Type II Phosphatidylinositol-4-Kinase β is a Cytosolic and Peripheral Membrane Protein that is Recruited to the Plasma Membrane and Activated by Rac-GTP

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Running Title: Plasma membrane recruitment of type II PtdIns 4-kinases by Rac
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Phosphoinositides have a pivotal role as precursors to important second messengers and as bona fide signaling and scaffold targeting molecules. Phosphatidylinositol 4-kinases (PtdIns 4-kinases or PI4Ks) are at the apex of the phosphoinositide cascade. Sequence analysis revealed that mammalian cells contain two type II PtdIns 4-kinase isoforms, now termed PI4KIIα and β. PI4KIIα was first cloned. It is tightly membrane associated and behaves as an integral membrane protein. In this study, we cloned PI4KIIβ and compared the two isoforms by monitoring the distribution of endogenous and overexpressed proteins, their modes of association with membranes, their response to growth factor stimulation or Rac-GTP activation, and their kinetic properties. We find that while the two kinases are coexpressed in many tissue culture lines, they have different properties. PI4KIIβ is primarily cytosolic and it associates peripherally with plasma membranes, endoplasmic reticulum and the Golgi. In contrast, PI4KIIα is primarily Golgi-associated. Platelet derived growth factor promotes PI4KIIβ recruitment to membrane ruffles. This effect is potentially mediated through Rac; overexpression of the constitutively active RacV12 induces membrane ruffling, increases PI4KIIβ translocation to the plasma membrane and stimulates its activity. The dominant negative RacN17 blocks plasma membrane association and inhibits activity. Although RacV12 also induces redistribution of PI4KIIα among membrane compartments, it does not boost PI4KIIα catalytic activity further, probably because it is constitutively membrane-bound and already activated. Membrane recruitment is an important mechanism for PI4KIIβ activation, because microsome-bound PI4KIIβ is sixteen times more active than cytosolic PI4KIIβ. Membrane-associated PI4KIIβ is as active as membrane-associated PI4KIIα, and has essentially identical kinetic properties. We conclude that PI4KIIα and β may have partially overlapping, but not identical, functions. PI4KIIβ is strongly activated by membrane association to stimulate PIP2 synthesis at the plasma membrane.
These findings provide new insight into how phosphoinositide cascades are propagated in cells.
INTRODUCTION

Phosphatidylinositol 4-kinases (PtdIns 4-kinases, PI4Ks) are at the apex of the phosphoinositide cascade. They phosphorylate PI to generate phosphatidylinositol 4 phosphate (PIP), which is an immediate precursor of important signaling and scaffolding molecules such as phosphatidylinositol 4,5 bisphosphate (PIP2), phosphatidylinositol 3,4 bisphosphate and phosphatidylinositol 3,4,5 trisphosphate (PIP3). In addition, genetic evidence in S. cerevisiae suggests that PIP may also have a direct structural and functional role in its own right. There is increasing evidence that phosphoinositides are synthesized in a spatially and temporally defined manner to regulate signaling, cytoskeletal dynamics and membrane trafficking.

Multiple PI4Ks have been identified. Yeast has two dominant PtdIns 4-kinases, Pik1 and Stt4, which are functionally nonoverlapping. Pik1 is important for cell viability and Golgi-to-plasma membrane transport that appears to be regulated primarily by PIP, and not by PIP2. In contrast, Stt4 maintains vacuole morphology and supply PIP/PIP2 for actin cytoskeletal regulation. Mammalian homologs of Pik1 and Stt4 are called type III PtdIns 4-kinase β and α (PI4KIIIβ and α), respectively. They are found in the cytosol, Golgi and endoplasmic reticulum, and neither is reported to be enriched in the plasma membrane or in lysosomes.

Biochemical studies suggest that another class of PtdIns 4-kinases, designated as type II kinases, accounts for most of the PtdIns 4-kinase activity in the plasma membrane of mammalian cells. Since phosphatidylinositol 4 phosphate 5-kinases (PtdIns 4P 5-kinases or PIP5Ks), which convert PIP to PIP2, are enriched in the plasma membrane and PIP2 is synthesized there in response to many stimuli, the type II kinases may be the major source of PIP at this critical interface. Until recently, type II PtdIns 4-kinases have been identified only as biochemical entities that are distinguished from type III kinases based on their different sensitivities to inhibitors such as adenosine and wortmannin.
We and another group recently cloned the first type II PtdIns 4-kinase, which is designated as PI4KIIα. Overexpression studies showed that PI4KIIα behaves like an integral membrane protein and is primarily organelle associated. Data base analyses reveal that humans have another type II PtdIns 4-kinase, that has a unique N-terminal 100 amino acids sequence and a highly homologous (58% identical and 75% homologous) downstream sequence to that of PI4KIIα. This kinase, designated as PI4KIIβ, was cloned recently by Balla et al. According to this group, overexpressed PI4KIIβ colocalizes with overexpressed PI4KIIα in endosomal vesicles. Neither isoform is obviously enriched in the plasma membrane or the Golgi apparatus, and their endogenous distribution was not reported.

