methods

Cyclodextrins enhance recombinant phosphatidylinositol phosphate kinase activity

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Abstract **Inositol lipid kinases have been studied extensively in both plant and animal systems. However, major limitations for in vitro studies of recombinant lipid kinases are the low specific activity and instability of the purified proteins. Our goal was to determine if cyclodextrins would provide an effective substrate delivery system and enhance the specific activity of lipid kinases. For these studies, we have used recombinant *Arabidopsis thaliana* phosphatidylinositol phosphate kinase 1 (*At*PIPK1). *At*PIPK1 was produced as a fusion protein with glutathione-*S*-transferase and purified on glutathione-Sepharose beads. A comparison of lipid kinase activity using substrate prepared in α-, β-, or γ-cyclodextrin indicated that β-cyclodextrin was most effective and enhanced lipid kinase activity 6-fold compared with substrate prepared in Triton X-100-mixed micelles. We have optimized reaction conditions and shown that product can be recovered from the cyclodextrin-treated recombinant protein, which reveals a potential method for automating the assay for pharmacological screening.**—Davis, A. J., I. Y. Perera, and W. F. Boss. **Cyclodextrins enhance recombinant phosphatidylinositol phosphate kinase activity. *J. Lipid Res.* 2004.** 45: **1783–1789.**

Supplementary key words *Arabidopsis thaliana* • automated assay • inositol lipids

Inositol lipid kinases function at the interface of the lipid bilayer and selectively phosphorylate the head group of inositol phospholipids (1). One of the limitations of in vitro lipid kinase assays is that the recombinant lipid kinases are often unstable and exhibit low activity when presented with lipid substrate as sonicated or Triton-mixed micelles. Our goal was to determine if cyclodextrins could increase recombinant lipid kinase activity by more effectively delivering lipid substrate. We used recombinant *Arabidopsis thaliana* phosphatidylinositol phosphate kinase 1 (*At*PIPK1) (2) fused to glutathione-*S*-transferase (GST) to investigate the effects of using cyclodextrins to deliver lipid substrate to the recombinant lipid kinase. GST-*At*PIPK1 phosphorylates phosphatidylinositol-4-phosphate (PtdIns4P) to form phosphatidylinositol-(4,5)-bisphosphate PtdIns(4,5)P_2 (3, 4).

Cyclodextrins are cyclic oligomers of α-D-glucopyranose that are produced naturally in bacteria. Their ring structures form a cone shape that has a hydrophilic outer surface and a hydrophobic inner core. There are three naturally occurring cyclodextrins: α-cyclodextrin (αCD) contains six glucopyranose units; β-cyclodextrin (βCD) contains seven glucopyranose units; and γ-cyclodextrin (γCD) contains eight glucopyranose units. In addition, naturally occurring cyclodextrins have been modified with various substitutions on the glucopyranose subunits to increase their efficacy in specific industrial and scientific applications (5).

Industrial applications of cyclodextrins include use in pharmaceuticals to enhance drug stability and delivery and in food additives to preserve flavors and enhance shelf-life (5). Recent studies in polymer sciences have used cyclodextrins to facilitate the formation of polymers and enhance the intercalation of small molecules into the polymer matrices for potential drug delivery (6). Cyclodextrins are also used in the cosmetics industry to create longer lasting fragrances and prevent the oxidation and degradation of oils (5). Laboratory applications include using cyclodextrins as size-exclusion columns, as artificial chaperones to remove detergents and facilitate the refolding of recombinant proteins (7, 8), as a vehicle to develop molecular machines (9), and as a means for the delivery and removal of lipids from membranes to study bilayers and lipid rafts (10–13).

We have taken advantage of the ability of cyclodextrins to bind lipids (11, 12, 14) and deliver them to cells (13) and asked whether cyclodextrin could be used to deliver inositol phospholipids to recombinant lipid kinases for in

Abbreviations: *At*PIPK1, *Arabidopsis thaliana* phosphatidylinositol phosphate kinase 1; αCD, βCD, and γCD, α-, β-, and γ-cyclodextrin; GST, glutathione-*S*-transferase; NBD-PtdInsP, D(+)-*sn*-1-*O*-[1-[6′-[6-[(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]hexanoyl]amino]-hexanoyl]-2-*O*-hexadecanoylglyceryl D-*myo*-phosphatidylinositol phosphate; PtdInsP, phosphatidylinositol phosphate; PtdIns(4,5)P_2, phosphatidylinositol-(4,5)-bisphosphate.

