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The Stereoselective Recognition of Substrates by Phosphoinositide Kinases

STUDIES USING SYNTHETIC STEREOISOMERS OF DIPALMITOYL PHOSPHATIDYLINOSITOL*

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Soluble phosphatidylinositol (PtdIns) 4- and 3-kinase activities were partially purified and characterized from human placental extracts. The placental PtdIns 4-kinase (type 3) has a K_m for ATP of 460 μM and is kinetically different to a partially purified human erythrocyte, membrane-bound, PtdIns 4-kinase (type 2). These three inositol lipid kinases were then used to compare their substrate specificities against the four synthetic stereoisomers of dipalmitoyl PtdIns. Only the placental 4-kinase was influenced by the chirality of the glycerol moiety of PtdIns. However, neither of the 4-kinases was able to phosphorylate L-PtdIns and, therefore, these kinases have an absolute requirement for the inositol ring to be linked to the glyceryl backbone of the lipid through the D-1 position. Phosphoinositide 3-kinase, on the other hand, was found to phosphorylate both D- and L-PtdIns. While the 3-kinase phosphorylated exclusively the D-3 position of D-PtdIns, further analyses demonstrated that the same enzyme phosphorylated two sites on L-PtdIns, namely the D-6 and D-5 positions of the inositol ring. Some implications of these findings are discussed.

The generation of second messengers from the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P_2)[1] by phosphoinositidase C has been implicated in the mediation of cellular responses to a variety of neurotransmitters, hormones, and growth factors (1, 2). The first step in the synthesis of PtdIns(4,5)P_2 from phosphatidylinositol (PtdIns) is catalyzed by PtdIns 4-kinase of which several isozymes are known to exist. One species which has been extensively characterized is membrane-bound, requires detergent for solubilization, and has a subunit molecular mass of around 55 kDa (3–6). This PtdIns 4-kinase has a relatively low K_m for ATP (20–60 μM) and is sensitive to inhibition by adenosine (K_i, 20–50 μM). Another subtype of PtdIns 4-kinase can be readily distinguished from the former as it has a much higher K_m for ATP (>250 μM) and is relatively resistant to inhibition by adenosine (3, 7).

The recent discovery of a PtdIns 3-kinase (8) has led to the emergence of a novel inositol phospholipid pathway with an, as yet, unknown functional significance (9). Although PtdIns has routinely been utilized as substrate for *in vitro* detection of 3-kinase activity, some confusion currently exists over the actual *in vivo* metabolism of the newly described 3-phosphorylated inositol phospholipids. For instance, in activated neutrophils the substrate for the 3-kinase appears to be PtdIns(4,5)P_2 (10), whereas in thrombin-stimulated platelets (11) and platelet-derived growth factor-stimulated 3T3 fibroblasts (12) it has been concluded to be solely PtdIns. It remains to be seen whether these apparent discrepancies can be accounted for by the existance of multiple inositol lipid 3-kinases with specific substrate preferences and tissue distributions. Until this time phosphoinositide 3-kinase would appear a more appropriate term for describing this activity.

PtdIns analogues possess two asymmetric centers and all naturally occurring analogues, whether extracted from mammalian or plant tissues, contain a *myo*-inositol (Ins) system esterified at the D-1 position and a glycerol moiety acylated at the *sn*-1- and 2-positions, although the fatty acid composition is variable (13). Whether this stereochemical arrangement is crucial for these phospholipids to be accepted as substrates is a question of fundamental importance for furthering our understanding of intracellular signaling processes and also as a basis for rational substrate-based inhibitor design. The recent synthesis of the four stereoisomers of PtdIns (14) has provided an opportunity to study the stereochemical requirements of enzymes which utilize PtdIns as substrate. We have therefore compared the rates of phosphorylation of these four synthetic dipalmitoyl PtdIns stereoisomers (see Fig. 4) using three different partially purified inositol lipid kinases. The results show that phosphoinositide 3-kinase and two PtdIns 4-kinases have distinct stereochemical requirements with respect to their phospholipid substrates.

MATERIALS AND METHODS AND RESULTS[2]

Substrate Specificity of Three Different PtdIns Kinases—Fig. 4 shows the structures of the four synthetic dipalmitoyl

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[1] The abbreviations used are: PtdIns(4,5)P_2, phosphatidylinositol 4,5-bisphosphate; PtdIns, phosphatidylinositol; PtdIns3P, phosphatidylinositol 3-phosphate; PtdIns4P, phosphatidylinositol 4-phosphate; GroPIns3P, glycerophosphoinositol 3-phosphate; GroPIns4P, glycerophosphoinositol 4-phosphate; Ins, *myo*-inositol; InsP, $InsP_2$, and $InsP_3$, inositol mono-, bis-, and trisphosphates, with positional isomerism of phosphoesters in parentheses; TLC, thin-layer chromatography; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; DTT, dithiothreitol; HPLC, high-performance liquid chromatography; PMSF, phenylmethylsulfonyl fluoride.

[2] Portions of this paper (including "Materials and Methods," part of "Results," Figs. 1–3 and Tables 1–3) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are included in the microfilm edition of the Journal that is available from Waverly Press.

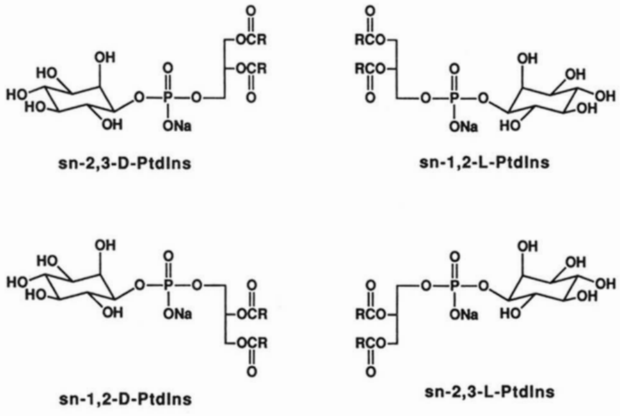

FIG. 4. **The four synthetic stereoisomers of dipalmitoyl PtdIns.**

TABLE 4

A comparison of the four synthetic stereoisomers of dipalmitoyl PtdIns as substrates for three different types of PtdIns kinase

Freshly isolated enzymes were simultaneously assayed for activity using 200 μg of each inositol lipid stereoisomer. Reactions were carried out at 30 °C as described under "Materials and Methods" in the miniprint section. The reaction rate for each partially purified kinase was quantified by measuring the ^{32}P radioactivity contained within the organic phase following phospholipid extraction. The values in parentheses represent the relative percentage rate for each kinase when compared with the synthetic analogue of natural *sn*-1,2-D-PtdIns.

