Interfacial Catalysis by Phosphoinositide 3′-Hydroxykinase

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ABSTRACT: Phosphorylation of phosphoinositides by phosphoinositide 3′-hydroxykinase (PI3K) occurs at a lipid/water interface. We have determined that highly purified recombinant human P13K binds tightly to vesicle interfaces composed primarily of phosphatidylinositol (PI) or 1,2-dimyristoyl-*sn*-glycero-3-phosphomethanol (DMPM). The rate of desorption of PI3K from the vesicle interface is slow and does not significantly affect the observed product formation kinetics. Observations which demonstrate that PI3K is tightly bound to the vesicle lipid/water interface include the following: (1) product formation plateaus rapidly, even in the presence of active enzyme and excess substrate; (2) total product formation is proportional to the amount of PI3K; (3) initial product formation rates are unaffected by bulk lipid concentration but are dependent on the interfacial substrate concentration; and (4) PI3K partitions with lipid vesicles in sedimentation gradients. This enzymatic profile has been referred to as catalysis in the "scooting" mode (Berg et al., 1991). A kinetic analysis of PI3K catalysis in the scooting mode is presented. The interfacial $K_{m,app}$ for PI was determined to be approximately 6.0 mol % in PI/DMPM vesicles. The ratio of specificity constants (k_{cat}/K_m) for PI, phosphatidylinositol 4-monophosphate (PIP), and phosphatidylinositol 4,5-diphosphate (PIP_2) utilization was determined to be near unity. These results provide a rigorous enzymological framework for the kinetic analysis of PI3K inhibitors.

Mammalian phosphoinositide 3′-hydroxykinase (PI3K) is involved in metabolic pathways, growth factor-stimulated mitogenesis, and cellular transformation [see Stephens et al. (1993) for a comprehensive review]. Because of its role in many essential cellular processes, PI3K may represent a therapeutic target for human diseases, including cancer (see below). Evaluation of inhibitors of PI3K requires a detailed understanding of the kinetic behavior of this complex enzyme. PI3K is subject to a number of potential regulatory mechanisms, including subcellular localization, protein/protein interactions, and posttranslational phosphorylation. We have undertaken a characterization of the fundamental kinetic properties of highly purified recombinant human (rh) PI3K to provide a foundation for the evaluation of regulatory mechanisms and inhibitors.

PI3K is a heterodimer composed of 85 and 110 kDa subunits (Carpenter & Cantley, 1990; Morgan et al., 1990; Shibasaki et al., 1991). The 85 kDa regulatory subunit (p85) has structural characteristics consistent with a number of regulatory mechanisms. The p85 subunit contains two src homology region 2 (SH2) domains (bind to phosphotyrosine residues) (Escobedo et al., 1991b; Skolnik et al., 1991; Otsu et al., 1991; McGlade et al., 1992; Klippel et al., 1992; Hu et al., 1993; Reedijk et al., 1992; Wood et al., 1992; Cantley et al., 1991; Parker & Waterfield, 1992), a src homology 3 (SH3) domain [binds poly(Pro) sequences], a proline-rich region (binds SH3 domains) (Kapeller et al., 1994; Pleiman et al., 1994), a bcr-homology domain (may activate GTPase activity in members of the rho family of small GTP binding proteins) (Fry, 1992; Heisterkamp et al., 1985; Otsu et al., 1991), and a serine residue which is susceptible to phosphorylation (Carpenter et al., 1993; Dhand et al., 1994). The p110 binding domain is located between the two SH2 domains (Klippel et al., 1993, 1994).

The 110 kDa catalytic subunit of PI3K contains a region of homology to other ATP binding proteins (Hiles et al., 1992) in the carboxy-terminal half of the protein. The p110 subunit also displays homology to the yeast proteins VPS34 and Tor2 (Hiles et al., 1992; Kunz et al., 1993), which are involved in efficient sorting of proteins to lysosome-like vacuoles (Schu et al., 1993) and cell cycle activation (Kunz et al., 1993), respectively.

PI3K catalyzes the transfer of the γ-phosphate of ATP to the inositol D-3 position of PI, PIP, and PIP_2 (Shibasaki et al., 1991; Carpenter & Cantley, 1990; Morgan et al., 1990). The primary products of growth factor mediated PI3K activation, $PI(3,4)P_2$ and $PI(3,4,5)P_3$, appear to represent novel second messengers. These phospholipids are not substrates for phospholipase C (Lips et al., 1989; Serunian et al., 1989) and do not appear to be directly involved in the elevation of intracellular calcium concentrations, although they are reported to activate certain isoforms of protein kinase C (Nakanishi et al., 1993).

Several lines of evidence suggest that PI3K is directly involved in mitogenesis and oncogenic transformation. As mentioned above, PI3K binds to phosphotyrosine residues of activated growth factor receptors and other oncogene products, followed rapidly by production of $PI(3,4)P_2$ and $PI(3,4,5)P_3$ (Auger et al., 1989; Hawkins et al., 1992; Kaplan et al., 1987). Numerous mutagenesis experiments of receptor and oncogene product kinase domain and phosphotyrosine residues support the essential role of PI3K in mitogenesis and oncogenic transformation. Mutations of PDGFr, Src, Abl, or polyoma middle T antigen which eliminate binding and activation of PI3K also abrogate the mutant protein's

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mitogenic or transforming activity (Escobedo et al., 1991a; Severinsson et al., 1990; Yu, 1991; Valius et al., 1993; Kazlauskas et al., 1992; Fantl et al., 1992; Kashishian et al., 1992; Kazlauskas & Cooper, 1989; Varticovski et al., 1991; Courtneidge & Heber, 1987; Fukui & Hanafusa, 1989; Kaplan et al., 1986; Whitman et al., 1985). Conversely, cells containing oncogenic Neu and c-Src mutants exhibit constitutively activated PI3K activity (Peles et al., 1992; Chan et al., 1990). The close association of PI3K activation with mitogenesis and the transforming activity of numerous oncogene products suggests that this enzyme plays an essential role in cellular proliferation.