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vitro lipid kinase assays. We found that using cyclodextrin for substrate delivery increased *At*PIPK1 specific activity 4- to 6-fold compared with sonicated substrate alone or Triton-mixed micelles, respectively. In addition, when cyclodextrin was used for substrate delivery, the product PtdIns(4,5)P_2 could be recovered with the recombinant GST-*At*PIPK1 beads. Long, arduous lipid extractions in organic solvents are an additional challenge when performing lipid kinase assays. The data presented here provide a basis for developing an environmentally friendly method that does not require organic solvents for the recovery of phosphorylated lipid products and uses a procedure that would be readily applicable for large-scale screening of kinase inhibitors.

METHODS

Cloning and expression of GST-AtPIPK1

The cDNA of *At*PIPK1 (At1g21980) was amplified by PCR using the sense primer AAACCCATGGGAATGAGTGATTCAGAAGAAG and the antisense primer GTTAAAAACTCGAGCCTTCTTGTCTTTAGCC to create *Nco*I and *Xho*I restriction sites, respectively, for directional cloning into pET-41a vector (Novagen, Madison, WI). The PCR product was digested and ligated into the pET-41a vector that had been digested with *Nco*I and *Xho*I. The sequence of the resulting construct, pET-41a5K1, was confirmed by DNA sequencing. The *Escherichia coli* expression strain BL21(DE3)pLysS (Novagen) was transformed with pET-41a5K1 and used to express the fusion protein GST-*At*PIPK1.

For recombinant protein expression, an overnight culture of BL21(DE3)pLysS carrying pET-41a5K1 was diluted 1:500 with fresh Lennox L broth (Invitrogen, Carlsbad, CA) medium and grown at 37°C with shaking until an OD_{600} of 0.3 was reached. At this point, isopropyl-β-D-thiogalactoside was added to a final concentration of 1 mM, and incubation continued at 25°C for 4 h with shaking. After 4 h, the cells were collected by centrifugation and the bacterial pellets were frozen at −20°C until the recombinant protein was to be used.

GST-AtPIPK1 purification

Bacterial pellets were thawed and resuspended in ice-cold PBS buffer (0.1 M KH_2PO_4, 0.1 M K_2HPO_4, 135 mM NaCl, and 2.7 mM KCl, pH 7.3) and sonicated on ice for 20 s. The sonicated solution was centrifuged at 12,000 *g* for 10 min. The supernatant was removed and combined with glutathione-Sepharose beads (Amersham Pharmacia Biotech, Pitscataway, NJ) or with magnetic glutathione-agarose beads (Novagen) preequilibrated with PBS. The mixture was incubated at 4°C for 2 h with continuous mixing followed by extensive washing of the beads with PBS. Protein concentration was determined using the Bio-Rad protein assay reagent (Bio-Rad, Hercules, CA) with BSA as a standard. Purified recombinant proteins bound to the glutathione-Sepharose beads or the magnetic glutathione-agarose beads were stored at 4°C until use in lipid kinase assays. The purified enzyme was not stored longer than 12 h before use. The storage time of the purified lipid kinase was correlated with a decrease in the specific activity of the enzyme, as seen when comparing Figs. 2 and 3. In Fig. 2, the purified lipid kinase was used immediately, and in Fig. 3, the purified enzyme was stored for 12 h.

Microsomal preparation

Microsomes were isolated from *A. thaliana* cells grown in suspension culture. Cells were harvested at 4 days after subculture by gravity filtration and immediately homogenized in an equal volume of ice-cold buffer containing 5 mM HEPES, pH 7, 10 mM $MgCl_2$, 2 mM EGTA, 8% (w/v) sucrose, 1 mM PMSF, 1 mg/100 ml leupeptin, and polyvinylpolypyrrolidone (0.1 g/g cells). The homogenate was centrifuged twice at 2,000 *g* for 6 min at 4°C. The resulting supernatant was centrifuged at 40,000 *g* for 60 min at 4°C to obtain the microsomal fraction. The microsomes were washed in 50 mM Tris, pH 7.5, and centrifuged at 40,000 *g* for 30 min at 4°C, and the final pellet was resuspended in 50 mM Tris, pH 7.5. Microsomes were placed on ice and used immediately for lipid kinase assays. Protein concentration was determined using the Bio-Rad protein assay reagent with BSA as a standard.