PtdIns	Erythrocyte PtdIns 4-kinase	Placental PtdIns 4-kinase	Placental PtdIns 3-kinase
	pmol PtdInsP formed/min/mg protein		
1,2-D-PtdIns	10,418 (100%)	767 (100%)	79 (100%)
2,3-D-PtdIns	9,020 (87%)	280 (37%)	85 (108%)
1,2-L-PtdIns	330 (3%)	18 (2%)	31 (39%)
2,3-L-PtdIns	232 (2%)	4 (0.5%)	27 (35%)

stereoisomers of PtdIns. The optical purity of the inositol moiety in the PtdIns analogues has previously been determined to be at least 99% (14). These phospholipid isomers were tested as potential substrates for the following three kinases: the erythrocyte, membrane-bound, PtdIns 4-kinase; the placental, soluble, PtdIns 4-kinase; and the placental, soluble, phosphoinositide 3-kinase. The aim was not to compare the absolute rates between enzymes but to examine the differences between their rates of phosphorylation of D- *versus* L-PtdIns. Results shown in Table 4 demonstrate that the erythrocyte PtdIns 4-kinase phosphorylated both *sn*-1,2-D-PtdIns (the synthetic analogue of natural PtdIns) and *sn*-2,3-D-PtdIns at similar rates. The chirality of the glycerol moiety did not, therefore, affect substrate recognition. In contrast, the two synthetic PtdIns counterparts which have the L-stereochemistry with respect to the inositol ring were found to be poor substrates for the erythrocyte 4-kinase. The findings with the placental 4-kinase were similar to those of the erythrocyte isozyme in that neither of the L-PtdIns isomers were effective substrates. The placental 4-kinase did, however, show more of a dependence on the stereochemistry of the glycerol moiety since *sn*-2,3-D-PtdIns was phosphorylated at approximately a third the rate of *sn*-1,2-D-PtdIns.

The data obtained by comparing the four synthetic PtdIns analogues as substrates for the placental 3-kinase was very different from that for the two 4-kinases (Table 4). Although the chirality of the glycerol moiety again made little difference for substrate recognition, the 3-kinase, unlike both 4-kinases, phosphorylated the two L-PtdIns stereoisomers at a significant rate which was around one third of that obtained for the corresponding D-PtdIns stereoisomers. As this somewhat surprising result was initially obtained using a partially purified 3-kinase preparation from human placenta, it was crucial to rule out the possibility that a contaminating lipid kinase was responsible for the data. To this end, a highly purified PtdIns 3-kinase isolated from bovine brain (see Ref. 25) was kindly provided by the Ludwig Institute of Cancer Research, London. This enzyme preparation was used as the source in the successful partial cloning of the 3-kinase recently published (26). Moreover, this brain PtdIns 3-kinase phosphorylated L-PtdIns at approximately 50% the rate at which it phosphorylated D-PtdIns (data not shown), and the products from both reactions were found to run with authentic D-PtdInsP upon TLC analysis (Fig. 5). All further experiments to characterize the products of the 3-kinase incubations were carried out with the highly purified enzyme from bovine brain.

Analysis of the Sites Phosphorylated by PtdIns 3-Kinase—To determine the sites phosphorylated, purified brain PtdIns 3-kinase was incubated with D- and L-PtdIns. Although the results reported here have been obtained through the use of *sn*-1,2-L-PtdIns, essentially identical results have been obtained for the *sn*-2,3-L-PtdIns diastereoisomer.

The synthetic lipid substrates were phosphorylated using [γ-^{32}P]ATP as the phosphate donor. The ^{32}P-containing lipids were then deacylated and deglycerated, generating ^{32}P-labeled inositol bisphosphates ($InsP_2$s). To characterize these samples, [^3H]$InsP_2$ standards were added and an aliquot analyzed by HPLC. The remainder of the sample was dephosphorylated by treatment with ammonia to obtain inositol monophosphates (InsPs) which were subsequently spiked with [^3H]InsP standards and subjected to HPLC. Fig. 6*A* shows the HPLC elution trace obtained for $InsP_2$s derived from the phosphorylation of *sn*-1,2-D-PtdIns. A single peak of ^{32}P-labeled material cochromatographed with the [^3H]Ins(1,3)P_2 standard. Fig. 6*B* shows the HPLC profile for the monophosphates derived from the dephosphorylation of this bisphosphate sample. The ^{32}P-labeled compound obtained cochromatographed with [^3H]Ins(1)P/Ins(3)P. As this phospholipid was synthesized by linking the inositol ring to the glyceryl backbone via the D-1 position (14), D-PtdIns was therefore phosphorylated exclu-

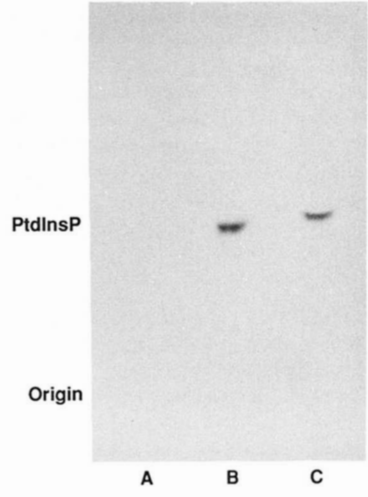

FIG. 5. **Phosphoinositide 3-kinase phosphorylates both D- and L-PtdIns.** A highly purified bovine brain 3-kinase was incubated for 20 min at 30 °C with 100 μM [^{32}P]ATP (5 μCi/nmol) and either enzyme alone (*lane A*), 200 μg/ml *sn*-1,2-D-PtdIns (*lane B*), or 200 μg/ml *sn*-2,3-L-PtdIns (*lane C*). ^{32}P-containing lipids were then extracted, run on TLC, and visualized by autoradiography as described under "Materials and Methods" in the miniprint section. Authentic D-PtdIns4P (Sigma) was run in a parallel lane and visualized with iodine vapor.

sively on the D-3 position of the inositol ring.