PI3K is a member of a class of enzymes which catalyzes product formation at a lipid/water interface. Enzymes which perform interfacial catalysis have unique kinetic properties. These properties, along with the difficulties which can arise if these properties are ignored, have been elegantly described for porcine phospholipase A_2 (PLA_2) (Berg et al., 1991; Jain et al., 1992). Most complications arise from uncertainties about the proportion of the enzyme which is bound to the interface at any one time. Conditions where dissociation of the enzyme from the interface is kinetically significant are referred to as "hopping", since the enzyme dissociates from the lipid vesicle after a number of turnovers. Conditions under which dissociation of the enzyme is kinetically insignificant are referred to as "scooting". For example, the presence of small amounts of anionic lipid in a zwitterionic lipid vesicle increases the amount of PLA_2 bound to the interface and results in a several thousand-fold increase in the rate of product formation due to the conversion of PLA_2 from the hopping mode to the scooting mode (Jain & Berg, 1989). Characterization of the enzyme when it is tightly bound to the interface (in the scooting mode) greatly simplifies the kinetic analysis.

In the current study, we describe PI3K catalysis at a lipid/water interface which is consistent with the enzyme functioning in the scooting mode. This knowledge enabled us to determine the PI3K interfacial catalytic rate constants for PI, substrate selectivity, and kinetic mechanism. These results demonstrate that PI3K product formation kinetics can be readily described in the "scooting" mode of interfacial catalysis.

EXPERIMENTAL PROCEDURES

Materials. Phosphatidylinositol (bovine liver), phosphatidylcholine (bovine liver), and phosphatidylethanolamine (bovine liver) were purchased from Avanti Polar Lipids, Pelham, AL. 1,2-Dimyristoyl-*sn*-glycerophosphomethanol and polymyxin B sulfate were from Calbiochem, La Jolla, CA.

PI3K Cloning, Expression, and Purification. The details of PI3K cloning and expression will be described elsewhere. Briefly, human p85α and p110 clones were isolated from cDNA libraries and cloned into baculovirus expression vectors. The p85 and p110 proteins were coexpressed in insect cells and then purified by standard chromatography techniques. The infected SF9 cells (1.2 L, 4×10^5/mL) expressing p85 and p110 were harvested at 200*g* for 10 min at 4 °C and then resuspended in 60 mL of lysis buffer [20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10% glycerol, 50 mM NaF, 10 mM benzamidine, 25 μg/mL 4-(2-aminoethyl)-benzenesulfonyl fluoride, 1 μg/mL pepstatin, 0.13 unit/mL aprotinin, 4 μg/mL *N*-tosyl-L-lysine chloromethyl ketone, 25 μg/mL leupeptin, and 4 μg/mL *N*-tosyl-L-phenylalanine chloromethyl ketone]. The cells were sonicated on ice until, by visual inspection, they were lysed. Cellular debris was pelleted at 12000*g*. The crude lysate was subjected to DEAE-Sepharose and S-Sepharose chromatography as described (Carpenter et al., 1990). The active fractions were loaded onto a Reactive-Blue 4 agarose column (Sigma Chemical Co.) equilibrated in 50 mM Tris-HCl, pH 7.4, 25 mM NaCl, 1.0 mM DTT, and 5% glycerol. PI3K was eluted with a salt gradient (25−500 mM NaCl). The highly purified rhPI3K, when dilute, was easily lost through absorption to plastic. This could be prevented by the inclusion of bovine serum albumin (BSA) at a concentration of 0.1 mg/mL. BSA was not used or needed in reactions described in this paper because concentrated PI3K was always added to a solution containing lipid. This procedure prevented PI3K absorption to vessel walls. Unless otherwise noted, purified rhPI3K was used in all studies. Bovine brain PI3K extraction and DEAE chromatography were performed as previously described (Carpenter et al., 1990).

PI3K Enzyme Assay. A stock solution of small unilamellar vesicles was prepared by drying chloroform stock solutions of the lipids in a polypropylene tube under a stream of nitrogen. Tris-HCl, pH 7.4, was added and the lipid suspended by vortexing. This solution was sonicated on ice for 30 s for each 200 μL of solution using a small probe sonicator (Kontes Micro-Ultrasonic cell disrupter). The sonicator was tuned according to the manufacturer's instructions, and a power setting was used which did not break the surface tension of the liquid. Vesicles were prepared fresh for each experiment. Unless otherwise noted, the assay buffer contained 50 mM Tris-HCl, pH 7.4, 1.0 mM DTT, 2 mM $MgCl_2$, 50 μM [γ-^{32}P]ATP, and 600 μM PI. The reaction was incubated at 25 °C for the times indicated. All reactions were run in a large excess of vesicles, as empirically determined from reaction progress curves. Lipid vesicles were titrated into the reaction until a further addition did not increase the amount of product formed after product production plateaued; a 10-fold excess over this amount was then used. Reactions were terminated and the lipid aggregated by the addition of an equal volume of 1.0 N HCl. The aggregated lipid was collected on a positively charged nylon membrane (Wallac Oy, catalog no. 1205-403) in a Betaplate (Wallac Oy, Turku, Finland) manifold. The filter was washed once with 1.0 N HCl and once with 0.5 M NaCl and then transferred to a shallow dish where it was washed with shaking for 15 min in 0.5 M NaCl (3 times). The membrane was then blotted dry, wrapped in plastic film, placed between two sheets of a solid scintillant (MeltiLex A, catalog no. 1205-441, Wallac Oy), and taped to the inside of the two halves of the counting cassette. The membrane was counted in a Betaplate 1205 liquid scintilation counter. Assays requiring the identification of the lipid product or increased sensitivity were analyzed by thin-layer chromatography on grooved Whatman Silica Gel 60A thin-layer plates. The plates were developed in a filter paper lined and equilibrated tank using 1-propanol/2.0 M acetic acid (13:7, by volume).