Lipid kinase assay

Phosphatidylinositol phosphate (PtdInsP) kinase activity was assayed in duplicate as described by Cho and Boss (15) with a final reaction volume of 50 μl. Each assay contained either 30 μg of microsomal protein or 10 μg of purified recombinant protein on glutathione-Sepharose beads washed once with 50 mM Tris, pH 7.5. Lipid substrate was prepared using PtdIns4P (porcine brain; Avanti Polar Lipids, Alabaster, AL), PtdIns3P (Matreya, Inc., Pleasant Gap, PA), or PtdIns5P (Echelon Biosciences, Inc., Salt Lake City, UT) from 1 mg/ml stocks. Lipids were divided into aliquots and dried under an N_2 stream to form a thin, even film in the bottom of the test tube. Dried lipid films were solubilized for use in the lipid kinase assays in the presence and absence of cyclodextrins. In the absence of cyclodextrins, lipids were sonicated for 10 s in 50 mM Tris, pH 7.5, or in a solution of Triton X-100 resulting in a final concentration of 0.1% Triton X-100 (v/v) in the final reaction volume and then incubated on ice for 10 min. Triton (0.1%) was determined to give optimal enzyme activity (D. Galanopolou, I. Y. Perera, and W. F. Boss, unpublished results). Lipids were also solubilized by vortexing in the presence of deoxycholate to give a final concentration of 1% in the final reaction volume, as described by Westergren et al. (4). Cyclodextrin solubilization was accomplished by adding αCD, βCD, or γCD (all from Sigma) from a 150 mM (saturated) stock solution to achieve the desired concentration in the 50 μl reaction volume. The final concentrations of cyclodextrin solutions produced from the stock solution were confirmed by comparison with the refractive indices of cyclodextrin solutions of known concentrations. The cyclodextrin solution was added to the dried lipid film, vortexed for 5 s, and incubated on ice for 10 min before use. The lipid concentration for each lipid kinase assay was 125 μM, except where noted. The reaction mixture contained final concentrations of 50 mM Tris, pH 7.5, 10 mM $MgCl_2$, 1 mM EGTA, 1 mM Na_2MoO_4, and 50 μM ATP (9 μCi of [^{32}P]ATP per reaction). In experiments performed at varying pH values, all proteins, lipids, and reaction mixtures were prepared in 50 mM Tris at the appropriate pH. Reactions were incubated at room temperature for 10 min with shaking, stopped by adding 1.5 ml of $CHCl_3$/methanol (1:2, v/v), and stored at 4°C until the lipids were extracted. Lipid extraction was performed using an acid solvent system as described previously (15). Extracted lipids were separated by TLC on silica gel plates (LK5D; Whatman, Clifton, NJ) using a $CHCl_3$/methanol/NH_4OH/water (90:90:7:22, v/v) solvent system. The ^{32}P-labeled phospholipids were quantified with a Bioscan System 500 imaging scanner.

Fluorescence experiments

Fluorescence spectroscopy was used to monitor lipid distribution during substrate preparation and during the lipid kinase assays. For the substrate preparation, 6.25 μg of D(+)-*sn*-1-*O*-[1-[6′-[6-[(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]hexanoyl]amino]hexanoyl]2-*O*-hexadecanoylglyceryl D-*myo*-phosphatidylinositol 4-phosphate (NBD-PtdIns4P; Echelon Biosciences, Inc.) was divided

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into aliquots for each reaction and dried under an N_2 stream to form a thin, even film in the bottom of the test tube. Lipids were solubilized as described above either by sonication in 50 mM Tris, pH 7.5, or in a Triton X-100 solution or by incubation with βCD and incubated on ice for 10 min. Triton X-100 and βCD were added to correspond to 0.1% Triton X-100 or 5 mM βCD in the 50 μl volume of a lipid kinase assay. The supernatant was removed. The supernatant and the residual, nonsolubilized lipid adhering to the test tube were extracted as described above. The extracts were dried under vacuum and reconstituted in 1 ml of chloroform. All samples were analyzed in a Hitachi F-2000 fluorescence spectrophotometer with an excitation wavelength of 470 nm and an emission wavelength of 530 nm. The relative amount of lipid solubilized with each method was calculated by comparing the fluorescence recovered in the supernatant with the total fluorescence recovered (supernatant plus residue).