Fig. 7*A* shows the corresponding HPLC data for [^{32}P]InsP_2 samples derived from the product of L-PtdIns phosphorylation by PtdIns 3-kinase. The majority of the ^{32}P radioactivity was found to cochromatograph with [^{3}H]Ins(1,4)P_2, however a small shoulder to this peak indicated the presence of at least one other ^{32}P-labeled InsP_2. When the major InsP_2 in this preparation was dephosphorylated it yielded a ^{32}P-labeled InsP which cochromatographed with the [^{3}H]Ins(4)P/Ins(6)P standard (Fig. 7*B*). As the inositol ring in the intact phospholipid was linked through the D-3 position to the glyceryl backbone, and its phosphorylation product contained a phosphate in either the D-4 or D-6 position, the only possible bisphosphates that could be obtained by the strategies outlined above are Ins(3,4)P_2 and Ins(3,6)P_2. The former can be eliminated since the unknown bisphosphate did not cochromatograph with an Ins(3,4)P_2 standard (see Fig. 7*A*) and the latter is the enantiomer of Ins(1,4)P_2, therefore explaining its cochromatography with the [^{3}H]Ins(1,4)P_2 standard. This identifies the D-6 (L-4) position of the inositol ring as the major site phosphorylated on L-PtdIns by PtdIns 3-kinase. Fig. 7*C* demonstrates an improved InsP_2 separation. Using this separation the major ^{32}P-containing bisphosphate cochromatographs precisely with the [^{3}H]Ins(1,4)P_2 standard. In addition, the shoulder seen in Fig. 7*A* has now been resolved and does not cochromatograph with any of the standards. Fractions containing this unknown peak were pooled and subjected to ammonia dephosphorylation. The resulting [^{32}P]InsP did not cochromatograph precisely with any of the included [^{3}H]InsP standards and by elimination must be Ins(5)P (Fig. 7*D*). The unknown bisphosphate must, therefore, have been Ins(3,5)P_2. Thus the D-5 position of L-PtdIns represents a second phosphorylation site for PtdIns 3-kinase. From the data depicted in Fig. 7*C*, the calculated relative amounts of radioactivity show that for L-PtdIns, PtdIns 3-kinase phosphorylated the D-6 position ~67% and the D-5 position ~33%.

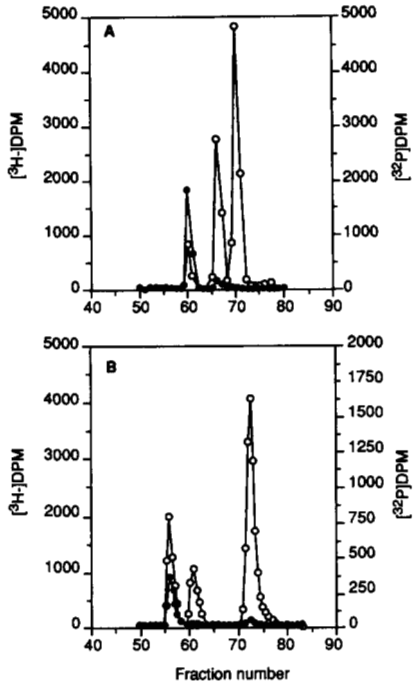

FIG. 6. **HPLC data for the separation of inositol bis- and monophosphates derived from D-PtdIns phosphorylated by phosphoinositide 3-kinase.** *A*, ^{32}P-labeled InsP_2s (*closed circles*) were derived from synthetic *sn*-1,2-D-PtdIns phosphorylated by PtdIns 3-kinase and separated by HPLC as described under "Materials and Methods" in the miniprint section. [^{3}H]InsP_2 standards (*open circles*) elute in the order Ins(1,3)P_2, Ins(1,4)P_2, Ins(3,4)P_2. *B*, ^{32}P-labeled InsPs were derived from the above InsP_2 sample and separated by HPLC as described under "Materials and Methods" (miniprint). [^{3}H]InsP standards (*open circles*) eluted in the order Ins(1)P/Ins(3)P, Ins(2)P, Ins(4)P/Ins(6)P.

DISCUSSION

We have used three different inositol lipid kinases to compare their substrate specificities against the four synthetic