Vesicle Floatation on Discontinuous Metrizamide Gradients. Large unilamellar vesicles of PI were prepared using a small-volume extrusion apparatus (LiposoFast; Avestin, Inc., Ottawa, Canada). PI from a chloroform stock solution

was dried in a stream of nitrogen and then under a vacuum for 1 h. The lipid was suspended in 50 mM Tris-HCl, pH 8.0, by vortexing. This emulsion was subjected to 10 freeze/thaw cycles on powdered dry ice and then extruded through 2 polycarbonate membranes (100 nm pore diameter) 19 times according to the manufacturer's instructions. Vesicles or vesicles plus PI3K were mixed with 60% metrizamide, 50 mM Tris-HCl, pH 8.0, 1.0 mM $MgCl_2$, and 1.0 mM DTT to a final metrizamide concentration of 45%. One milliliter of this solution was placed in the bottom of a 4.5 mL centrifuge tube, and 2.0 mL of 30% metrizamide prepared from the 60% stock was layered carefully on top. Buffer (1.5 mL) was layered on top. These discontinuous gradients were centrifuged at 100000*g* at 4 °C for 16 h. Aliquots (400 μL) were removed from the gradients by careful pipetting from the top of the gradient. Aliquots of 50 μL were assayed and the products analyzed by thin-layer chromatography as described above.

PI3K Specificity Constants for PI, PIP, and PIP_2. Specificity constant ratios were determined on vesicles containing 95 mol % DMPM and pairs of substrates (2.5 mol % each) according to the following relationship (Fersht, 1985; Ghomashchi et al., 1991):

$$\frac{\ln[1 - \mathrm{CPM}^1(t)/\mathrm{CPM}^1(\infty)]}{\ln[1 - \mathrm{CPM}^2(t)/\mathrm{CPM}^2(\infty)]} = \frac{(k_{cat}/K_{MS})_1}{(k_{cat}/K_{MS})_2}$$

where $\mathrm{CPM}^1(t)$ is the number of counts incorporated into product 1 at time t and $\mathrm{CPM}^1(\infty)$ is the maximum number of counts which can be incorporated onto product 1. The products of the reactions were separated by TLC, scraped from the plate, and then quantitated by scintillation counting. The products were analyzed at several times during the reaction including 1 h after product formation had plateaued.

RESULTS

PI3K Expression and Purification. PI3K subunits (p85 and p110) were cloned in baculovirus expression vectors and coexpressed in SF9 insect cells. The active heterodimer was purified as described under Experimental Procedures. Active fractions from each step of the purification were pooled and analyzed by SDS−PAGE and Coomassie staining (Figure 2). The identities of the purified proteins were verified by "Western" blot analysis with anti-p85 and anti-p110 antisera. The final yield from 2.0 L of culture was 2.4 mg, and the PI3K heterodimer was greater than 95% pure. PI3K activity was detected only when the two subunits were coexpressed. No expression of the catalytic p110 subunit was detected in single infection experiments.

Interfacial Catalysis by PI3K. A model for PI3K catalysis at a lipid−water interface is presented in Figure 1. In this model, the lipid substrate, PI3K, and lipid products are bound at the interface and remain there throughout the course of the reaction. ATP and ADP are presumed to be soluble in the aqueous phase except when bound to enzyme. The PI3K added to the reaction must interact with the interface to access the lipid substrate. The fraction of PI3K found in the aqueous phase compared to that at the interface at any given time will have significant kinetic consequences. When PI3K is bound to the interface, it is postulated that a Michaelis−Menten kinetic model is obeyed.

PI3K Progress Curves. According to the model presented in Figure 1, affinity of PI3K for the substrate vesicle will

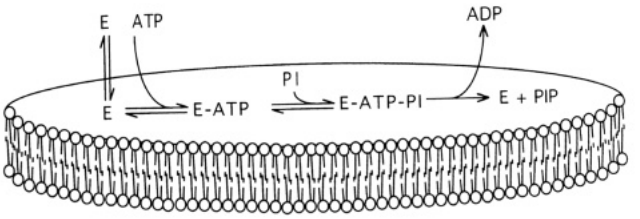

FIGURE 1: Model bireactant system for PI3K interfacial catalysis is described by the equilibria shown above. The order of substrate addition has not been determined and is shown as ordered for simplicity of presentation. Data presented in this paper are consistent with a sequential reaction mechanism. All species enclosed by the ellipse in the diagram are in or bound to the lipid bilayer, and those outside the ellipse are free in solution. E represents an enzyme (PI3K) which can be free in solution or bound to the interface. E−ATP and E−ATP−PI are the transitory complexes present in the interface. Once PI3K is bound to the interface, product formation is postulated to follow normal Michaelis−Menten kinetics. Product formation can be affected in two general and nonmutually exclusive ways. Compounds or conditions can affect the catalytic steps occurring in the interface, or they can shift the equilibrium in the amount of PI3K bound to the interface and that free in solution. We describe conditions in which PI3K is bound tightly to the interface. This system greatly simplifies a rigorous determination of rate constants and reaction mechanism.