Recovery of lipid from GST-*At*PIPK1 beads treated with cyclodextrin was also monitored by fluorescence spectroscopy. NBD-PtdIns4P was divided into aliquots and dried under an N_2 stream for each experiment. Lipid was prepared by adding 50 mM Tris, pH 7.5, and sonicating or by vortexing in 5 mM βCD to yield a final concentration of 6.25 μg of NBD-PtdIns4P per 10 μl of solution. For each experiment, 5 μl magnetic glutathione-Sepharose beads, 2 μg of purified GST on magnetic glutathione-Sepharose beads, or 2 μg of purified GST-*At*PIPK1 on magnetic glutathione-Sepharose beads was incubated with 1.25 μg of the prepared lipid, either in Tris buffer or in βCD. To assay for lipid kinase activity, purified GST-*At*PIPK1 and prepared lipid were mixed, ATP was added to a final concentration of 0.5 mM, and the reaction was incubated with mixing for 1 h. To stop the reaction, 2 ml of 50 mM Tris, pH 7.5, was added. The beads were retained with a magnet, and the solution was removed and discarded. This washing procedure was repeated once. After the final wash, the fluorescence was monitored microscopically or the lipids were extracted as above. After the extraction, lipids were reconstituted in $CHCl_3$:methanol:water (2:1:0.01, v/v) and spotted on a TLC plate. The plate was developed in the same solvent system as described above. After the TLC plate was dry, the regions where PtdIns4P and PtdIns(4,5)P_2 migrated were scraped and the lipids were eluted from the silica gel with two washes of 500 μl of $CHCl_3$:methanol:NH_4OH:water (90:90:7:22, v/v). The eluted lipids were analyzed in a Hitachi F-2000 fluorescence spectrophotometer with an excitation wavelength of 470 nm and an emission wavelength of 530 nm.

RESULTS

To determine whether adding PtdIns4P in the presence of cyclodextrins increased enzyme activity, we compared the specific activity of the purified recombinant GST-*At*PIPK1 in the presence and absence of cyclodextrins. αCD, βCD, or γCD was added to the lipid substrate as described in Methods to achieve a concentration of 0–30 mM cyclodextrin in the final reaction mixture. The specific activity of the lipid kinase was compared with that obtained using PtdIns4P solubilized in Triton X-100 or by sonication. The results of this experiment (**Fig. 1**) indicate that under identical reaction conditions, the PtdInsP kinase activity was approximately four to six times greater when the substrate was delivered in a solution of βCD compared with sonication or Triton, respectively. The optimal cyclodextrin concentration was 5 mM βCD. Although 5 mM αCD or γCD also enhanced enzyme activity compared with Triton or sonication, neither of these cyclodextrins enhanced activity to the extent of βCD, and at higher concentrations they decreased enzyme activity. Because βCD gave the highest enzyme activity and because it

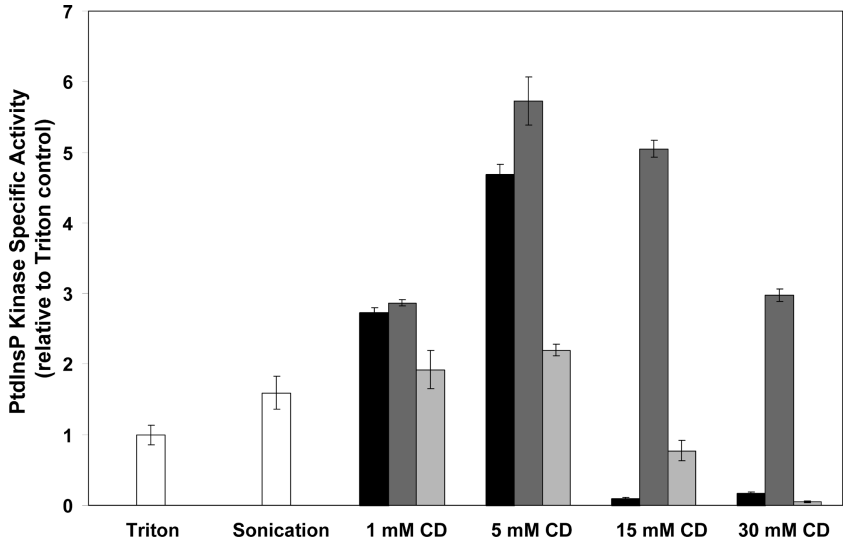

Fig. 1. The activity of purified recombinant glutathione-*S*-transferase-*Arabidopsis thaliana* phosphatidylinositol phosphate kinase 1 (GST-*At*PIPK1) was measured with lipid substrate prepared as 0.1% Triton X-100-mixed micelles, sonicated micelles, or with various concentrations of α-cyclodextrin (αCD; black bars), β-cyclodextrin (βCD; dark gray bars), or γ-cyclodextrin (γCD; light gray bars). Ten micrograms of purified recombinant protein was assayed, and the lipid concentration was kept constant at 125 μM phosphatidylinositol 4-phosphate (PtdIns4P). The values shown are averages of two independent experiments and are reported as fold increases in specific activity of GST-*At*PIPK1 compared with the specific activity using PtdIns4P-Triton micelles. Error bars in all figures indicate the SD of at least four values from two independent experiments.