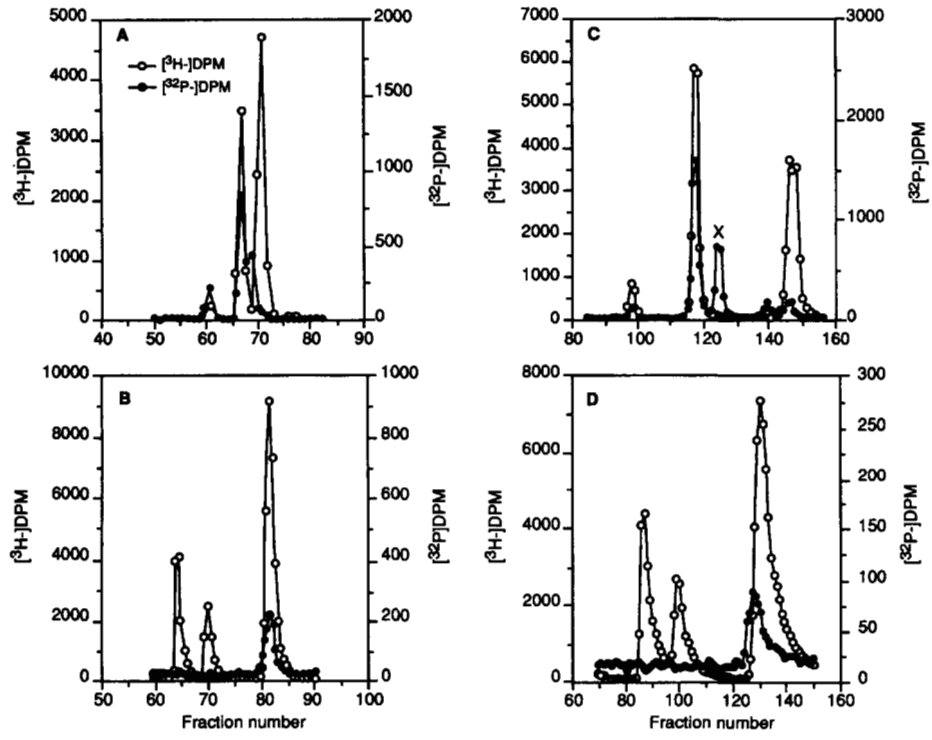

FIG. 7. **HPLC data for the separation of inositol bis- and monophosphates derived from L-PtdIns phosphorylated by phosphoinositide 3-kinase.** *A*, ^{32}P-labeled InsP_2s (*closed circles*) were derived from synthetic *sn*-1,2-L-PtdIns phosphorylated by phosphoinositide 3-kinase and separated by HPLC. [^{3}H]InsP_2 standards (*open circles*) elute in the same order as for Fig. 6*A*. *B*, ^{32}P-labeled InsPs were derived from the above InsP_2 samples and separated by HPLC. [^{3}H]InsP (*open circles*) eluted in the same order as Fig. 6*B*. *C*, in an attempt to resolve the shoulder seen in *A* the HPLC separation of the ^{32}P-labeled InsP_2s derived from L-PtdIns was optimized as outlined under "Materials and Methods" in the miniprint section. [^{3}H]InsP_2 standards eluted in exactly the same order as described in the legend to Fig. 6*B*. *D*, fractions containing the peak marked *X* were pooled, desalted, and the sample subjected to ammonia dephosphorylation as described under "Materials and Methods" in the miniprint section. ^{32}P-Labeled InsPs (*closed circles*) derived from this sample were analyzed by HPLC. [^{3}H]InsPs (*open circles*) eluted in the same order as in Fig. 6*B*.

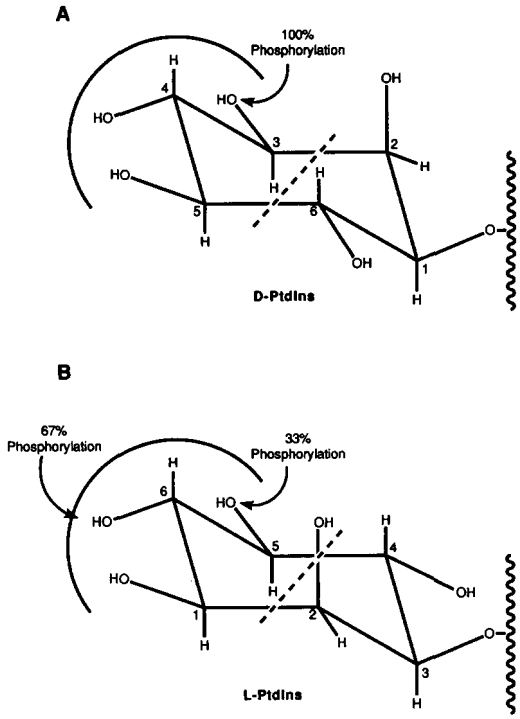

FIG. 8. **Relative orientation of the inositol head groups in both D- and L-PtdIns.** Here we show diagramatically the relative orientations of the inositol head group in (*A*) D-PtdIns and (*B*) L-PtdIns. Bisecting the inositol ring, as illustrated by the *dotted line*, shows the portion of the ring containing C3-C4-C5 in D-PtdIns and C5-C6-C1 in L-PtdIns to be in an identical configuration, as indicated within the two semicircles. The portions of the inositol rings to the *right* of the *dotted line* are obviously very different, particularly with respect to the relative positions of the axial 2-hydroxyl groups.

stereoisomers of dipalmitoyl PtdIns. The partially purified human erythrocyte, membrane-bound, PtdIns 4-kinase appears kinetically indistinguishable from a widely reported isozyme in that it has a relatively low K_m for ATP (49 μM) and is inhibited by adenosine (3–6). In contrast, the PtdIns 4-kinase isolated from human placental extracts has a much higher K_m for ATP (460 μM) and is closer in its properties to activities which have been characterized from bovine brain (3) and bovine uterus (7). The adenosine-resistant, placental phosphoinositide 3-kinase, on the other hand, with a K_m for ATP of 47 μM is similar to activities which have been recently purified to apparent homogeneity (25, 27) and subsequently partially cloned (26, 28, 29).

The finding that phosphoinositide 3-kinase (both human placental and bovine brain), but neither of the 4-kinases, phosphorylated L-PtdIns is a novel finding and of obvious interest. Before discussing this observation further we must first consider the potential limitations of having phospholipid substrates contaminated with less than or equal to 1% the wrong enantiomer. More specifically, could the rate of phosphorylation of L-PtdIns by 3-kinase be accounted for by D-PtdIns contamination? Under the assay conditions used for 3-kinase, its K_m for D-PtdIns is 48 μM (25). As we used 240 μM of each PtdIns isomer in our assays, this means that the enzyme is working at 83% of V_{max} for D-PtdIns. If we were to assume the worst scenario that the L-isomer contains 1% the D-isomer, then the enzyme's rate would be 4.8% of the V_{max} for D-PtdIns. As the 3-kinase actually phosphorylated L-PtdIns at 50% the rate at which it phosphorylated D-PtdIns, then the vast majority of product formed must have been L-PtdInsP. When similar calculations are performed for the erythrocyte 4-kinase which has a K_m of approximately 100 μM under the assay conditions used, then a 1% contamination of L-PtdIns with D-PtdIns would give a rate of around 2% the V_{max} for D-PtdIns. This is almost exactly the proportion of the activity of D-PtdIns that was found using L-PtdIns as the substrate for this PtdIns kinase. Thus we cannot eliminate the possibility that the phosphorylation of the L-PtdIns preparation by the 4-kinase was due to the presence of less than or equal 1% D-PtdIns. However, we can be certain from the arguments presented above that the majority of the products of the phosphorylation of the L-PtdIns preparation by the 3-kinase must be L-PtdInsPs. One obvious consequence of this observation is that L-PtdIns can be used as a specific substrate for 3-kinase activity.