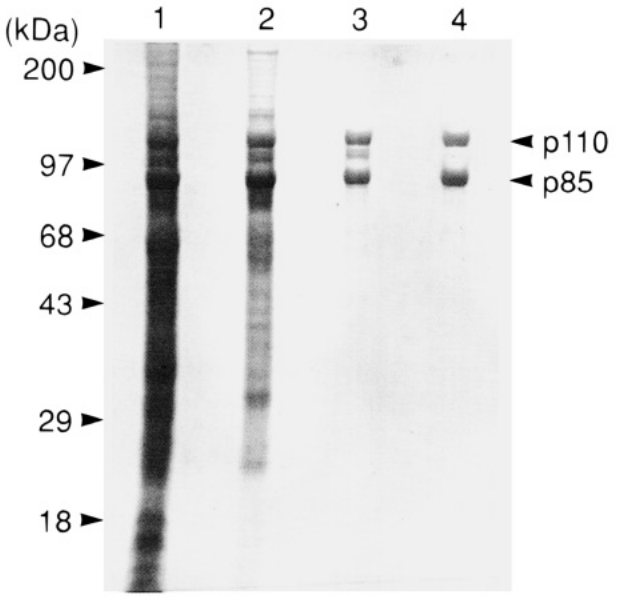

FIGURE 2: Coomassie-stained SDS−PAGE of rhPI3K containing protein pools following chromatography steps. p110 and p85 copurify and are indicated by arrows. The crude extract (lane 1), DEAE chromatography (lane 2), S-Sepharose chromatography (lane 3), and reactive blue-4 chromatography (lane 4) pools are shown.

have dramatic kinetic consequences. In the case where PI3K is tightly bound to the interface and does not dissociate over the course of the reaction, the substrate present in enzyme-containing vesicles will be utilized while the rest of the substrate remains inaccessible. If the enzyme is not tightly bound, all of the substrate present on all of the vesicle surfaces in the reaction will be accessible. Under conditions where the concentration of enzyme is much less than the concentration of vesicles, and where vesicle fusion does not occur, these two kinetic extremes are readily distinguishable.

Typical progress curves for the phosphorylation of PI are shown in Figure 3. A large excess of lipid vesicles was present in this experiment as described under Experimental Procedures. In all cases, the progress curve plateaued when only a small amount (0.001%) of the available substrate had

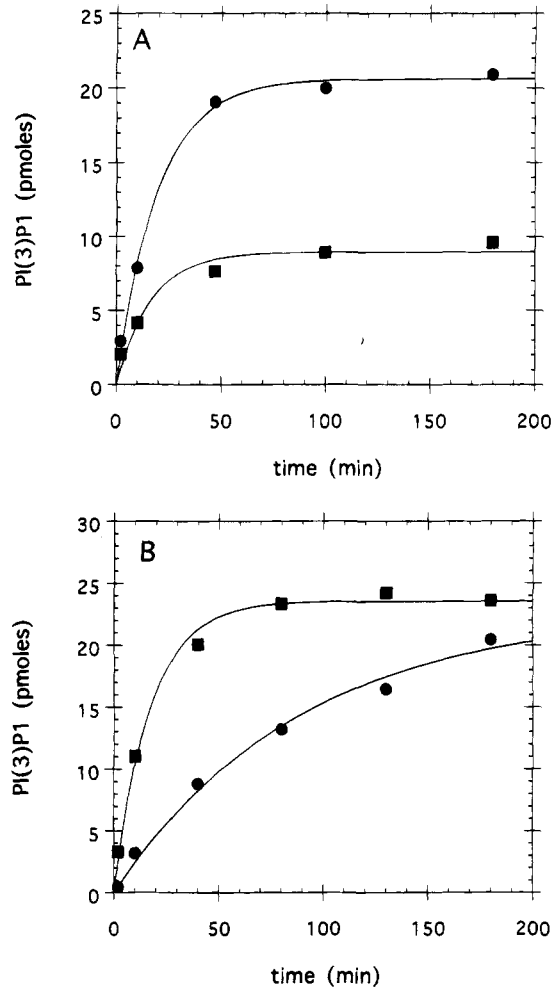

FIGURE 3: Reaction progress curves for the phosphorylation of PI by rhPI3K and bPI3K. Reactions contained small sonicated vesicles of PI/DMPM (90:10, panel A) or PI/DMPM (20:80, panel B). The total lipid concentration was 1.0 mM. The reactions shown in panel A were initiated by the addition rhPI3K (squares, 30 fmol; circles, 60 fmol). In panel B, the reactions were initiated by the addition of 60 fmol of rhPI3K (circles) or 4 μL of the partially purified bPI3K.

been converted. The total amount of substrate converted was proportional to the amount of enzyme present in the reaction. The observed rate constant to reach a plateau in product formation (k_{obs}) was proportional to the mole fraction of substrate lipid in the vesicle (compare filled circles, Figure 3A,B). These observations suggest that the enzyme is tightly bound to the interface and that substrate is not being replenished over time by vesicle fusion.

Alternative explanations for the plateau in product formation include enzyme inactivation or bulk product inhibition. To address the possibility of enzyme inactivation, we immunoprecipitated the PI3K obtained after the reaction had plateaued with an anti-p85 antibody, and then reassayed the immunoprecipitate under initial velocity conditions. Compared to control immunoprecipitates, approximately 90% of the PI3K activity was recovered from samples in the plateaued reaction mixtures, and the efficiency of recovery did not vary with the time of incubation in the initial reaction (data not shown). These observations suggest that the plateau in product formation observed in Figures 3 and 4 is not due to irreversible inactivation of the enzyme.

Another technique used to rule out PI3K inactivation was to fuse vesicles using calcium or polymyxin A (Figure 4).

