (high affinity) but poorly phosphorylates PtdIns(4)P (low Vₘₐₓ) as shown in Table I.

The N-terminal Portion of the Iβ Activation Loop Is Required for in Vivo Plasma Membrane Association—Our previous results (19) suggested that the Iβ activation loop also provides a high affinity membrane-binding site that is required for targeting and/or anchoring of Iβ to the plasma membrane. Because mutations in Iβ that abolish PtdIns(4)P binding also abrogate plasma membrane localization, a direct link between substrate binding and membrane association appeared possible (19). To test this hypothesis further, we investigated whether the membrane- and substrate-binding sites within the Iβ activation loop could be functionally separated from each other. To determine whether plasma membrane localization is dependent on the N-terminal, substrate binding, or the C-terminal portion of the activation loop, we made use of the Iβ-IIβ and Iβ-IIₐ chimeras. Accordingly, FLAG-tagged Iβ-IIβ and Iβ-IIₐ chimeras together with the Iβ-Iβloop chimera and wild-type controls were transiently transfected into MG63 fibroblast cells, and the subcellular localization of the expressed proteins was assessed by confocal immunofluorescence microscopy. As expected, FLAG-tagged Iβ localized to the plasma membrane and, occasionally, dot-like internal structures, whereas IIβ was only detected in the cytoplasm and the nucleus (Fig. 6, A–D) (19). The Iβ-IIβ chimera that still contained the N-terminal portion of the Iβ activation loop predominantly localized to the plasma membrane (Fig. 5, E and F), although a minor fraction of the kinase was consistently found in the cytoplasm in the majority of transfected cells. By contrast, the Iβ-IIβ chimera, in which the N-terminal portion of the Iβ activation loop was replaced with the corresponding region of IIβ, was largely delocalized from the plasma membrane and detected throughout the cytosol and the nucleus (Fig. 5, C and D). Consistent with its ability to localize to the plasma membrane and to generate PtdIns(4,5)P₂, the Iβ-IIβ chimera, but not the Iβ-IIβ chimera, induced actin cytoskeleton reorganization upon overexpression (Fig. 5, C–F). Taken together, these data suggest that the N-terminal portion of the Iβ activation loop, which also determines substrate specificity, is required for stable plasma membrane association and in vivo function. Furthermore, because the N-terminal portion of the activation loop appears to contain a high affinity membrane-binding site, a second, low affinity membrane-interaction site may be present in the C-terminal portion of the loop.

To test whether the N-terminal region of the Iβ activation loop is also sufficient to mediate plasma membrane association, as was observed previously (19) for the full-length Iβ activation loop, we characterized the subcellular localization of the IIβ-Iβ and IIβ-Iβ chimeras. Immunofluorescence analysis of MG63 cells transfected with these FLAG-tagged chimeras revealed that neither could promote plasma membrane associa-
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The data are representative of two independent sets of experiments. Units for kinetic values are μM for $K_m$ and pmol/min per mg of purified protein based on a Bradford assay for $V_{max}$.

| Enzymes       | PtdIns(3)P     | PtdIns(4)P     | PtdIns(5)P     |
|---------------|----------------|----------------|----------------|
| $K_m$         | $V_{max}$      | $V_{max}/K_m$  | $K_m$          | $V_{max}$ | $V_{max}/K_m$ | $K_m$ | $V_{max}$ | $V_{max}/K_m$ |
| β             | 20             | 100            | 5              | 24          | 100          | >100  | ND          | ND          |
| IIβ           | 24             | 18             | 1              | 680         | 680          | >80   | ND          | ND          |
| IIβ-1βloop    | 9              | 80             | 8.9            | 18          | 656          | 38    | ND          | ND          |
| IIβ(A381E)    | 22             | 34             | 1.5            | 745         | 39           | >100  | ND          | ND          |
| IIβ(E362A)    | 3              | 27             | 9              | 156         | 28           | 4     | 680         | 169         |

ND, not determined.

The presence and positioning of the invariant glutamate residue within the loop is consistent with the substrate selectivity of the type I kinases. A glutamate at this position within the activation loop is found in all PIP kinases that phosphorylate the D-5 position of polyphosphoinositides. These comprise all known or suspected type I PIP kinases that phosphorylate PtdIns(4)P and PtdIns(3)P, as well as the type III PIP kinases that phosphorylate PtdIns(3)P. Thus it appears that the glutamate residue in the activation loop serves to prevent access of phosphoinositide substrates that are phosphorylated at the D-5 position of the inositol head group. One mechanism for this specificity could be electrostatic repulsion between the negatively charged side chain of glutamate and the negatively charged phosphate present at the D-5 position of the inositol head group. In agreement with such an hypothesis, mutation of Glu-362 in the Iβ(E362A) mutant to an uncharged alanine residue would eliminate the potential for electrostatic repulsion and result in the dual specificity for PtdIns(4)P and PtdIns(5)P observed in our experiments. Alternatively, the glutamate in Iβ could interact with a positively charged residue in an ion pair and thereby stabilize a specific confirmation of the activation loop such that it specifically interacts with PtdIns(4)P but not with PtdIns(5)P. Because the kinase core is not required for stereo-specific substrate recognition, the glutamate may interact with one or more of the invariant lysine residues in the loop. In agreement with the conservation of the glutamate residue in all type I PIP kinases, our site-directed mutagenesis studies demonstrate that an aspartate residue at this same position can only partially compensate for glutamate. Thus, although the IIβ(A381D) mutant is able to phosphorylate PtdIns(4)P, it does not exclude binding or phosphorylation of PtdIns(5)P.