Analyses of the phosphorylation sites indicated that phosphoinositide 3-kinase phosphorylates the D-5 and D-6 positions of the inositol ring in L-PtdIns. In order to evaluate this finding further, the relative head group orientations for D- and L-PtdIns which are depicted diagramatically in Fig. 8 should be considered. A number of features with respect to the configuration of the inositol ring for the two lipids are illustrated: if the inositol ring is bisected at the dotted line, the orientations of the hydroxyl groups linked to C3-C4-C5 in D-PtdIns, and those linked to C5-C6-C1 in L-PtdIns are identical; in contrast, there is very little similarity between portions of the inositol rings to the right of the dotted lines. We have also demonstrated that the stereochemistry of the glyceryl moiety has little or no discernible effect on either phosphorylation rates for D- or L-PtdIns, or on the sites phosphorylated by the 3-kinase. It seems likely then that there is a structural feature contained within the hydroxyl group orientation along the C6-C1-C2 axis in D-PtdIns that directs the specificity of the enzyme and this feature is not in the required configuration in L-PtdIns. The most obvious candidate is the axial 2-hydroxyl group in D-PtdIns, whereas there is an equatorial hydroxyl in the comparable 4-position in L-PtdIns. Finally, phosphoinositide 3-kinase can phosphorylate PtdIns4P and PtdIns(4,5)P_2 as well as PtdIns *in vitro*, and recent data suggest that PtdIns(4,5)P_2 may be the important substrate in agonist-stimulated cells (9, 10). The data described here cannot, unfortunately, be extrapolated to make a definitive comment on this possibility.

As mentioned previously, when these synthetic lipids were tested as substrates for two subtypes of PtdIns 4-kinase, both enzymes had an absolute requirement for the inositol ring to be linked to the glyceryl backbone of the lipid through the D-1 position. It seems that these 4-kinases also recognize some structural feature contained within the hydroxyl group orientations along the C6-C1-C2 axis in D-PtdIns that is not found in L-PtdIns. Unlike the phosphoinositide 3-kinase, however, this structural feature appears to be an absolute requirement for catalysis by the PtdIns 4-kinases. Of additional interest was the observation that the placental soluble PtdIns 4-kinase, but not the erythrocyte membrane-bound isozyme, had a pronounced preference for the *sn*-1,2- configuration of the glyceryl moiety.

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Supplemental material to: **The stereoselective recognition of substrates by phosphoinositide kinases: Studies using synthetic stereoisomers of dipalmitoyl phosphatidylinositol.**

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MATERIALS AND METHODS

Materials: *myo*-[2-^{3}H(N)]-inositol (15.4 Ci/mmol), [2-^{3}H(N)]-inositol 4-phosphate (10 Ci/mmol), and [2-^{3}H(N)]-inositol 1,3,4-trisphosphate (30 Ci/mmol) were purchased from NEN Research Products; [γ-^{32}P]-ATP (3,000Ci/mmol) was bought from Amersham International plc; Triton X-100 and octyl-β-D-glucoside were from Calbiochem; hydroxylapatite from Bio-Rad Laboratories Ltd.; DEAE-cellulose and phosphocellulose were obtained from Whatman; leupeptin, pepstatin A, antipain, PMSF, PtdIns, and PtdInsP were purchased from Sigma; DEAE sephacel was bought from Pharmacia. All other laboratory reagents were analytical-grade.

Isolation of human placental soluble PtdIns kinases: Two different soluble PtdIns kinase activities were isolated from human placental tissue as follows. A fresh placenta was obtained from a local hospital and processed within 2 hr of delivery. Tissue was cut away from the supporting membrane, cubed, and washed extensively in 10mM Tris-HCl (pH 7.4, 4°C), 150mM NaCl. This tissue was subsequently homogenized in a Waring blender, 2 x 15 sec, in 2 volumes of a buffer containing 20mM Tris-HCl (pH 7.6, 4°C), 0.25M sucrose, 5mM EDTA, 5mM EGTA, 1mM DTT, 150μM PMSF, and 2μg/ml each of leupeptin, pepstatin A, and antipain. The homogenate was centrifuged at 27,000 x g for 60min and the resultant supernatant filtered through glass wool. This supernatant was batch adsorbed onto 300ml of DEAE cellulose which was equilibrated in 20mM Tris-HCl (pH 7.5, 4°C), 2mM EDTA, 1mM DTT (buffer A). Following gentle stirring for 3hr at 4°C, the slurry was poured into a 15cm Buchner funnel and washed under mild suction with up to 15 bed volumes of buffer A. The resin was then poured into a 5cm diameter column and eluted with a 1.5-litre linear NaCl gradient (0-0.5M) in buffer A. Fractions were collected, assayed for PtdIns kinase, and the fractions which made up two different peaks of activity were pooled as depicted in Fig. 1. The two pools of activity were then treated identically as follows. They were first dialysed against 20mM Tris-HCl (pH7.5,4°C), 1mM DTT, 1mM EDTA, plus protease inhibitors (buffer B) before loading onto a 30ml phosphocellulose column pre-equilibrated in the same buffer. The column was eluted with a linear salt gradient from 0-0.7M NaCl in a total volume of 250ml in buffer B. Active fractions were pooled and dialysed against 10mM KH_2PO_4 (pH7.4, 4°C), 1mM DTT, 10% glycerol (buffer C). The dialysed pool was filtered (0.22μm) before loading onto a hydroxylapatite HPLC column (2μm particle size, 7.5 x 50 mm, Pentax) pre-equilibrated in buffer C. Kinase activity was eluted with a 45ml linear salt gradient from B0-0.5M KH_2PO_4 at a flow rate of 0.5 ml/min. Peak fractions were then used as a source of PtdIns kinase activity.