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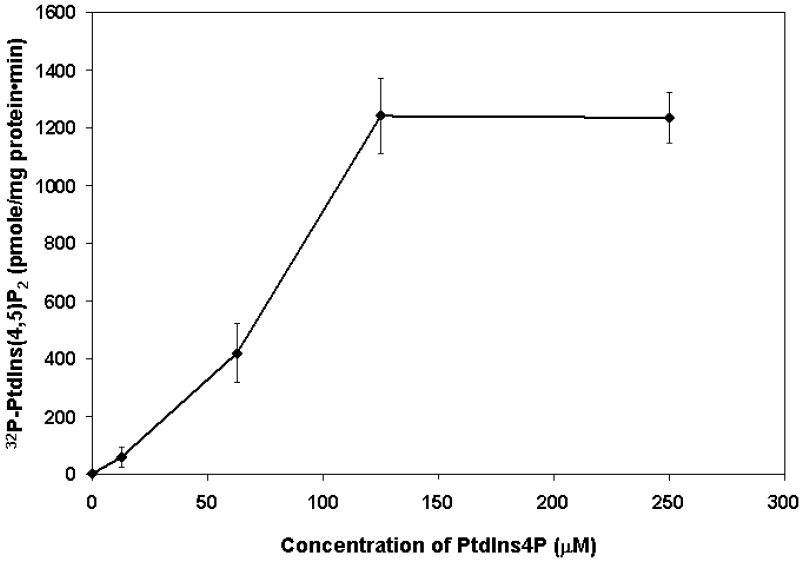

Fig. 2. Purified recombinant GST-*At*PIPK1 (10 μg) was assayed using increasing concentrations of PtdIns4P at a constant substrate-to-βCD ratio of 1:40 (w/w). The results are reported as averages of two independent experiments. PtdIns(4,5)P_2, phosphatidylinositol-(4,5)-bisphosphate.

is the most cost-effective delivery system, we focused on using βCD to optimize conditions for enzyme activity.

*At*PIPK1 had previously been shown to have a much greater preference for PtdIns4P than for PtdIns3P or PtdIns5P as the substrate (4). To determine if the addition of βCD to the lipid kinase assays altered the substrate specificity of *At*PIPK1, the activity of the recombinant enzyme was compared using PtdIns4P, PtdIns3P, and PtdIns5P prepared in 5 mM βCD. The specific activity of the lipid kinase with PtdIns4P was 22 times greater than that of the enzyme with PtdIns3P (data not shown). The low level of activity when PtdIns3P was used as the substrate confirmed previous results (4). There was no significant difference in lipid kinase specific activity when PtdIns3P was prepared by sonication or in 5 mM βCD, and there was no detectable activity when PtdIns5P was used as the substrate with either method under our assay conditions (data not shown). These results indicate that PtdIns4P is the preferred substrate for *At*PIPK1 and that βCD does not affect the substrate specificity of the enzyme.

To determine the optimal concentration of substrate, we altered the concentration of substrate, keeping the ratio of substrate to cyclodextrin constant. At 5 mM βCD, the PtdIns4P-to-βCD ratio was 1:40 (w/w); therefore, for each concentration of PtdIns4P, the molar ratio of PtdIns4P to βCD was kept at 1:40 (w/w). The lipid kinase activity increased sharply from 0 to 125 μM PtdIns4P but did not increase further at 250 μM PtdIns4P (**Fig. 2**). By keeping the concentration of βCD constant at 5 mM and changing the concentration of lipid, we were able to determine that the optimal PtdIns4P concentration is between 125 and 250 μM (**Fig. 3**). The K_m and V_{max} values for PtdIns4P were calculated using two different concentrations of βCD (Fig. 3), Triton X-100, and deoxycholate (data not shown). The calculations indicate a K_m of 69 μM and a V_{max} of 600 pmol PtdIns(4,5)P_2/mg·min at 5 mM

