Characterization and Purification of Membrane-associated Phosphatidylinositol-4-phosphate Kinase from Human Red Blood Cells*

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The membrane-bound form of phosphatidylinositol-4-phosphate (PtdInsP) kinase was purified 4,300-fold from human red blood cells to a specific activity of 117 nmol min⁻¹ mg⁻¹. Although this enzyme copurified with red blood cell membranes, it was solubilized by high salt extraction in the absence of detergent indicating that it is a peripheral membrane protein. The major protein seen in the most purified preparation migrated at 53,000 daltons on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The major PtdInsP kinase activity in this preparation was also coincident with this 53,000-dalton band upon renaturation of activity from SDS-PAGE. To test further whether the 53,000-dalton protein contained PtdInsP kinase activity, antibodies were prepared against the gel-purified 53,000-dalton protein. This antiserum was able to precipitate both the 53,000-dalton peptide and PtdInsP kinase activity from red blood cell membranes. The apparent size of the native enzyme in the most purified preparation was determined to be 150,000 ± 25,000 daltons by gel filtration. This PtdInsP kinase activity was at least 100-fold more active in phosphorylating PtdInsP than phosphatidylinositol monophosphate was easily separated from the red cell membrane phosphatidylinositol kinase by salt extraction. Analysis of the reaction product, phosphatidylinositol 4,5-bisphosphate, indicates that the enzyme phosphorylates phosphatidylinositol 4-phosphate specifically at the 5'-hydroxyl of the inositol ring. The apparent Kₐ for ATP was 2 μM, and the concentrations of Mg²⁺ and Mn²⁺ giving half-maximal activity were 2 and 0.2 mm, respectively. Mg²⁺ supported 3-fold higher activity than Mn²⁺ at optimal concentrations. The enzymatic activity was inhibited by its product, phosphatidylinositol 4,5-bisphosphate and enhanced by phosphatidylserine.

Agonist-activated phosphoinositide turnover generates two second messengers, inositol trisphosphate which elicits a rise in intracellular calcium and diacylglycerol which activates protein kinase C (Ca²⁺/phospholipid-dependent enzyme) (1–5). Although it is clear that these second messengers arise

from hydrolysis of PtdInsP₃ by an agonist-activated, phosphoinositide-specific phospholipase C, PtdInsP kinase, the enzyme which phosphorylates PtdInsP to PtdInsP₃, also appears to be important to the production of second messengers. Increased synthesis of PtdInsP₃ is necessary during second messenger generation because only a minute amount of hormone-sensitive PtdInsP₃ is present in cells, and upon agonist-activated phosphoinositide turnover this population is quickly depleted (6, 7). Quantitative measurements show that inositol trisphosphate produced during activated phosphoinositide turnover greatly exceeds that which is available from PtdInsP₃ present prior to stimulation (8–10). Taylor et al. (10), for example, determined that the rate of PtdInsP₃ synthesis during concanavalin A activation of thymocytes must be stimulated 10-fold to account for the amount of inositol trisphosphate produced.

Although the PtdInsP₃ kinase is responsible for synthesis of PtdInsP₃, a rate limiting component in second messenger production, few studies have focused directly on this enzyme and its role in phosphoinositide turnover. Early work on PtdInsP₃ kinase in rat brain homogenates defined an ATP- and divalent cation-dependent enzyme activity capable of phosphorylating PtdInsP to PtdInsP₃ (11). Structural studies of PtdInsP₃ and PtdInsP formed in rat brain tissue showed that PtdInsP₃ was phosphorylated at the 4' and 5' positions of the inositol ring, whereas PtdInsP was phosphorylated only at the 4' position (12). Thus, it was hypothesized that PtdIns₃ is phosphorylated first by PtdInsP kinase at the 4' position followed by phosphorylation at the 5' position by PtdInsP kinase (13–15). However, recent findings with the PtdIns₃ kinase suggest that there are at least two types of this enzyme with different positional specificities. Purified PtdIns₃ kinase from bovine uterus was shown to phosphorylate at the expected 4' position (16), but a new class of PtdIns₃ kinase activity which associates with the middleT/pp60⁺⁺ transforming protein and activated platelet-derived growth factor receptor was discovered to phosphorylate PtdIns₃ at the 3' position (17, 18). Furthermore, two types of PtdIns₄-kinase activity have been defined in bovine brain (19). These findings indicate that there are at least three distinct PtdIns₃ kinase activities which can be divided into two classes by their ability