The apparent low abundance of type II PI4Kα and β at the plasma membrane is surprising in view of previous biochemical data suggesting that they are enriched there. This paradoxical finding also raises questions about whether PIP and PIP2 synthesis is coupled at the plasma membrane, and if so, how. One possibility that is modeled after yeast is that PIP is synthesized primarily in the Golgi, while PIP2 is synthesized primarily at the plasma membrane. The two processes are therefore not necessarily coupled. Another possibility is that PIP is synthesized in situ at the plasma membrane by PtdIns 4-kinases that are recruited there by an as yet unidentified mechanism. There is already evidence for the plasma membrane and/or internal membrane recruitment of PIP5Ks and PI4KIIIβ by Rho, Rac, and Arf small GTPases.

In this paper, we compared the intracellular localization of endogenous and overexpressed PI4KIIα and β, and examined the effect of growth factor stimulation and Rho family GTPases on their distribution and kinase activity. Our results are significantly different from those reported recently by Balla et al. for overexpressed PI4-kinases. The differences and possible explanations are discussed at the end of this paper.
EXPERIMENTAL PROCEDURES

**cDNA Cloning and Transfection**—The human PI4KIIβ cDNA was cloned by PCR from the Invitrogen Full-length Genoscope FL1001. The primers used were 5′ gac acg cgt tag aag atc cct ccg ag 3′ and 5′ ggc tct aga tta cta cca gga gga 3′. The cDNA was subcloned into the pCMV5-myc2 or HA vectors between the MluI and XbaI sites. The rat myc-PI4KIIα clone is as described previously 15. Myc-tagged or untagged RacV12 and RacN17 were gifts of A. Hall (MRC, Univ. London). Cells were transiently transfected using Lipofectamine and used between 8-18 hours post transfection. In cotransfection studies with Rac, the Rac expression plasmids were used at between a seven- to three-fold excess over the PtdIns 4-kinase plasmids. GFP-expression plasmids were used in control samples.

**Antibody Production**—Synthetic peptides encompassing residues 2-17 of human PI4KIIα and residues 22-36 of human PI4KIIβ and conjugated to keyhole limpet hemocyanin via an extra N-terminal cysteine residue were used to immunize rabbits. The antibodies were affinity purified by absorption against peptides attached to nitrocellulose filters 25.

**Immunofluorescence Localization**—Cells with and without transfection were fixed either in paraformaldehyde and permeabilized with Triton X-100, or with cold methanol as described previously 26. Cells were stained with antibodies and images were collected by confocal fluorescence microscopy. In some cases, cells were stimulated with PDGF (AB, 100 ng/ml for 15 min.) prior to fixation. Cells with distinct plasma membrane staining were scored in randomly chosen fields.

**Golgi Fractionation**—Golgi-enriched membranes were obtained using a discontinuous gradient fractionation protocol 27. COS7 postnuclear supernatant was adjusted to 1.3 M sucrose (cytosol, 8 ml), placed on top of a 2.4 M sucrose button and overlaid with 1.2 M and 0.8 M sucrose. Each tube had a total volume of 37 ml. After centrifugation, the upper part of the 0.8
M sucrose layer was collected from the top in bulk. The fractions surrounding the interface between 0.8 M and 1.2 M sucrose (interface III) were collected as 1 ml fractions, and labeled as fractions 1, 2, 3 and 6). Interface III which is enriched for Golgi light membranes was collected in two 3 ml fractions, called 3 and 4. The remaining 1.2 M and 1.3 M sucrose layers, and interfaces III and I were collected in bulk. These interfaces are enriched for ER and heavy membranes (lysosomes, plasma membrane and mitochondria), respectively 27.

Membrane Association

Membrane association was analyzed in two ways. First, microsomal membranes were isolated by a one step fractionation procedure 15. Cells were scraped from the petri dish in a hypotonic lysis solution containing 0.25 M sucrose, 50 mM Tris-HCl (pH 8.0), 50 mM NaCl, 2 mM EDTA, 1 mM dithiothreitol (DTT) and a protease inhibitor cocktail. They were lysed by 2 freeze and thaw cycles, and unbroken cells and nuclei were removed by centrifugation at 1,000 x g for 5 min. to obtain a postnuclear fraction. The supernatant was recentrifuged at 190,000 x g for 15 min. to obtain cytosol (supernatant) and microsome (pellet) fractions. The 190,000 x g pellets were washed once in the lysis buffer and recentrifuged. The pellets were boiled and analyzed on SDS-polyacrylamide gels. In some cases, the pellets were further extracted for 10 min. on ice either with 1 M NaCl in lysis buffer, or with 1 M NaCl/0.1 M Na2CO3 (pH 10). Membranes and insoluble material were collected by centrifugation at 190,000 x g for 15 min.

A second procedure employing multiple centrifugation steps was used to examine the partitioning of these kinases in different crude organelle fractions 28. Cells were lysed by freeze-thawing as above, and centrifuged at 19,000 x g for 20 min. at 4 C. The 19,000 x g pellet was resuspended in 100 µl lysis buffer, and overlaid onto a 0.8 ml cushion of 1.12 M sucrose. After centrifugation at 100,000 x g for 1 hr., the membrane layer at the top of the
sucrose cushion was collected with a long needle and sedimented at 40,000 x g for 20 min. This fraction was enriched in plasma membrane markers and is designated as PM 28. The 19,000 x g supernatant obtained from the first step was sequentially centrifuged at 41,000 x g for 20 min. and at 180,000 x g for 80 min. to collect the low speed pellet (LSP) and the high speed pellet (HSP). The partitioning of PtdIns 4-kinases in these fractions was determined by analyzing equal fraction of each sample by western blotting with anti-myc antibody.