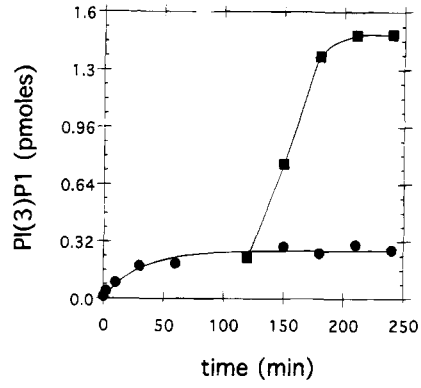

FIGURE 4: rhPI3K activation by substrate replenishment following vesicle fusion. rhPI3K was assayed on small sonicated vesicles (PI/PE, 20:80, 1.0 mM total lipid, circles). After 120 min, half of the reaction was adjusted to 100 μM polymyxin B sulfate (squares) and the reaction continued. Products were separated by thin-layer chromatography, scraped, and quantitated by liquid scintillation counting.

Vesicle fusion replenishes substrate-depleted vesicles and dilutes the product, allowing resumption of the product formation reaction. Vesicle fusion is intrinsically difficult to control. Vesicles can also aggregate, depending on the concentration of the fusogen and the length of time it is present. Aggregated vesicles are poor substrates for PI3K and mask the resumption of PI3K catalysis. Despite these problems, we were able to demonstrate a resumption of PI3K activity following substrate replenishment by vesicle fusion using either calcium (data not shown) or polymyxin B (Figure 4). The polymyxin B was added to half of the reaction after 120 min, and product formation resumed immediately. Product formation in the polymyxin B treated reaction plateaued after an additional 120 min. Again, less than 0.001% of the available substrate was utilized. Neither calcium nor polymyxin B affects PI3K initial velocities (data not shown). These data demonstrate that replenishment of substrate and dilution of product induced by vesicle fusion are sufficient to initiate resumption of product formation, consistent with a model in which the enzyme is tightly bound to the vesicle interface. This experiment also demonstrates that inhibition by bulk product does not account for the observed plateau. As mentioned above, it is possible that product inhibition within the individual enzyme-bound vesicle does contribute to the observed plateau in product formation.

Another experiment demonstrates that tight binding of PI3K to anionic vesicles is not dependent on the presence of substrate. PI3K was added to homogeneous vesicles of DMPM or PI. After 15 min, vesicles of the other lipid were added so that the final reaction conditions were identical in the two samples. The only difference was the lipid present when PI3K was added. The reactions were started by the addition of ATP, and product formation was measured at 5 and 60 min. The amount of product formed in the reaction in which the PI3K was added to DMPM vesicles first was <5% of that formed when PI3K was added to PI vesicles first, demonstrating that PI3K binds tightly to DMPM vesicles even in the absence of substrate.

Vesicle-Bound and Unbound PI3K Can Be Separated by Floatation on Discontinuous Metrizamide Gradients. Analysis of PI3K reaction progress curves indicates PI3K is tightly bound to the vesicle interface and that association/dissocia-

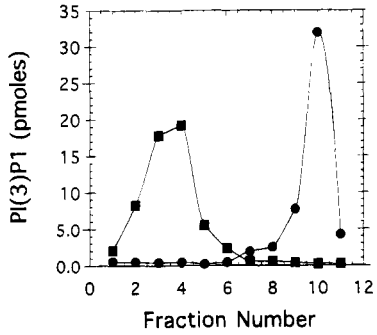

FIGURE 5: Floatation of rhPI3K on discontinuous metrizamide gradients. Free rhPI3K (circles) or rhPI3K bound to extruded PI vesicles (squares) was analyzed on discontinuous metrizamide gradients. The top of the gradient is on the left.

tion from the interface does not contribute to the overall reaction rate. To address this point further, we sought a technique to physically separate vesicle-bound PI3K from free PI3K. Figure 5 shows the distribution of rhPI3K in a discontinuous metrizamide gradient in the presence or absence of PI vesicles. PI3K or PI3K plus PI vesicles were adjusted to 45% metrizamide and placed in the bottom of a centrifuge tube. A layer of 30% metrizamide was added followed by buffer. After centrifugation, the PI vesicles banded near the buffer/30% metrizamide interface. When PI3K was mixed with PI vesicles, greater than 90% of the PI3K activity loaded on the gradient comigrated with the PI vesicles (Figure 5). In the absence of PI vesicle, the PI3K activity remained near the bottom of the tube. These results demonstrate that the PI3K/vesicle complex is stable over the course of several hours required for the vesicles to reach their density equilibrium, and support the hypothesis that recombinant human PI3K is tightly bound to the vesicle interface.

$K_{m,app}$ Determinations for ATP and PI. The $K_{m,app}$ for ATP was determined to be 50 μM for rhPI3K (Figure 6A). This is consistent with the previously reported values for the bovine and rat heterodimers assayed on PI (Carpenter et al., 1990; Woscholski et al., 1994).

Rigorous determination of the lipid substrate K_m is more complex. One of the consequences of the model presented in Figure 1 is that if PI3K is tightly bound to the interface then product formation will be unaffected by the bulk lipid substrate concentration, since under conditions where the concentration of enzyme is limiting each enzyme molecule "sees" only the substrate in the vesicle to which it is bound. The effect of the bulk lipid concentration on total product formation under conditions where the vesicle concentration exceeds that of PI3K is presented in Figure 7. Initial velocities were measured at bulk lipid concentrations varying from 30 μM to 3.0 mM, and they did not vary significantly over this range. These results demonstrate that the amount of product formation from PI3K is insensitive to bulk lipid concentration, and are consistent with a model in which PI3K is tightly bound to the vesicle interface.