Partial purification of human erythrocyte membrane-bound PtdIns kinase: Highly purified human erythrocyte plasma membranes were prepared as outlined by Hawkins et al (15). Membranes from 550ml of blood were stirred for 1hr in the following solubilising buffer; 20mM Tris-HCl (pH7.4, 4°C), 10% glycerol, 1mM EGTA, 1mM DTT (buffer D), containing 0.5% (v/v) Triton X-100 plus 2μg/ml each of leupeptin, pepstatin A, and antipain. Solubilised membranes were centrifuged, 100,000 x g for 30min, and the supernatant applied to a 150ml DEAE sephacel column. The column was washed with an equal volume of buffer D and fractions collected for activity determinations. The 'flow-through' activity pool was then loaded onto a 7ml hydroxylapatite column and Triton X-100 exchanged for octyl glucoside with sequential 1.5 column volume washes of the following solutions: buffer D containing 0.1% (w/v) octyl glucoside; buffer D containing 0.1% octyl glucoside plus 0.5M KH_2PO_4; buffer D containing 0.1% octyl glucoside; buffer D containing 1.2% octyl glucoside. The column was then eluted with a linear gradient of 0-0.8M KH_2PO_4 in buffer D containing 1.2% octyl glucoside. The activity fractions were pooled, dialysed, and concentrated using an Amicon ultrafiltration unit fitted with a YM30 membrane. This concentrated sample was then applied to pre-equilibrated gel filtration, Superose 12 (Pharmacia), column and eluted at a flow rate of 0.3ml/min using 20mM Tris-HCl (pH7.4, 4°C), 0.2M NaCl, 1mM EGTA, 1mM DTT, 1.5% octyl glucoside. Peak fractions of activity were then used as a source of enzyme.

PtdIns kinase assays: Two types of kinase assays were carried out which depended on the particular enzyme being assayed. The two soluble enzymes isolated from placental tissue used PtdIns sonicated in 50mM Hepes (pH7.4, 30°C) whereas the erythrocyte PtdIns 4-kinase, originally membrane-bound, utilised sonicated detergent(0.3% Triton X-100)/PtdIns mixed micelles. Unless otherwise stated, assays were conducted at 30°C for 5-10 min in a volume of 200μl using a buffer which contained 50mM Hepes (pH7.4, 30°C), 225μM PtdIns, 10mM $MgCl_2$, 1mM EGTA, 1mM DTT, 0.1M NaCl, and 50μM [^{32}P]-ATP (0.2-0.5 μCi/nmol). Incubations were linear over the range of times and dilutions used and terminated by the addition of 750μl of chloroform/methanol/concentrated HCl (40/80/1, by vol.). Two phases were obtained by adding 250μl chloroform and 250μl of a solution containing 0.1M HCl, 5mM EDTA, 0.5M NaCl. After mixing and centrifugation the upper phase was carefully aspirated and the lower phase washed twice with 600μl of a synthetic upper phase with composition, chloroform/methanol/0.1M HCl, 5mM EDTA, 0.5M NaCl (3/48/47, by vol.). Portions of this organic phase were Cerenkov counted for [^{32}P]-radioactivity. Studies demonstrated that even when using the crudest enzyme sample, greater than 70% of the [^{32}P]-radioactivity in the organic phase was attributed to [^{32}P]-PtdInsP (data not shown). The [^{32}P]-radioactivity in the organic phase was therefore routinely used as a rapid measurement of enzyme activity when assaying chromatography profiles.

Analysis of lipid products: To identify the [^{32}P]-lipid products of the kinase reaction, the organic phase was dried under vacuum and deacylated according to the method described by Hawkins et al. (16). The resulting glycerophosphoinositol phosphates (GroPtdInsP's) were subjected to HPLC on a Whatman Partisphere SAX anion-exchange column (5μm particle size; 12.5cm cartridge) eluted with a gradient of water (A) and 1.25M-$(NH_4)_2HPO_4$, pH3.8 (B) as follows: 0 min, 0% B; 5 min, 0% B; 45 min, 12% B; 60 min, 30% B; 61 min, 100% B; 65 min, 100% B; 66 min, 0% B. In some experiments the products of the kinase assay were separated on silica gel 60 F-254 TLC plates (Merck) prior to deacylation and HPLC analyses. In brief, samples were dried and resuspended in 30μl chloroform prior to applying to a TLC plate which had been pre-run in methanol/1% (g/vol) potassium oxalate (2/3, v/v) and baked for 30 min at 110°C. The TLC plate was immediately developed in chloroform/methanol/2.6M NH_4OH (9/7/2, v/v/v) and radiolabelled phospholipids detected by autoradiography on Kodak X-Omat RP film. Deglyceration of phospholipids was achieved according to the method outlined by Brown and Stewart (17).

Preparation of standards and chromatography: [^{3}H]-GroPtdIns4P and [^{3}H]-GroPtdIns3P were prepared from [^{3}H]-Ins - labelled cells as previously described (18). [^{3}H]-Ins(1,3)P_2 and [^{3}H]-Ins(3,4)P_2 were prepared from [^{3}H]-Ins(1,3,4)P_3 essentially as described in (19). In brief, rat brain cerebral cortex from one brain was dissected on ice then homogenised in 5ml of 10mM Hepes (pH7.2, 4°C), 70mM KCl, 20mM NaCl. The homogenate was diluted to 10ml with the same buffer and centrifuged at 50,000 rpm, using a 70.1Ti rotor in a Beckman L8-M ultracentrifuge, for 35 min at 4°C. The resultant supernatant was taken and used without further treatment. [^{3}H]-Ins(1,3,4)P_3 was incubated in a final volume of 200μl containing 10mM Hepes (pH7.2, 37°C), 5mM $MgCl_2$ with 20μl of the cortex supernatant for 15 min. The reaction was terminated with 250μl of ice-cold 6.5% (v/v) perchloric acid. After 15 min on ice the mixture was microfuged for 5

min at full speed and the supernatant neutralised with octylamine/freon as previously described (20).