**In vitro PtdIns 4-Kinase Assay**—Kinase activity was measured by phosphorylation of exogenous PtdIns using [γ-32P]ATP (10 mCi/mmol) as a phosphate donor. Phospholipids were extracted and resolved by thin layer chromatography (TLC) in n-propyl-alcohol/H2O/NH4OH (65:20:15). 32P-labeled PIP was detected by Phosphorimager analysis and kinase activity was normalized against kinase content, determined by western blotting with anti-myc. Kinase activity was assayed in three different preparations. First, myc-tagged kinases were immunoprecipitated and used for activity assays. Cells overexpressing myc-tagged PtdIns 4-kinases together with untagged RacV12 or RacN17 (at 1:4 weight ratio) were lysed in a buffer containing 25 mM Tris-HCl, pH 7.5, 0.15 M NaCl, 5 mM MgCl2, 1 mM EGTA, 10% glycerol, 1 mM DTT, 1 mM sodium vanadate, 0.5% NP40 and protease inhibitors. The kinases were immunoprecipitated with anti-myc bound to protein A Sepharose and used directly for in vitro kinase assays in the absence of additional NP40. The kinase assay buffer contains 25 mM Hepes, pH 7.2, 25 mM NaCl, 2.5 mM MgCl2, 0.2 M sucrose, 1 mM DTT, 0.5 mM ATP, protease inhibitors, 5 mM creatinine phosphate, and 10 U/ml creatinine kinase. Second, cells overexpressing myc-PI4KIIβ were broken by cycles of freeze-thaw, and separated into the 190,000xg microsome pellet and soluble cytosol. Myc-PI4KIIβ was immunoprecipitated from each fraction after solubilization with 1% Triton X-100. Third, the 190,000 x g pelletable
microsome-associated kinases was assayed directly (without immunoprecipitation) after extraction for 15 min. on ice with a solution containing 20 mM Tris-HCl, pH 7.5, 10% glycerol, 0.1 M NaCl, 1% Triton X-100, 1 mM DTT and protease inhibitors. Insoluble material was removed by centrifugation at 200,000 x g for 15 min., and the supernatant, representing the detergent-extractable membrane-bound pool, was analyzed by immunoblotting and assayed for PtdIns 4-kinase activity.

RESULTS AND DISCUSSION

PI4KIIα and β Protein Expression—We cloned the human PI4KIIβ cDNAs, and confirmed that its sequence is almost identical to that in the database. The Genebank accession no. of our clone is AY091514. The antibodies generated by immunizing rabbits with the unique N-terminal sequences are isoform specific, based on western blotting with overexpressed proteins (data not shown). Each recognizes a single band in western blots in the three tissue culture cell lines tested (Fig. 1A). PI4KIIα, which has a slightly smaller predicted mass than PI4KIIβ (54,022 vs 54,744 daltons, respectively), migrates slightly ahead of PI4KIIβ. Among the cells tested here, HeLa cells have the weakest PI4KIIα signal and strongest PI4KIIβ signal. Since the antibody is directed against the human peptide sequence, the lower intensity of the HeLa PI4KIIα band suggests that HeLa cells have less PI4KIIα than the two monkey derived cells, COS7 and CV1. However, because we do not know if monkeys and humans have completely identical PI4KIIα sequences, it is not possible to draw conclusions about the relative amounts of PI4KIIα between the monkey and human-derived cells at this point.

Immunofluorescence Localization—HeLa cells were used for immunolocalization of endogenous PI4KIIβ. PI4KIIβ has a diffuse cytosolic staining pattern (Fig. 1B, top panels), with some concentration in a perinuclear region and at the plasma membrane of lamellipodia.
(highlighted by arrows, Fig. 1B). The perinuclear staining coincides with that of βCOP1 and TGN46, which are markers for the cis and trans Golgi network, respectively. This pattern was observed irrespective of whether cells were fixed with formaldehyde and permeabilized by Triton X-100 or fixed and permeabilized with cold methanol (data not shown).

Myc-tagged PI4KIIβ exhibited a similar staining pattern (Fig. 1B, bottom panels). PI4KIIβ is concentrated at the perinuclear Golgi region that is also positive for TGN46 in cells with low level overexpression (Fig. 1B, bottom panels, cell on the upper left). The inset shows that PI4KIIβ and TGN46 staining overlaps almost completely in this region. At higher level overexpression, myc-PI4KIIβ is predominantly cytosolic with prominent plasma membrane staining at the leading edge (Fig. 1B, bottom panels, arrow).