While it is impossible to obtain phospholipid K_m's by varying the bulk lipid concentration, it is theoretically possible to obtain a $K_{m,app}$ by varying the interfacial concentration of PI. The interfacial PI concentration (mole percent) was varied by diluting the PI with the nonsubstrate lipid, DMPM. The PI $K_{m,app}$ for rhPI3K was determined by measuring the initial velocities of product formation at

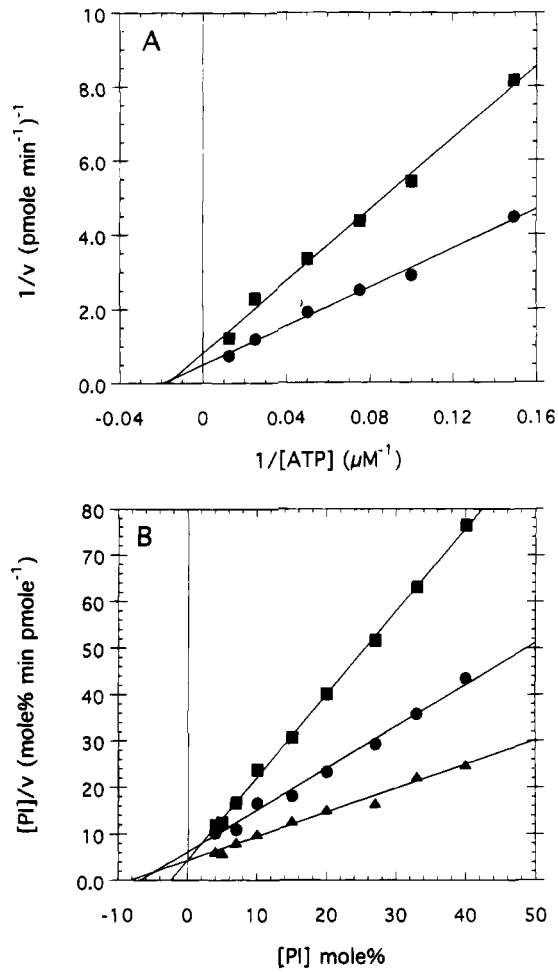

FIGURE 6: Determination of ATP and PI $K_{m,app}$ for rhPI3K and bPI3K. Panel A shows a double-reciprocal plot from which the ATP $K_{m,app}$ values for rhPI3K (circles) and bPI3K (squares) were determined. Panel B shows a double-reciprocal plot from which the PI $K_{m,app}$ for rhPI3K was determined using DMPM (circles) or PC (triangles) as the diluent. The PI $K_{m,app}$ determination for bPI3K (DMPM, squares) is also shown.

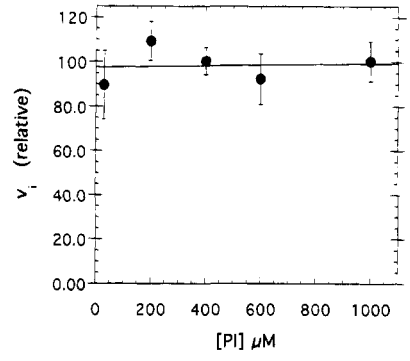

FIGURE 7: rhPI3K relative initial velocities measured as a function of total lipid concentration. Initial velocities were measured using a constant amount of enzyme (2 fmol) on small sonicated vesicles (PI/DMPM, 90:10). The total lipid concentration was varied from 30 μM to 1.0 mM.

several interfacial PI concentrations. Initial velocities increased as the interfacial PI concentration increased (Figure 6B). The PI $K_{m,app}$ for rhPI3K was 6.2 mol %. We were unable to measure the $K_{m,app}$ for PIP and PIP_2 due to vesicle aggregation.

The validity of the vesicle dilution approach for measuring lipid substrate K_m's depends on the assumption that the

Table 1: Comparison of PI3K Specificity Constants (k_{cat}/K_m) for PI, PIP, and PIP_2[a]

substrates (A vs B)	$(k_{cat}/K_m)^A/(k_{cat}/K_m)^B$
PI vs PIP	0.94
PI vs PIP_2	1.06
PIP vs PIP_2	1.12

[a] All studies were carried out on vesicles of DMPM as described in Figure 2. The total substrate concentration was 5 mol %. Calculation of specificity constants has been described (Fersht, 1985; Ghomashchi et al., 1991).

diluent lipid does not interact with the lipid binding site of PI3K, *i.e.*, that it is a "neutral diluent" (Jain et al., 1991a). If the lipid diluent competes with PI for the active site, then the decrease in velocity seen as the PI concentration is decreased may result, in part, from the increased concentration of the potential competitive inhibitor (DMPM). To investigate this possibility, we determined the $K_{m,app}$ for PI using PC as the diluent (Figure 6B). DMPM and PC vary significantly in their headgroup size, charge, acyl chain length, and degree of chain saturation. As with the PI/DMPM vesicles, the rhPI3K progress curve on vesicles of PI/PC (50:50) plateaued when only a small fraction of the available substrate had been converted (data not shown). This suggests that rhPI3K binds tightly to vesicles containing PC as well as to those containing DMPM. The $K_{m,app}$ for PI using PC as the diluent was 8.0 mol %. The similarity in the $K_{m,app}$'s determined using two structurally diverse lipid diluents (6.2 and 8.0 mol %) suggests that they do not compete for the lipid substrate binding site, or do so to a similar extent.

PI3K Selectivity for PI, PIP, and PIP_2. PI3K will phosphorylate PI, PIP, and PIP_2 *in vitro*, but the pattern of substrate utilization is much different *in vivo*. *In vivo*, the primary product of PI3K catalysis following growth factor stimulation is PI(3,4,5)P_3 (Hawkins et al., 1992; Stephens et al., 1991, 1993). One possible explanation for this apparent discrepancy is that *in vitro* experiments are not representative of the *in vivo* situation because vesicles comprised exclusively of each lipid substrate will exhibit dramatically different interfacial properties, resulting in different fractions of enzyme bound to the different vesicles (Ghomashchi et al., 1991). To avoid this concern, the substrate specificity of rhPI3K was studied under conditions of tight binding to the interface where the interfacial properties of the vesicles would be similar for all three substrates (*e.g.*, low mole percent of the substrates in a vesicle of a nonsubstrate lipid, DMPM). By comparing the relative rates of product formation between pairs of lipid substrates, the intrinsic selectivity of rhPI3K for PI, PIP, and PIP_2 was assessed. As shown in Table 1, the ratios of specificity constants were close to unity for all three pairs of phosphoinositide substrates, suggesting that under these conditions PI3K does not discriminate between them.