[^{3}H]-Ins(1,4)P_2 was prepared by the action of a partially purified turkey erythrocyte inositol lipid-specific phospholipase C (PIC) on authentic [^{3}H]-PtdIns4P. The PIC was isolated as described by Morris et al (21), up to and including the Heparin-Sepharose step. Finally, a mixture of [^{3}H] -Ins1P, -Ins2P, -Ins3P was prepared exactly as described in (22).

InsP_2 isomers were separated initially using the following gradient based on A (water) and B (1.25M $(NH_4)_2HPO_4$, pH3.8 with H_3PO_4): 0 min, 0% B; 10 min, 0% B; 12 min, 10% B; 72 min, 10% B; 75 min, 100% B. The column used was a Partisphere SAX with anion guard and the flow rate was 1 ml/min. In some experiments isomers were resolved more efficiently using the following gradient: 0 min, 0% B; 10 min, 0% B; 12 min, 5% B; 72 min, 5% B; 75 min, 100% B.

InsP isomers were separated using the following gradient based on buffer A (water) and B (0.2M CH_3COONH_4, pH3.75 with CH_3COOH) at a flow rate of 1 ml/min: 0 min, 0% B; 10 min, 0% B; 12 min, 40% B; 72 min, 45% B; 75 min, 100% B. Separations were performed on a Partisphere SAX column with anion guard in the acetate form. Later separations were run isocratically at 40% B. As with all HPLC separations 0.45ml fractions were collected. Eluates containing phosphate were counted for radioactivity, after addition of 1ml of ethanol, in 4ml Optiphase HiSafe scintillant (Pharmacia). Acetate containing eluates were counted in 4ml Optiphase Hi-Safe3 only.

Ammonia dephosphorylation: [^{32}P]-InsP_2 samples derived from lipids were dried in small Reacti-vials (Pierce) and 0.4ml ammonia solution added to commence dephosphorylation. The vials were tightly capped and heated in a dry block at 110^{0}C for 12 hrs, after which time they were dried again. Each sample was redissolved in 5mM EDTA (pH7.5) and spiked with [^{3}H]-InsP standards.

Protein determinations: Proteins were determined by the method of Lowry et al (23), using bovine serum albumin (Fraction V, Sigma) as standard.

RESULTS

Characterisation of human placental PtdIns 3- and 4-kinases. DEAE-cellulose chromatography routinely resulted in two or three peaks of PtdIns kinase activity being resolved. Two pools of enzyme activity were taken for further characterisation (Fig. 1). All of the fractions which comprised the activity peak eluting at ~0.1M NaCl (i.e., pool 1) were taken in addition to a portion of the much larger activity peak which eluted at ~0.25M NaCl (i.e., pool 2). Chromatography of pool 1 on phosphocellulose afforded both a good purification step as well as a separation of two activities (Fig. 2A). The first and major peak of activity was finally resolved and concentrated on a hydroxylapatite column (Fig. 2B). When the product of this partially purified PtdIns kinase was subsequently analysed it was found to be solely PtdIns3P (Fig. 3B). Table 1 summarises the partial purification of this soluble PtdIns 3-kinase. It should be noted that when placental supernatant is assayed for PtdIns kinase activity the majority of the product formed is in the form of PtdIns4P (Fig. 3A).

When the kinase activity of pool 2 from DEAE cellulose was sequentially taken through phosphocellulose and hydroxylapatite chromatography, a highly enriched PtdIns 4-kinase activity resulted (Fig.3C). As only a fraction of the total 4-kinase activity was utilised from DEAE chromatography the extent of purification was not tabulated.

Partial purification of a human erythrocyte, membrane-bound, PtdIns 4-kinase. Table 2 shows a typical purification of a PtdIns 4-kinase derived from highly purified human erythrocyte plasma membranes. Gel filtration chromatography in the presence of 1.5% (w/v) octyl glucoside indicated that the kinase eluted very close to bovine serum albumin giving it a detergent/protein mixed micellular molecular weight of around 66kDa (data not shown). Moreover, the product of the kinase reaction was found to be exclusively PtdIns4P (data not shown).

Kinase ATP recognition sites. The erythrocyte, membrane-bound, PtdIns 4-kinase and the placental 3-kinase had similar Km's for ATP of 49 and 47μM respectively (Table 3). The placental soluble PtdIns 4-kinase on the other hand was found to have a Km for ATP approximately ten fold higher at 460μM. Table 3 also demonstrates that whereas the 3-kinase was relatively insensitive to inhibition by adenosine or the more potent adenine analogue 9-cyclohexyladenine (24), the erythrocyte 4-kinase was found to be sensitive to inhibition by these compounds.

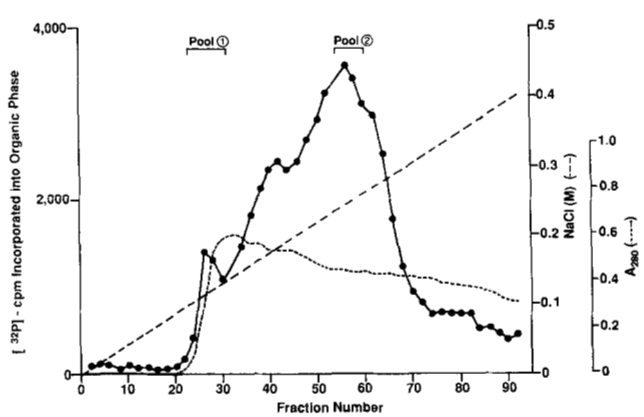

Fig 1. **DEAE-cellulose chromatography of a human placental extract.** Fractions were eluted with a linear 0-0.5M NaCl gradient and assayed for PtdIns kinase by measuring the [^{32}P]-radioactivity content (o) of the organic phase, following phospholipid extraction, as outlined in Methods. The A_{280} was determined using an on-line U.V. detector. The fractions which comprised the two pools of activity which were taken for further analysis are also shown.