Unlike PI4KIIβ, PI4KIIα is located primarily in the Golgi, and there is no obvious plasma membrane staining (Fig. 1C). The mitotic cell in the middle of the field has no Golgi staining by either anti-PI4KIIα or anti-TGN46, consolidating the conclusion that the perinuclear PI4KIIα is Golgi-associated. The colocalization of both PtdIns 4-kinase isoforms in the Golgi and their differences in the cytosol and plasma membrane are confirmed by coexpression (Fig. 1D). Overexpressed PI4KIIα is primarily organelle associated; it is present on the Golgi apparatus and on vesicular structures outside of the perinuclear region as well (Fig. 1D, inset). The latter is consistent with Balla et al.’s report on overexpressed PI4KIIβ 17. Some overexpressed PI4KIIβ is Golgi-associated, but it is much more cytosolic and more obviously plasma membrane associated than myc-PI4KII. The difference in plasma membrane association is confirmed by direct counting of cells in randomly chosen fields. Overexpressed PI4KIIβ is found in the plasma membrane of 39% of the cells, while overexpressed PI4KIIα is found in only 19% of the cells (Table 1).

Subcellular Fractionation—Subcellular fractionation studies also confirmed the
predominant Golgi localization of PI4KIIα and the differential localization of the two type II PtdIns
4-kinases. We employed a well-characterized protocol that is widely used to study COP1 coat
formation 27 and Arf1 dependent recruitment of regulatory proteins to Golgi-enriched membranes
23. PI4KIIα is highly enriched in Golgi fractions 3 and 4 (interface III) (Fig. 2) and the surrounding
fractions as well. TGN38 is almost exclusively in fractions 3 and 4, and this tighter distribution
profile is consistent with TGN38’s primary localization in the TGN. COP1, which cycles between
the cytosol and the Golgi, is found in these fractions, as well as in interfaces II (ER-enriched)
and I (heavy membranes). The large amount of cytosolic COP1 is consistent with its transient
and GTP-dependent association with membranes. PI4KIIβ has a similar distribution,
suggesting that it is transiently associated with the Golgi as well. PI4KIIβ appears as a doublet
in some of the fractions, possibly due to differential phosphorylation (manuscript in preparation).

Modes of Membrane Association—The immunofluorescence localization and subcellular
fractionation results suggest that PI4KIIα is primarily Golgi associated while PI4KIIβ is both
cytosolic and organelle associated. We used a quick one step microsome preparation to
compare and characterize the organelle association of endogenous PtdIns 4-kinases. Following
high-speed centrifugation, 38% of endogenous PI4KIIβ was recovered in the 190,000 x g light
microsome pellet fraction (P, Fig. 3A), while >99% of endogenous PI4KIIα was membrane
bound, as reported previously for overexpressed PI4KIIα 15;16.

Significantly, the membrane-associated PI4KIIα and β are differentially sensitive to salt
and alkaline extraction (Fig. 3A). Microsome-associated PI4KIIβ was 95% extractable with 1 M
NaCl and Na₂CO₃/NaCl did not extract more. Thus, PI4KIIβ behaves like a peripheral
membrane protein. In contrast, PI4KIIα was resistant to carbonate extraction (Fig. 3A), behaving
like an integral membrane protein.
Similar results were observed with overexpressed PtdIns 4-kinases, except that more myc-PI4KIIβ is microsome-associated (ranging from 20 to 50% in COS7 cells in a total of 5 experiments, and more in 293 cells (data not shown)), and myc-PI4KIIβ was less extractable by high salt or carbonate than the endogenous protein (Fig. 3B). We believe that variable recovery and decreased extractability are due to overexpression and therefore limited the overexpression time to less than 18 hrs.

Effects of Growth Factor Stimulation on PtdIns 4-Kinase Localization—The existence of a large cytosolic pool of PI4KIIβ and its association with the plasma membrane as a peripheral protein raises the possibility that it may be recruited to the plasma membrane to promote the in situ sequential synthesis of PIP and PIP2. Many agonists stimulate PIP2 dependent actin polymerization responses at the plasma membrane 12;21;30, and some growth factors promote actin polymerization to generate membrane ruffles. After PDGF treatment, 51% of the cells have prominent plasma membrane staining of endogenous PI4KIIβ (Fig. 4, Table 1) and 25% of the cells have membrane ruffles (data not shown). In contrast, only 14% and 5% of the serum-starved untreated cells have plasma membrane PI4KIIβ and membrane ruffles, respectively. Importantly, all the cells with plasma membrane ruffles also have plasma membrane PI4KIIβ staining. These results suggest that PI4KIIβ translocation to the plasma membrane is upstream of membrane ruffling, and that although PI4KIIβ translocation per se is not sufficient to generate ruffles, it is probably an early step in the orchestration of the PIP2-dependent actin polymerization response.

Effects of RacV12 Overexpression on Type II PtdIns 4-Kinase Localization—PDGF activates the small GTPase Rac1 to stimulate membrane ruffling, and Rac1 20;21 and other small GTPases 12;31 recruit PIP5Ks to the plasma membrane. We therefore examined the possibility that small GTPases may regulate the plasma membrane association of type II PtdIns 4-kinases by
cotransfection studies.