Comparison of Kinetic Properties of rhPI3K with Bovine Brain PI3K. The interfacial kinetic properties of partially purified bovine brain PI3K were compared to those of the highly purified recombinant human PI3K. On vesicles composed primarily of PI (PI/DMPM 90:10), the reaction progress curves have similar shapes (data not shown). At lower PI mole fractions (PI/DMPM 20:80), however, the bovine PI3K progress curve plateaus after about 60 min while the progress curve for the human PI3K has not completely plateaued after 180 min (Figure 3B). One possible explanation for the difference in substrate utilization between these two enzymes is that, while the $K_{m,app}$ for ATP was virtually identical for PI3K from both sources (Figure 6A), the PI $K_{m,app}$ for the bovine enzyme was 3-fold lower than that of the recombinant human enzyme (6.2 mol % vs 2.1 mol %; Figure 6B).

DISCUSSION

PI3K Interfacial Catalysis in the Scooting Mode. A model for PI3K interfacial catalysis is presented in Figure 1. PI3K involved in substrate utilization partitions between the lipid/water interface and the aqueous phase based on the association/dissociation constants of the enzyme for the interface. It is only in the bound state that PI3K can access the long-chain phosphatidylinositol substrates. Once PI3K is bound to the interface, the enzyme is postulated to undergo normal Michaelis–Menten kinetics. The model is diagrammed as an ordered sequential Bi-Bi reaction for simplicity, however, it has not been determined if the addition of the substrates is ordered or random. In this model, the central complexes are present in the interface, and the lipid product remains in the interface. If PI3K is not tightly bound to the interface, the rate of adsorption and desorption will contribute significantly to the overall rate of catalytic turnover and complicate the determination of the individual rate constants. Using highly purified rhPI3K and partially purified bPI3K, we have identified reaction conditions where PI3K binds tightly to substrate-containing vesicles for long periods of time, and this has allowed us to begin a detailed kinetic analysis of PI3K.

There are several observations which indicate that PI3K binds tightly to the lipid/water interfaces described in this report. The properties of the reaction progress curves presented in Figures 3 and 4 are characteristic of those obtained using enzymes known to undergo interfacial catalysis in the scooting mode (Berg et al., 1991; Jain & Gelb, 1991). These reaction progress curves are completely described by the Michaelis–Menten model system when the molar ratio of vesicles to enzyme is large (Berg et al., 1991). One of the unusual characteristics of these progress curves was that they plateaued when less than 0.001% of the available (outer layer of the vesicle) substrate has been consumed. This is the expected result for a large molar excess of vesicles over enzyme where the enzyme is tightly bound to the interface. Substrate conversion ceases when the substrate in the outer vesicle layer has been depleted. Similar observations were made when PI3K reactions were run on PI/PE (20:80) vesicles. The progress curves plateau when only a small amount of the substrate had been utilized. Substrate utilization resumed following vesicle fusion and substrate replenishment. Additional evidence for tight binding of PI3K to DMPM and PI vesicles comes from the order of addition experiments. When PI3K was preabsorbed onto DMPM vesicles, PI in subsequently added vesicles was not phosphorylated. The plateauing in the progress curves when only a small amount of the lipid substrate has been utilized and the lack of product formation when PI3K is sequestered on nonsubstrate-containing vesicles suggest that PI3K is tightly bound to the anionic lipid vesicles and that substrate replenishment is not occurring through vesicle fusion.

In addition to the reaction progress curves, order of addition experiments, and vesicle fusion experiments, there

is further kinetic evidence for PI3K catalysis in the scooting mode. Figure 6B shows a Hanes−Woolf plot of initial velocities with changing interfacial PI concentrations. Initial velocities increase as the interfacial PI concentration increases from 4 to 40 mol %. The bulk PI concentration varies from 24 to 240 μM. In contrast, the data presented in Figure 7 indicate that initial velocities are independent of bulk PI concentrations over a range of 30 μM to 1 mM when the interfacial PI concentration remains constant. These results are expected if PI3K is tightly bound to the interface; initial velocities are insensitive to increases in the bulk PI concentration because PI3K is bound to the interface and the addition of more vesicles is of no consequence. There have been reports of increasing PI3K initial velocities with increasing bulk lipid substrate concentrations (Carpenter et al., 1990; Shibasaki et al., 1993; Whitman et al., 1987; Woscholski et al., 1994). This can be explained if the vesicle concentration is not in large excess over the PI3K concentration, or if reaction conditions utilized drive PI3K into the hopping mode.

Enzyme denaturation was eliminated as an alternative explanation for the product formation plateau by showing that vesicle fusion and substrate replenishment resulted in a resumption of product formation, and by demonstrating that PI3K immunoprecipitated from reaction mixtures where product formation had ceased retained catalytic activity. The vesicle fusion and substrate replenishment experiments also demonstrate that the bulk product concentration was not responsible for the plateau in the progress curves. Finally, quantitative analysis of the amount of PIP formation yielded values consistent with nearly complete phosphorylation of all PI in the outer membrane layer of the substrate vesicles containing bound enzyme (Jain et al., 1991b), based on the assumption that the enzyme binds tightly to the substrate vesicle (data not shown).

To complement our kinetic analysis of PI3K interfacial catalysis in the scooting mode, we employed sedimentation studies to demonstrate that rhPI3K was tightly bound to lipid vesicles. PI3K comigrated with lipid vesicles to a point near the top of a discontinuous metrizamide gradient, while PI3K in the absence of vesicles remained near the bottom of the gradient. In the vesicle-containing experiments, there was no detectable PI3K in the lower part of the gradient, suggesting that PI3K bound tightly to the vesicles over the course of the several hours that were required to float the vesicles.