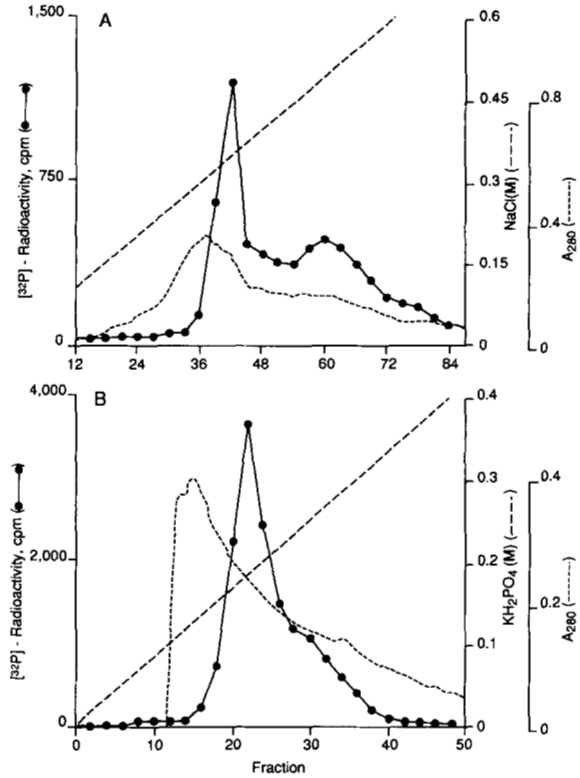

Fig 2. **Phosphocellulose and hydroxylapatite chromatography of pool 1.** Pool 1 from Figure 1 was sequentially chromatographed on phosphocellulose (A) and hydroxylapatite (B) columns eluted with NaCl and KH_2PO_4, respectively. PtdIns kinase activity was measured as in Figure 1.

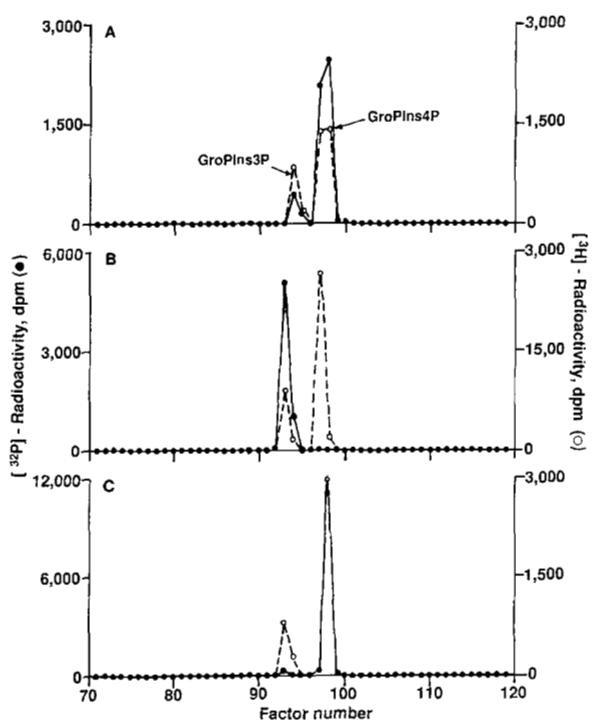

Fig 3. **Product analysis of placental PtdIns kinases.** Assays were carried out using placental extract (A) and the partially purified kinases originally derived from pools 1 (B) and 2 (C) of Figure 1. The [^{32}P]-containing lipid products were deacylated, mixed with [^{3}H]-standards and subjected to HPLC analysis as outlined in Methods.

Table 1

Partial purification of placental phosphoinositide 3-kinase.

The partial enzyme purification from one human placenta is presented. Each step was carried out as described in the Methods section. The product of the assay, [^{32}P]-PtdIns3P, was quantified by HPLC analysis following phospholipid extraction and deacylation.

STEP	PROTEIN (mg)	TOTAL ACTIVITY (pmol/min)	SPECIFIC ACTIVITY (pmol/min/mg)	PURIFICATION (fold)
Supernatant	8,665	2,808	0.32	1
DEAE Cellulose	597	820	1.4	4
Phosphocellulose	20	333	17	53
Hydroxylapatite	4.6	266	58	181

Table 2

Partial purification of the erythrocyte, membrane-bound, PtdIns 4-kinase.

The purification began with highly purified erythrocyte plasma membranes isolated from 550ml of fresh human blood. Columns were run as described in the Methods section and activity determined at 30°C using Triton X-100/PtdIns mixed micelles.

STEP	PROTEIN (mg)	TOTAL ACTIVITY (nmol/min)	SPECIFIC ACTIVITY (nmol/min/mg)	PURIFICATION (fold)
Solubilised Membranes	745	210	0.28	1
Supernatant	162	151	0.93	3
DEAE Sephacel	17	72	4.3	15
Hydroxylapatite	2.6	26	10	36
Gel Filtration	0.8	20	25	89

Table 3

Characteristics of the ATP recognition sites of three different PtdIns kinases.

The Km values for ATP were determined from Lineweaver-Burk plots in which the concentration of [γ-^{32}P]-ATP was varied. Assays were conducted as described in Methods and each value represents the average of triplicate determinations with a SEM of not greater than 10%. Inhibitor assays were conducted in the presence of 100 μM ATP.

		IC_{50} (μM)	
PtdIns Kinase	Km ATP (μM)	Adenosine	9-Cyclohexyladenine
Erythrocyte 4-kinase	49	30	5
Placental 4-kinase	460	580	43
Placental 3-kinase	47	830	120