COS7 cells that were serum starved overnight have some HA-PI4KIIβ staining of the plasma membrane, while cells that were cotransfected with the constitutively active myc-RacV12 (at a 1:4 ratio) have much more pronounced plasma membrane staining (Fig. 4B). As expected, myc-RacV12 is enriched at the plasma membrane where it colocalizes with HA-PI4KIIβ (Fig. 4B). RacN17, which is inactive, did not induce PI4KIIβ translocation to the plasma membrane. Under the conditions of our experiment, 39% of control cells displayed plasma membrane staining of HA-PI4KIIβ, 79% and 12% of cells had plasma membrane staining when transfected with RacV12 or RacN17 (Table 1). These results establish that active Rac promotes PI4KIIβ translocation to the plasma membrane, while dominant negative Rac inhibits.

RacV12 also recruited endogenous PI4KIIβ to the plasma membrane (Fig. 4B, bottom panels). In the field shown, cells not transfected with RacV12 have predominantly cytosolic PI4KIIβ staining, while the cell expressing RacV12 has pronounced PI4KIIβ staining at the plasma membrane.

Membrane recruitment of PI4KIIβ is specific for Rac1 because other small GTPases, such as Rho, Arf6 and Cdc42 did not alter the distribution of PI4KIIβ (data not shown). Since PDGF and RacV12 both recruit PI4KIIβ to the plasma membrane, and Rac is a known downstream effector in the growth factor stimulation pathway for increased PIP and PIP2 synthesis, we suggest that PDGF induces PI4KIIβ recruitment to the plasma membrane by activating Rac1.

Likewise, PI4KIIα is recruited to the plasma membrane by RacV12 (Fig. 4C). This effect is GTP-dependent, because cells RacN17 does not increase plasma membrane recruitment (Fig. 4C, bottom panels). The increase in plasma membrane staining is corroborated by comparing cells with plasma membrane association (Table 1). However, RacN17 does not
reduce the percent of cells with PI4KIIα membrane localization to below control level, unlike what was observed for PI4KIIβ. This difference suggests that the two isoforms may be enriched at the plasma membrane through different mechanisms.

**Biochemical Characterization of the Effects of RacV12 on PI4KIIβ Membrane Association**—The immunofluorescence results are corroborated by biochemical experiments (Fig. 5A). We employed a differential fractionation protocol that was optimized to establish the Golgi and plasma membrane localization of syntaxin 6 28. The cell lysates were separated into a cytosolic fraction (CYT), a low-speed pellet (LSP) that is enriched in Golgi membranes and early endosomes, a high-speed pellet (HSP) that is enriched for lysosomes and late endosomes and a plasma membrane rich fraction (PM).

This fractionation technique confirmed that the two kinases are differentially localized. 47% and 2% of the total PI4KIIβ and PI4KIIα, respectively, are cytosolic (Fig. 5A). Of the membrane bound myc-PI4KIIβ, most (35% of soluble and insoluble enzyme) was found in the HSP fraction which is enriched for lysosomes/late endosomes. Some was associated with the PM fraction (6%) and the Golgi/early endosome-enriched LSP fraction (12%). In contrast, most of the PI4KIIα was in the LSP (68%), and smaller amounts were present in the HSP (11%) and PM (19%) fractions. The ratios of PI4KIIβ in the PM: LSP: HSP fractions are 1:2:6, while that for PI4KIIα is 1:4:1. These results establish that the two isoforms are preferentially associated with different organelles at steady state, and corroborate data obtained by gradient centrifugation (Fig. 2), one step fractionation (Fig. 3) and by immunofluorescence microscopy (Fig. 1). From the large difference in kinases partitioning between the Golgi and plasma membrane, we conclude that PI4KIIα may be primarily responsible for the synthesis of PIP at the Golgi, while PI4KIIβ may have a more important role elsewhere, such as shuttling between the cytosol, endosomes, lysosomes and the plasma membrane.
We next confirmed that RacV12 increases PI4KIIα and β association with the plasma membrane. As expected, RacV12 is found predominantly in the PM fraction (Fig. 5A). RacV12 increases in PI4KIIβ in the PM (2.5 fold increase) and LSP (1.6 fold increase) fractions, and slightly decreases it in the HSP and cytosol (to 0.7 and 0.8 fold of control, respectively). Some PI4KIIα is recruited to the plasma membrane (1.6 fold increase), but the most dramatic change is a 22-fold decrease in HSP association. The different responses of PI4KIIα and β to RacV12 suggest that the observed redistributions do not merely reflect a generalized change in vesicular trafficking that alters the amount of membranes recovered in each fraction. If this were the case, both kinases would be expected to shift from one compartment to another to a similar extent. Our data suggest that PI4KIIβ is recruited from the cytosolic fraction to the membranes by RacV12, whereas PI4KIIα, which is constitutively membrane associated, is increased at the plasma membrane by redistribution among various membrane compartments.

**RacV12 Increases PI4KIIβ, but not PI4KIIα, Activity**—The effect of Rac on PtdIns 4-kinase activity was tested by immunoprecipitating myc-tagged PtdIns 4-kinases from cells that cotransfected with an excess of untagged RacV12 or RacN17 cDNAs. The myc-PI4KIIβ immunoprecipitated from cells coexpressing RacV12 had a 1.5 fold higher kinase specific activity than those precipitated from cells without Rac, and RacN17 decreased activity to 0.6 of control levels (Fig. 5B). These effects are specific for PI4KIIβ, because Rac-GTP had minimal effect on PI4KIIα activity, even though it changes its intracellular distribution.