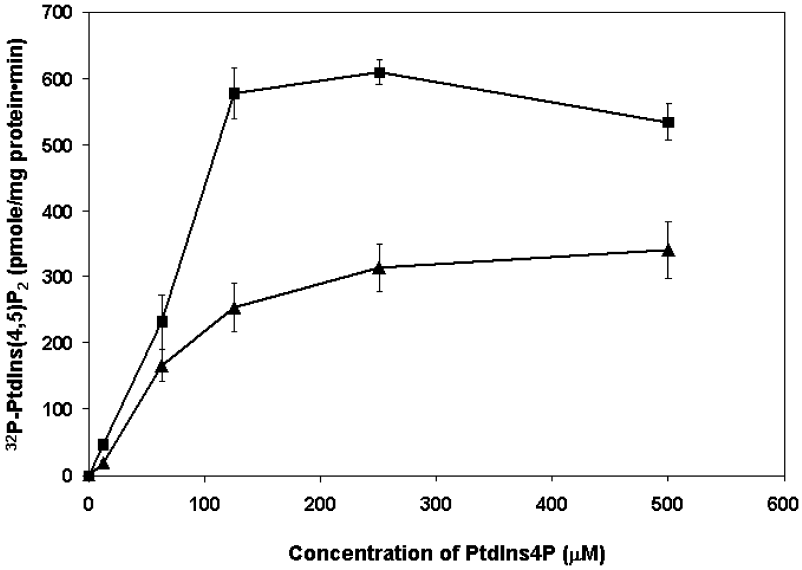

Fig. 3. Purified recombinant GST-*At*PIPK1 (10 μg) was assayed using increasing concentrations of PtdIns4P and a constant concentration of 5 mM βCD (black squares) or 30 mM βCD (black triangles). The results are reported as averages of two independent experiments.

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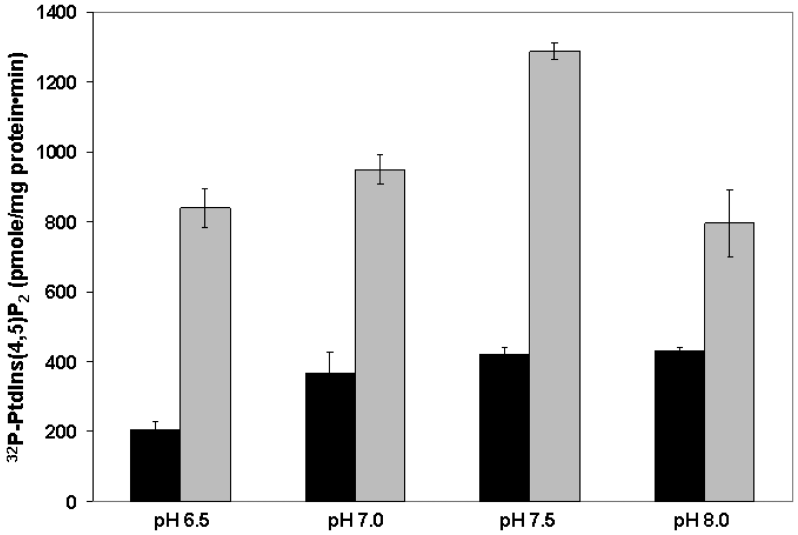

Fig. 4. Purified recombinant GST-*At*PIPK1 (10 μg) was assayed with 125 μM PtdIns4P prepared by sonication (black bars) or in 5 mM βCD (gray bars) at various pH values. Data are averages of two independent experiments.

βCD. At 30 mM βCD, the K_m did not change significantly but the V_{max} was decreased to 340 pmol PtdIns(4,5)P_2/mg·min. The solubilization of PtdIns4P in the detergents Triton X-100 and deoxycholate did not significantly change the K_m from that of 5 mM βCD, but the V_{max} was decreased to 100 and 79 pmol PtdIns(4,5)P_2/mg·min, respectively (data not shown). These results are consistent with the idea presented by Harper, Easton, and Lincoln (16) that cyclodextrins can be used as a reservoir of substrate and to facilitate substrate delivery for enzymes. However, at 30 mM βCD, cyclodextrins may be complexing with the inositol head group and forming aggregates (14). If aggregates formed at the higher concentrations and made the head group less accessible for modification, this would contribute to the reduction in V_{max} of GST-*At*PIPK1 with 30 mM βCD. Because a lipid-to-cyclodextrin molar ratio of 1:40 (w/w) and a substrate concentration of 125 μM were optimal for *At*PIPK1, 5 mM βCD and 125 μM PtdIns4P were used in subsequent experiments.

To determine if the optimum pH was altered with the addition of cyclodextrins, lipid kinase assays were performed at pH 6.5, 7.0, 7.5, and 8.0 with lipid substrate prepared in 5 mM βCD or sonicated. When cyclodextrin was used for substrate delivery, the specific activity increased up to pH 7.5 and then decreased at higher pH (**Fig. 4**). The decrease in specific activity at higher pH may reflect increased aggregation of βCD-PtdIns4P complexes. The decrease in specific activity was not observed using sonicated PtdIns4P.