Comparison of rhPI3K and bPI3K. The utility of studying PI3K in the scooting mode and varying the interfacial concentration of the lipid substrate in the kinetic analysis is illustrated by our comparison of PI3K from two different sources. The highly purified, insect cell-expressed rhPI3K and the partially purified bPI3K appear to have similar first-order relaxation constants when assayed on PI/DMPM vesicles containing 90 mol % PI (Figure 3A). However, when progress curves were performed on vesicles containing only 10 mol % PI, the first-order relaxation constants are different. The $K_{m,app}$ values for PI determined from a Hanes−Wolfe plot for rhPI3K and bPI3K are 6.2 and 2.1 mol %, respectively. This 3-fold difference in the $K_{m,app}$ for PI may contribute to the differences seen in the shape of the progress curves when the assay is performed on vesicles containing low mole percents of the substrate lipid. Post-translational modification or unconserved amino acids may also contribute to these differences.

PI3K Substrate Specificity. The substrate utilization by PI3K observed *in vivo* cannot be explained by the properties of rhPI3K or bPI3K determined *in vitro*. Using the approach outlined above, we determined that rhPI3K utilizes PI, PIP, and PIP_2 equally well (Table 1). Because there is 5−10 times as much PI in cells as PIP or PIP_2 (Majerus et al., 1984, 1986; Stephens et al., 1991), one would expect to see preferential production of $PI(3)P_1$ upon enzyme activation. Following growth factor stimulation and PI3K activation, however, the initial and predominant lipid product detected is $PI(3,4,5)P_3$. $PI(3,4)P_2$ levels also increase but seem to lag behind the production of $PI(3,4,5)P_3$, consistent with a model in which $PI(3,4)P_2$ production is the result of phosphatase action on $PI(3,4,5)P_3$ (Auger et al., 1989; Stephens et al., 1989, 1991). Multiple explanations for the apparent discrepancy between *in vitro* and *in vivo* substrate utilization patterns are possible. First, PI3K substrate specificity may be modulated by posttranslational modification and/or by interaction with phosphotyrosine-containing proteins through SH2 domains. Second, PI3K may be recruited to an area of the membrane where the local concentration of PIP_2 is higher than the surrounding regions. For instance, the local concentration of PIP_2 could be increased by PIP_2 binding proteins which are present in the complex; in fact, the PIP_2/protein complex could be the actual substrate. Alternatively, the local concentration of PIP_2 could be high because it is being synthesized in the PI3K-containing compartment.

PI3K Interfacial Catalysis in the Scooting Mode and the Evaluation of Inhibitors and Regulatory Mechanisms. We have identified reaction conditions where rhPI3K binds tightly to lipid substrate vesicles, and catalyzes product formation in the scooting mode. These results do not imply that PI3K is constitutively bound to the cytoplasmic membrane *in vivo*; indeed, several studies have demonstrated that unactivated PI3K is found predominantly in the cell cytoplasm, migrating to the membrane only after phosphorylation of tyrosine kinase receptors [reviewed in Cantley et al. (1991) and Stephens et al. (1993)]. This observation does not diminish the importance of studying the enzymology of PI3K in the scooting mode, however. The evaluation of inhibitors of enzymes which act at the lipid/water interface is known to be problematic. For example, Jain et al. have compiled a list of compounds which are known to inhibit pancreatic PLA_2 by changing the quality of the interface and not by competing with substrate for the active site (Jain et al., 1992). Inhibitors that work by changing the quality of the interface and preventing PI3K from binding are of little pharmacological significance. Inhibition by this means would be expected to have a significant toxic effect on cells because it would require modifying the properties of cellular membranes. The experiments outlined above can distinguish between a compound which interferes with PI3K binding to the interface and one that competes with one or both substrates. Understanding that the interfacial lipid substrate concentration must be varied, rather than the bulk lipid substrate concentration, enables one to determine if an inhibitor competes with the lipid substrate and to determine relative K_i values and structure/activity relationships for inhibitors. All the standard enzymological techniques can be applied

once it has been determined that the enzyme is functioning in the scooting mode.

Accounting for the lipid/water interface is also important for the analysis of interfacial diluents, substrates, and regulatory interactions. In general, any substance being evaluated for its effects on PI3K interfacial catalysis has the potential to affect the quality of the interface. Magnesium, for example, is required for PI3K utilization of ATP, but divalent cations are potent vesicle fusigens and can result in vesicle aggregation (Nir et al., 1983; Eastman et al., 1992; Lentz et al., 1989). Peptides are another class of molecules which are known to cause vesicle fusion and aggregation (Kubesch et al., 1987; Sundler & Bondeson, 1990), while detergents may inhibit PI3K by diluting the interfacial lipid substrate concentration and reducing the size of vesicle through the formation of micelles. Substrate specificity for PI3K and other enzymes which utilize a substrate present in the interface is best determined in a system where the enzyme is tightly bound to the interface. Under these conditions, substrates can be evaluated side by side in the same vesicle at low mole percents by determining the ratio of the specificity constants (k_{cat}/K_m) for each substrate. Vesicle size, curvature, and sensitivity to divalent cation-induced fusion or aggregation may also vary with interface composition.

PI3K binding to an interface may be required for substrate utilization. This contention is supported by the observation that the aqueous soluble inositol, phosphoinositols, and glycerophosphoinositols were not utilized by PI3K at detectable levels (data not shown) and the glycerol and fatty acid moieties of PI are not recognized by PI3K (Macphee et al., 1992). Localization to the interface, therefore, may be a major regulatory mechanism of PI3K.

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