We do not know how RacV12 activates myc-PI4KIIβ. The mostly likely possibility is that when PI4KIIβ is recruited to the plasma membrane by Rac, it is exposed to activating enzymes or ligands located at the plasma membrane. In this scenario, in vivo activation of PI4KIIβ at the plasma membrane could be much higher than the 1.5 fold increase detected in the immunoprecipitates, because only 15% of the total PI4KIIβ (Fig. 5A) is at the plasma membrane.
where it can be exposed to the activating ligands/enzymes. PI4KIIβ activation by phosphorylation/dephosphorylation is an attractive possibility. However, our preliminary experiments showed that alkaline phosphatase treatment did not alter the activity of either membrane-associated or cytosolic myc-PI4KIIβ kinase (data not shown).

*Membrane Association Increases the Activity of Myc-PI4KIIβ*—To test the possibility that PI4KIIβ is activated by membrane association, we compared the specific activity of myc-PI4KIIβ immunoprecipitated from the 190,000 x g pelletable microsomal fraction and from the supernatant fraction obtained from cells grown in serum-containing medium (i.e. activating conditions) (Fig. 6A). The membrane-bound PI4KIIβ is 16 times more active than an equivalent amount of soluble PI4KIIβ in the experiment shown. Similar results were obtained in three independent experiments, and activation can reach as high as 20 fold. Assuming that only 6% of PI4KIIβ in resting cells is membrane-associated (Fig. 5A&B), translocation of the entire cytosolic pool (94%) to the membrane would be predicted to increase overall cellular kinase activity by approximately 15-fold. Thus, translocation of 9% of the total PI4KIIβ upon Rac stimulation should increase activity by 1.4 fold, which is remarkably similar to the 1.5 fold value obtained experimentally (Fig. 5B). These results establish that PI4KIIβ is strongly activated by membrane association.

Membrane associated PI4KIIβ has almost identical kinetic properties as PI4KIIα which is constitutively membrane-associated. They have similar apparent K_m for ATP (65 µM vs. 86 µM for PI4KIIβ and α, respectively) and for PtdIns (21 vs. 35 µM) (Fig. 6B). The shallow drop in PI4KIIβ activity at high PtdIns concentration was consistently observed, raising the possibility that this isoform is more sensitive to substrate inhibition than PI4KIIα. The physiological significance of this remains to be explored. PI4KIIβ, like PI4KIIα, was inhibited by micromolar
adenosine, but not by wortmannin (data not shown), in agreement with Balla et al. 17. PI4KIIβs differential sensitivity to these inhibitors justifies its classification as a bona fide type II PtdIns 4-kinase.

In conclusion, our results establish that both PtdIns 4-kinases are recruited to the plasma membrane by Rac in a GTP-dependent manner, but PI4KIIβ, a predominantly cytosolic protein, is activated by membrane recruitment. Since Rac has already been shown previously to recruit and activate PIP5K at the plasma membrane 20:21, the simultaneous recruitment and activation of PI4KIIβ and PIP5Ks suggest that Rac coordinately regulates the sequential synthesis of PIP and PIP2 to increase membrane ruffling, endocytosis and exocytosis. Our result would therefore be consistent with the possibility of coupled PIP and PIP2 synthesis by PI4KIIβ and PtdIns 5-kinases at the plasma membrane of mammalian cells.

Comparison of Our Results with Those of Balla et al. 17While this work was in progress, Ballas group published a report comparing the intracellular localization, membrane association and kinase activities of PI4KIIα and β. Although we agree with some of their conclusions, we also find major differences. Their findings were based exclusively on the behavior of overexpressed PtdIns 4-kinases, and are different from ours in the following respects: First, fluorescence imaging showed that overexpressed PI4KIIβ, tagged at the C-terminus with GFP, is associated with organelles almost to the same extent as overexpressed PI4KIIα. Moreover, both kinases were preferentially associated with early endosomes (detected by anti-EEA1) but not with the Golgi (based on Golgi staining with anti-gm130). Second, biochemical fractionation showed that most of the overexpressed PI4KIIβ-HA, like PI4KIIα-HA, is recovered in the high-speed microsome fraction, suggesting that both are predominantly membrane bound. Third, in vitro kinase studies showed that immunoprecipitated PI4KIIβ-HA is only about 30% as active as PI4KIIα-HA.
In contrast, we studied both endogenous and overexpressed PtdIns 4-kinases, and presented new information about significant differences in the localization, regulation and mode of membrane association of PI4KIIα and β. First, immunofluorescence localization studies showed that PI4KIIβ is both cytosolic and organelle associated while PI4KIIα is predominantly Golgi associated. Furthermore, the endogenous and overexpressed forms of both kinases are enriched in the Golgi, and colocalize with two different Golgi markers that are located in the cis and trans Golgi (Fig. 1B). Similar results were obtained using two different methods to fix and permeabilize the cells, suggesting that the localization is unlikely to be due to fixation artifacts. Second, biochemical fractionation studies showed that PI4KIIβ is predominantly recovered in the cytosol fraction, while PI4KIIα is almost completely Golgi-associated (Fig. 2 and 5). Furthermore, PI4KIIβ associates with membrane as a peripheral protein, while PI4KIIα behave as an integral membrane protein (Fig. 3). Third, PI4KIIβ is recruited and activated at the plasma membrane by PDGF and by Rac in a GTP-dependent manner (Fig. 5). Fourth, membrane recruitment stimulates PI4KIIβ activity significantly (Fig. 6A).