A fluorescent PtdIns4P analog, NBD-PtdIns4P, was used to monitor the relative efficiency of substrate solubilization. Lipids solubilized by sonication in buffer, with Triton X-100, or with βCD were removed after a 10-min incubation on ice. The lipids in solution and the lipids that remained adhering to the glass were extracted and quantified. The results (**Table 1**) indicate that each method solubilized similar amounts of lipid. Therefore, the increase in enzyme activity was not attributable to an increase in solubilized substrate with cyclodextrin but rather to the presentation of the substrate to the enzyme.

In the presence of cyclodextrin, fluorescent lipids bound to GST-*At*PIPK1 could be readily detected using fluorescence microscopy (data not shown). The recovery of fluorescent lipids from glutathione-Sepharose beads was quantified after TLC separation. NBD-PtdIns4P binds more effectively to the purified recombinant protein when added with βCD (**Table 2**). Four percent of the NBD-PtdIns4P added was recovered from the *At*PIPK1 beads after an aqueous wash. There was no detectable lipid binding to glutathione-Sepharose beads and very little binding to purified GST. Because the data suggested that cyclodextrins delivered substrate more effectively to the lipid kinase, we investigated whether the product of the reaction, PtdIns(4,5)P_2, also might be trapped in the cyclodextrin. When ATP was added to the mixture of βCD plus NBD-PtdIns4P and GST-*At*PIPK1, NBD-PtdIns(4,5)P_2 was recovered even after washing the beads with buffer (Table 2). The fact that product could be recovered from the beads suggested that cyclodextrins could be used in a method for high-throughput analysis of lipid kinases that would not require extensive organic extractions.

TABLE 1. Comparison of lipid solubilization methods using NBD-PtdIns4P

Method	Total Lipid	Solubilized Lipid	Percent Solubilized
Sonication	2,868 ± 46.3	2,248 ± 65.1	78.4 ± 1.2
Sonication with Triton X-100	3,681 ± 48.2	3,009 ± 32.3	81.7 ± 1.3
Incubation with β-CD	3,240 ± 38.8	2,590 ± 28.1	79.9 ± 0.8

βCD, β-cyclodextrin. Solubilization of D(+)-*sn*-1-*O*-[1-[6′-[6-[(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]hexanoyl]amino]-hexanoyl]-2-*O*-hexadecanoylglyceryl D-*myo*-phosphatidylinositol-4-phosphate (NBD-PtdIns4P) was quantified by extracting the solubilized and nonsolubilized lipids from each method of lipid preparation. Extracted lipids were analyzed by measuring the fluorescence in a spectrofluorometer with an excitation wavelength of 470 nm and an emission wavelength of 530 nm. The values shown are percentages of the fluorescent lipid solubilized relative to the total amount of fluorescent lipid recovered from the supernatant (solubilized) plus the residual (nonsolubilized) lipid adhering to the tube. Plus-minus values indicate the SD of four values from two independent experiments.

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TABLE 2. Cyclodextrin enhances the recovery of NBD-PtdIns4P and NBD-PtdIns(4,5)P_2 from GST-*At*PIPK1 beads

Sample	Recovered Fluorescence	
	NBD-PtdIns4P	NBD-PtdIns(4,5)P_2
Glutathione-Sepharose beads	1	1
Glutathione-Sepharose beads + βCD	2	2
Purified GST	3	1
Purified GST + βCD	4	1
Purified GST-*At*PIPK1	16	1
Purified GST-*At*PIPK1 + βCD	78	2
Purified GST-*At*PIPK1 + ATP	12	12
Purified GST-*At*PIPK1 + βCD + ATP	73	47

Fluorescence was quantified by extracting the lipids from beads after a 1 h incubation with the fluorescent lipid preparations. Extracted lipids were separated by TLC. The fluorescent lipids were quantified by eluting the lipids from the silica matrix in the regions where PtdIns4P and phosphatidylinositol-(4,5)-bisphosphate PtdIns(4,5)P_2 were present and measuring the fluorescence in a spectrofluorometer with an excitation wavelength of 470 nm and an emission wavelength of 530 nm. Four percent of the NBD-PtdIns4P added was recovered from glutathione-*S*-transferase-*Arabidopsis thaliana* phosphatidylinositol phosphate kinase 1 (GST-*At*PIPK1) with βCD.