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1. The abbreviations used are: PtdInsP, phosphatidylinositol biphosphate; PtdIns, phosphatidylinositol; PtdInsP₃, phosphatidylinositol 4,5-bisphosphate; PtdSer, phosphatidylserine; EGTA, [ethylenebis(oxyethylene)nitritol]tetraacetate acid; MES, (2-[N-morpholino]ethanesulfonic acid); HEPES, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid; DTT, dithiothreitol; PMSF, phenylmethylsulfonyl fluoride; PBS, phosphate-buffered saline; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; HPLC, high pressure liquid chromatography; PtdIns(4)P, phosphatidylinositol 4-phosphate; GroPtdIns(4,5)P₂, glycerylphosphorylinositol 4,5-bisphosphate; Ins(1,4,5)P₃, inositol 1,4,5-trisphosphate.
Characterization and Purification of Membrane-associated PtdInsP

**TABLE I**

Summary of purification data

<table>
<thead>
<tr>
<th>Activity (nmol/min)</th>
<th>Cell lysate</th>
<th>Membranes</th>
<th>NaCl extract</th>
<th>DEAE peak</th>
<th>SP peak</th>
<th>SP peak (second half)</th>
</tr>
</thead>
<tbody>
<tr>
<td>% Yield</td>
<td>64.6</td>
<td>34.7 ± 18.2</td>
<td>37.4 ± 11.5</td>
<td>6.0 ± 0.5</td>
<td>2.9 ± 0.3</td>
<td>2.3 ± 0.8</td>
</tr>
<tr>
<td>Specific activity</td>
<td>100</td>
<td>41</td>
<td>44</td>
<td>16</td>
<td>4.5</td>
<td>3.6</td>
</tr>
<tr>
<td>(nmol/min/mg)</td>
<td>0.0035</td>
<td>0.038 ± 0.012</td>
<td>0.346 ± 0.127</td>
<td>1.02 ± 0.33</td>
<td>8.75 ± 0.60</td>
<td>14.9 ± 3.7</td>
</tr>
<tr>
<td>Specific activity*</td>
<td>(nmol/min/mg)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>117 ± 13</td>
</tr>
<tr>
<td>Protein concentra-</td>
<td>ment (mg/ml)</td>
<td>24.171</td>
<td>1,432 ± 460</td>
<td>118.5 ± 38.6</td>
<td>7.0 ± 1.2</td>
<td>0.336 ± 0.016</td>
</tr>
</tbody>
</table>
| to phosphorylate at the 4’ or 3’ position. It is not known whether different types of PtdInsP kinase exist. PtdInsP kinase is found both in soluble and particulate fractions of cell homogenates (20–24), but the relationship between the soluble and particulate enzymes is unclear. Currently, only the soluble enzyme from rat brain has been purified and characterized (22–24). Further studies of PtdInsP kinase including purification, characterization, and development of immunological or pharmacological techniques are needed to resolve this issue and to elucidate the function and regulation of PtdInsP kinase in vivo. As a preliminary step toward understanding the function and regulation of this important enzyme of the phosphoinositide turnover pathway, we purified the PtdInsP kinase from human red blood cell membranes 4300-fold and did an initial characterization of this enzyme.

**EXPERIMENTAL PROCEDURES**

**Materials**—[γ-32P]ATP (10 mCi ml−1), [3H]Ins(1,4,5)P3, [3H]inositol 1,2,4,5-tetrakisphosphate, and [3H]phosphatidylinositol 4,5-bisphosphate were purchased from Du Pont-New England Nuclear. PtdIns, PtdSer, and phosphatidylcholine (crude egg lecithin) were obtained from Calbiochem. Molec-}

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**FIG. 1. DEAE-Sephacel separation of membrane extract.** The membrane extract was loaded onto DEAE-Sephacel and eluted with a linear NaCl gradient from 0 to 0.4 M (solid line). 4-ml fractions were collected and assayed for PtdInsP (PIP) activity using method A (open squares). Protein content in each fraction was determined by absorbance at 280 nm (solid diamonds).