What is the basis for the discrepancies between ours and their results? At least some of the differences may be ascribed to the different placement of the epitope tags. Balla’s group places the GFP or HA tag at the C-termini; we attach a myc or HA tag to the N-termini. We chose to examine N-terminally tagged expressed PtdIns 4-kinases because we found that deletion of as few as seven residues from the C-terminus of PI4KIIα dramatically decreases its enzymatic activity whereas removal of even 91 residues from the N-terminus had no effect on catalysis (manuscript in preparation). A second potential explanation is the different levels of overexpression used in Balla’s and our experiments. We routinely used short overexpression time (between 8 to 18 hours post-transfection), because we found that myc-PI4KIIβ becomes more organelle-associated and insoluble with prolonged overexpression (data not shown), and
cells that overexpress at a high level (e.g. 293 cells) have more membrane-bound myc-PI4KIIβ kinases than those that express at moderate level (e.g. CV1 or COS7 cells). We also found that overexpressed PI4KIIα becomes increasingly associated with endosomes and lysosomes (data not shown) even though it is initially predominantly Golgi-associated (Fig. 1C & D). Balla’s group routinely used cells overexpressing the kinases for 24-48 hours and found that GFP-tagged PtdIns 4-kinases partially colocalize with anti-gm130 and GFP fluorescence is also present in surrounding granules that are not positive for anti-gm130. We suggest that their higher level of protein expression increases non-Golgi vesicle staining and obscures authentic Golgi staining. Perhaps the most important consideration here is that, in our studies, immunofluorescence localization and biochemical fractionation of overexpressed kinases merely served to confirm the behavior of endogenous kinases as detected with isoform specific anti-peptide antibodies. Finally, we cannot explain the large discrepancies in specific activities of PI4KIIα and β between our two groups. This difference cannot be due to a difference in membrane association, because their overexpressed PI4KIIβ is as pelletable as their PI4KIIα. Perhaps PI4KIIβ is more readily denatured when expressed at a high level. It remains to be seen if the different placement of the epitope tags can also account for this difference.
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    1018
Table 1. Immunofluorescence Evidence for Stimulus-Dependent Plasma Membrane Translocation of PtdIns-4 Kinases

| Treatments            | Percent Cells with Plasma Membrane Staining | PI4KIIβ | PI4KIIα |
|-----------------------|---------------------------------------------|---------|---------|
| Endogenous kinase     |                                             |         |         |
| None                  |                                             | 14      | ND      |
| PDGF                  |                                             | 51      | ND      |
| Overexpressed kinase  |                                             |         |         |
| None                  |                                             | 39      | 19      |
| RacV12                |                                             | 79      | 74      |
| RacN17                |                                             | 12      | 22      |

Cells with obvious plasma membrane staining were counted in randomly chosen fields. Between 75-100 cells are counted in each category per experiment, and the data is average of two experiments.
Fig. 1. Immunofluorescence localization of endogenous and overexpressed PI4KIIα. Western blotting of several types of cells with isoform specific anti-peptide antibodies. Cells were lysed in RIPA buffer, and equal amount of proteins was loaded in each fraction. B. Immunofluorescence localization of endogenous and overexpressed PI4KIIβ. Top panels, distribution of endogenous PI4KIIβ. HeLa cells were stained with anti-PI4KIIβ and with anti-βCOP1 (a cis Golgi marker) after fixation with formaldehyde/Triton X-100. Bottom panels, distribution of overexpressed HA-PI4KIIβ and TGN46 (a trans Golgi marker). Inset shows merged image in the perinuclear Golgi region of the cell in the upper left of the frame. C. Immunofluorescence localization of endogenous PI4KIIα. D. Comparison of the immunofluorescence localization of overexpressed HA-PI4KIIβ and myc-PI4KIIα. Inset shows merged image in the perinuclear Golgi region of the cell on the right hand side of the frame.

Fig. 2. Comparison of the partitioning of PtdIns 4-kinase isoforms in subcellular fractions. 10 µl of each fraction collected as described in EXPERIMENTAL PROCEDURES were analyzed by western blotting with isoform specific antibodies to PI4KIIα and β, and antibodies to COP1 and TGN38.

Fig. 3. Comparison of PtdIns 4-kinase α and β membrane association. “One step” fractionation into high speed supernatants (S) and microsomal pellets (P). COS7 cells that were not transfected or transfected with myc-tagged PtdIns 4-kinase overexpression plasmids were used and equal fractions were analyzed by western blotting with isoform specific anti-PI4KII
antibodies or with anti-myc. When indicated, the microsomal pellets were further extracted with 1 M NaCl or with 0.1 M Na₂CO₃ (pH 10) in 0.1 M NaCl. A. Endogenous PtdIns 4-kinases. B. Overexpressed PtdIns 4-kinases. The numbers below each panel are percent of total, and are representative of more than 4 experiments.