After characterizing the effects of cyclodextrins on the activity of purified recombinant PtdInsP kinase, we examined the effect of cyclodextrins on the membrane-associated lipid kinase activity found in *A. thaliana* membrane fractions. *A. thaliana* membranes have endogenous PtdInsP kinases as well as PtdIns4P; therefore, PtdInsP kinase activity was assayed in the presence and absence of exogenous substrate. The results (**Fig. 5**) show that even when excess substrate was added, cyclodextrin decreased the specific activity of the membrane-associated PtdInsP kinase relative to adding substrate as Triton micelles or sonicated vesicles. αCD had the least effect on the specific activity, and γCD had the greatest effect. The decrease in specific activity when cyclodextrin is added to the membranes is the opposite of the effect when the purified recombinant protein is used, suggesting that cyclodextrin either preferentially binds to other lipids so that the cyclodextrin-PtdIns4P concentration is less than anticipated (i.e., there is not as much substrate available) or that the cyclodextrin removes a factor from the membranes that normally enhances PtdInsP kinase activity. Cyclodextrins are often added to membranes or cells to remove cholesterol (17); however, they will also bind to other lipids and can effectively remove phospholipids from membranes (13).

CONCLUSION

The results of this study indicate that cyclodextrins are useful tools for delivering the negatively charged phospholipid, PtdIns4P, to the recombinant PtdInsP kinase. βCD was the most effective cyclodextrin for delivering PtdIns4P to purified, recombinant GST-*At*PIPK1. Using βCD did not alter the substrate specificity of *At*PIPK1 and did not alter the K_m compared with assays using Triton X-100 or deoxycholate. We hypothesize that the cyclodextrin takes up the PtdInsP substrate and facilitates its delivery. Based on work by Fauvelle et al. (14) and Anderson et al. (18), we propose that the fatty acid from PtdIns4P enters the cyclodextrin core and allows the enzyme access to the head group for phosphorylation. The affinity of cyclodextrin for lipids in general may explain, in part, the differences in the activities of recombinant GST-*At*PIPK1 and lipid kinases present in *A. thaliana* microsomes. Based on the fact that cyclodextrins are used to deplete cholesterol from membranes, we hypothesize that when membranes are used, the lipid substrate prepared in cyclodextrin is competitively displaced by other lipids in the membrane fractions.

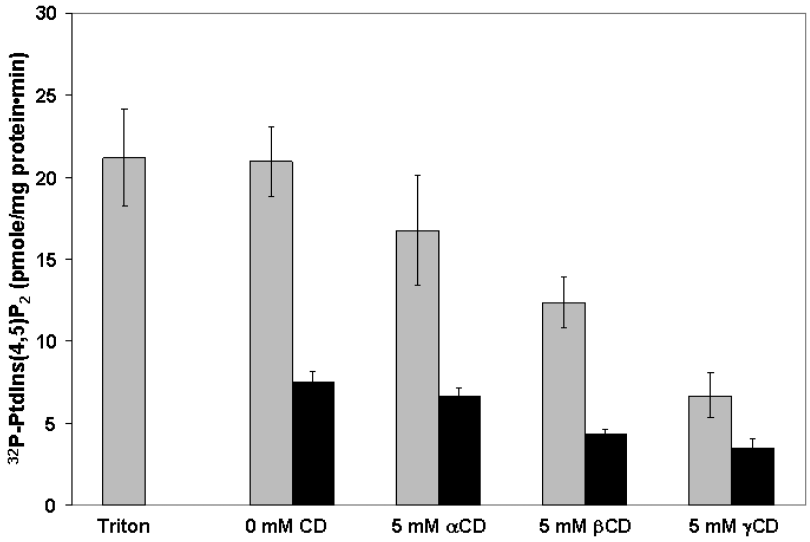

Fig. 5. The endogenous PtdInsP kinase activity of 10 μg of microsomal proteins was assayed in the presence of endogenous substrate or with 125 μM exogenous PtdIns4P. The activity with endogenous substrate (black bars) was measured in the presence of buffer or 5 mM α-, β-, or γ-CD as indicated. The exogenous substrate (gray bars) was prepared by sonication in Triton or buffer or by incubation in 5 mM α-, β-, or γ-CD as indicated. Data are averages of two independent experiments.

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When added to purified recombinant enzyme, βCD enhances lipid kinase activity. An additional advantage of this protocol is that the product could be recovered from enzyme bound to magnetic beads after a mild aqueous wash, which not only decreases the use of toxic chemicals but also can be easily modified for automated pharmaceutical screening of lipid kinases.

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