Our kinetic data further indicate that the activation loops have a somewhat promiscuous binding specificity for substrate but that residues within the loop are key for orienting the substrates in the kinase core for efficient catalysis (see Table I). This is emphasized by the Iβ(E362A) that has similar $K_m$ values for all substrates, whereas the $V_{max}$ values vary for substrates by more than 10-fold. Thus, whereas one key function provided by the loop is to stereo-specifically bind the substrate, the loop must also play an important role in orienting the substrate properly in the catalytic core for specific phosphotransfer to the correct hydroxyl. Glu-362 appears to be key.
for the orientation of PtdIns(4)P in the catalytic site for phosphorylation.

The data presented here also further emphasize the role of the type I activation loop in mediating membrane association as suggested by our earlier findings (19). We show that the primary membrane-binding site is contained within the N-terminal portion of the Iβ activation loop. Exchange of this sequence, but not of the C-terminal portion of the loop, with the corresponding region of IIβ completely abrogates plasma membrane association. In agreement with this finding, the conserved basic dilysoine motif that we previously identified as being critical for plasma membrane localization and PtdIns(4)P binding (19) is located within this N-terminal portion of the activation loop.

We speculated previously that plasma membrane localization of Iβ may, at least in part, be dependent upon binding of PtdIns(4)P, which may be enriched at the plasma membrane.
(19). We have further investigated whether membrane localization can be correlated with substrate binding by making use of I β activation loop chimeras and point mutants that exhibit modified substrate preference. Our combined data suggest that association of I β with the plasma membrane requires the ability to bind PtdIns(4)P. However, our data also show that PtdIns(4)P by itself is not sufficient to induce membrane recruitment of I β. This contention is supported by the data showing that the II β-IA β Loop, II β(A381E), and I β(E362A) variants have similar $K_m$ values for PtdIns(4)P, yet only the II β-IA β Loop chimera is targeted to the plasma membrane. By assuming that the $K_m$ for substrate is proportional to the substrate binding affinity, then membrane binding requirements within the loop must only partially be dependent upon affinity for PtdIns(4)P. Nevertheless, it is compelling that the point mutant I β(E362A) is much less plasma membrane-targeted compared with wild-type I β.

How does the small activation loop sequence provide both high affinity substrate and membrane-binding sites? Our studies clearly demonstrate that altogether the activation loop of both type I and type II PIP kinases is highly sensitive to mutations and that all the conserved residues, whether type-specific or invariant, play an important catalytic or structural role. Thus, we speculate that although the activation loop is disordered in the II β structure (9), it must adopt a highly ordered set of structures upon substrate and membrane binding. Indeed, secondary structure prediction using the PHD program indicates that the N-terminal portion of type I and type II activation loops has a high tendency of forming an amphipathic α-helix that would fit between the substrate and the kinase core. It is thus possible that the activation loop may undergo a structural change that leads to an increase in α-helix content upon binding of substrate and interaction with the membrane. Such a model is also attractive because a transition from a more random coil to a helical structure would result in a spring effect pulling the substrate into the kinase core.

Phosphoinositide recognition involving an amphipathic α-helix would be distinct from the β-strands that form the phosphoinositide-binding sites in pleckstrin homology and FYVE (where FYVE is Fab1p, YOTB, Vac1p, and EEA1 (early endosomal antigen 1) domains (41–45). According to such a model, the basic side of the amphipathic helix could interact with negatively charged head group of the phosphoinositide substrate as well as with other acidic membrane lipids. This would be consistent with our data showing that all conserved basic residues in the II β activation loop are required for wild-type kinase activity (Fig. 1) and would also provide an explanation as to why mutations in basic residues and in residues required for substrate binding abrogate membrane association.

The hydrophobic face of the helix, in contrast, may intercalate into the interface of the membrane and further stabilize the kinase at the membrane. Indeed, preliminary studies indicate that substitution of hydrophobic residues within the helical portion of the I β activation loop to acidic residues abolishes plasma membrane localization, indicating that these residues may support the association of type I PIP kinases with the plasma membrane.

It is generally difficult to engineer the ligand specificity of proteins because high affinity ligand binding requires the cooperation of multiple interactions within the ligand binding pocket. As a result, how can a single amino acid substitution have such dramatic consequences for substrate binding without completely abolishing substrate phosphorylation? It is clear from the crystal structure of I β that the active core of the kinase provides a relatively shallow substrate-binding pocket that accommodates binding of multiple substrates (9). The main substrate recognition site is created from the flexible activation loop that folds over the active site. Because the loop appears to control access of the phospholipid substrate to the catalytic site relatively independently from the catalytic core, the alanine to glutamate substitution in I I β can radically affect the type of phosphorylated phosphoinositide bound, apparently without drastically altering the phosphotransfer mechanism. This may also provide a possible explanation for how different phosphoinositide-binding affinities among the three classes of PIP kinases may have evolved through single or successive changes in amino acid composition in the activation loop.

Recent studies (46, 47) indicate that PI 3-kinase activation loops function in an analogous manner to determine the substrate specificity among the members of this kinase superfamily. Although a comparison of PI 3-kinase and PIP kinase activation loops reveals no sequence homology, the distinct substrate specificities of class I enzymes, which phosphorylate PtdIns, PtdIns(4)P, and PtdIns(4,5)P₂, class II enzymes, which prefer phosphatidylinositol and PtdIns(4)P, and class III enzymes, which only phosphorylate PtdIns, can be manipulated by amino acid substitutions in the activation loop (47). Thus, activation loops in these two sequence-diverse superfamilies of lipid kinases are functionally homologous and have evolved to provide distinct signaling functions by discriminating between stereoisomeric phosphoinositide substrates.

An important implication of our studies is the ability to engineer PIP kinase variants with altered substrate specificity by changing amino acid residues in the activation loop. The engineering of PIP kinase mutants with restricted substrate usage should greatly help to clarify the in vivo roles of the different substrate specificities of these kinases.

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