**Fig. 4. Effects of PDGF and Rac on the intracellular localization and activity of PtdIns 4-kinases.** A. COS7 cells were stimulated with 100 ng/ml PDGF or buffer control for 15 min., and stained with anti-PI4KIIβ. In panels B-C, cells were transiently transfected with HA-PtdIns 4-kinases, alone or together with myc-RacV12 or myc-RacN17, and cultured in 0.5% serum containing medium overnight. B. Immunofluorescence localization of overexpressed (top three panels) and endogenous PI4KIIβ (bottom panels). C. Immunofluorescence localization of overexpressed HA-PI4KIIα.

**Fig. 5. Effect of Rac1 on the partitioning and activity of overexpressed PtdIns 4-kinases.** A. Multistep fractionation. Lysates prepared from COS7 cells transfected with either myc-PtdIns 4-kinase cDNA alone (control, left panels) or together with myc-RacV12 cDNA (at a 1:4 ratio, right panels) were centrifuged sequentially at different speeds to obtain organelle/membrane fractions that are referred to as PM, which is enriched in plasma membranes, LSP, low speed pellet, HSP, high speed pellet and CYT, cytosol. Fractions were blotted with anti-myc to detect overexpressed proteins. Top panels, myc-RacV12 distribution in cells transfected with PI4KIIβ cDNA, with RacV12 (right panel) or without RacV12 (control, left panel). Middle panels, myc-PI4KIIβ distribution. Bottom panels, myc-PI4KIIα distribution. The relative distribution of kinases in each fraction is shown in the bar graphs. Values given are mean+/−SEM of 3 independent
experiments.  

B. Effects of RacV12 (not tagged) or RacN17 on the activity of immunoprecipitated myc-PI4KIIis. Cells were cotransfected with myc-PI4KII and Rac cDNAs. The amount of immunoprecipitated kinase was determined by western blotting with anti-myc and the western blot for one such experiment is shown. The faint lower band in the PI4KIIα western blot is due to the IgG heavy chain, which weakly crossreacts with the secondary antibody. Kinase activity data is obtained from 3 independent experiments, each done in duplicate. The kinase activity values for each control sample were set at 100%, and those exposed to RacV12 or RacN17 were expressed as a percent of control +/- SEM, after adjustment for the small variations in kinase content.

**Fig. 6. Comparison of the kinase activity of cytosolic and membrane-associated PIP4Kβ.** Cells overexpressing myc-tagged PtdIns 4-kinases were subjected to freeze-thawing and centrifuged at 190,000 x g. Kinase activity was assayed as described in EXPERIMENTAL PROCEDURES, and normalized to the amount of myc-kinase as determined by western blotting. A. Activity of immunoprecipitated myc-PI4KIIβ from the 190,000 x g supernatant (S) and pellet (P). A western blot of the immunoprecipitates used in this experiment is shown. Kinase activity is expressed in arbitrary units after normalization for the amount of kinase immunoprecipitated. Values are mean +/- SEM of triplicate samples from a single experiment. B. Activity of microsome-associated myc-PI4KIIβ and α. The 190,000 x g microsome pellet was extracted with Triton-X100 and used directly for kinase assays. The activity of myc-PI4KIIα is expressed in pmol/min./unit protein, and compared with that of a similar amount of myc-PI4KIIβ. Activities of extracts from untransfected cell membranes, which were at least 10-fold lower than those from transfected cells, were subtracted from each data point. Data shown are mean +/- SEM from 2 experiments, each done in triplicate. Top, dependence on ATP concentration at a
fixed PtdIns concentration of 0.5 mM. $K_m$ values for PI4KIIα and β were 86 and 65 µM, respectively. Bottom, dependence on PtdIns concentration, at a fixed ATP concentration of 0.5 mM and a 1:10 molar ratio of PtdIns: Triton X-100. These data give apparent $K_m$s of 35 and 21 µM for PI4KIIα and β, respectively.
Fig 2
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A

Endogenous

|          | One Step | NaCl  | Na₂CO₃ |
|----------|----------|-------|--------|
|          | S        | P     | S      | P       |
|          |          |       |        |         |
|          | 0        | 100   | 0      | 100     |
|          | 62       | 38    | 95     | 5       |
|          |          |       | 95     | 5       |

% PI4KIIα

% PI4KIIβ

B

Overexpressed

|          | One Step | NaCl  | Na₂CO₃ |
|----------|----------|-------|--------|
|          | S        | P     | S      | P       |
|          |          |       |        |         |
|          | 0        | 100   | 0      | 100     |
|          | 67       | 33    | 56     | 44      |
|          | 80       | 20    |        |         |

% PI4KIIα

% PI4KIIβ
**Fig 6**

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Type II phosphatidylinositol 4-kinase b is a cytosolic and peripheral membrane protein that is recruited to the plasma membrane and activated by Rac-GTP

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