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The concentrated extract was then dialyzed exhaus-}tively into lysis buffer and finally into DEAE buffer (25 mM Tris, 5 mM MgCl₂, 1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 0.1 mM PMSF, 5% glycerol, pH 7.5).

**DEAE-Sephacel Chromatography of Membrane Extract**—The salt extract from membranes was applied to a 25-ml DEAE-Sephacel column. After collection of the flow-through material, the column was washed with 60 ml of DEAE buffer and eluted with 120 ml of the same buffer with a linear gradient of NaCl from 0 to 0.4 M. 4-ml fractions were collected and assayed under standard conditions as described below.

**SP-Sephadex Chromatography of DEAE-Sephacel Peak**—The pooled peak of PtdInsP kinase activity from DEAE-Sephacel chromatography was dialyzed into SP buffer (20 mM MES, 5 mM MgCl₂, 1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 0.1 mM PMSF, 5% glycerol, pH 7.5).

**PtdInsP Kinase Assays and Thin Layer Separation of Phospholipids**—Standard PtdInsP kinase assays were performed as follows: 20 μl of PtdInsP kinase was mixed with 20 μl of a sonicated solution of phosphoprotein (0.1% Triton X-100). The reaction was initiated with 10 μl of 0.5 mM [γ-32P]ATP (2000 Ci mol⁻¹). The assay conditions employed depended upon the purpose of the assay. Method A was used for assays during purification and measured both PtdInsP and PtdInsP kinase activities. These reactions contained 0.04% Triton X-100, 25 mM Tris, 5 mM MgCl₂, 1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 0.1 mM PMSF, 5% glycerol, pH 7.4, and were incubated for 5 min at room temperature (23–25°C). Under these conditions, the
incorporation of $^{32}$P into PtdInsP$_2$ was linear for 7 min (data not shown). Optimal conditions for PtdInsP kinase activity were employed in method B. The conditions were essentially the same as for method A except that the assays were done with 1 mg ml$^{-1}$ PtdInsP alone and were incubated for 2.5 min at 30 °C. Reactions were stopped by adding 0.5 ml of methanol: n HCl (1:1) and extracted with 0.3 ml of chloroform. The organic layer was dried under oxygen, resuspended in chloroform:methanol:water (2:1:0.01), and spotted onto 0.2-mm oxalate-pretreated Silica Gel 60 plates (Merck). Plates were developed in chloroform:methanol:water:37% ammonium hydroxide (45:35:8:5:1.5), and phospholipids were visualized with iodine vapor after chromatography. The reaction products were visualized by autoradiography and identified by comparison with unlabeled standard phospholipids. Radioactivity in phospholipids was determined by exciting the appropriate piece of TLC plate and counting it in liquid scintillation mixture.

**SDS-Polyacrylamide Gel Electrophoresis**—Electrophoresis samples were either added directly to Laemmli sample buffer or precipitated with trichloroacetic acid and resuspended in sample buffer before loading. Electrophoresis was performed on SDS-polyacrylamide gels with an 8.5% or 10% separating gel and 4% stacking gel by the method of Laemmli (25).

**Renaturation of PtdInsP Kinase Activity from SDS-PAGE**—Samples containing 75 μl of SP-Sephadex-purified PtdInsP kinase (approximately 20,000 fmol/min) or 10 μl of SP-Sephadex-purified PtdInsP kinase (25–50 ng of 53,000-dalton protein) were incubated in SDS sample buffer (1% SDS (recrystallized), 10 mM DTT, 1% glycerol, 20 mM Tris, pH 6.8) for 5–10 min at room temperature. The samples were subjected to SDS-PAGE on 8% gels as described above except that the gels were polymerized with 2.5 μg/ml riboflavin in place of ammonium persulfate and electrophoresed at 5–10 °C. The lanes containing 10 μl of purified PtdInsP kinase were silver-stained and scanned by laser densitometry. The lanes containing 75 μl of the purified material were transferred to nitrocellulose. After transfer (2–3 h, 4 °C), the nitrocellulose strip was washed briefly in 0.1% Triton X-100, 10% glycerol, 1 mM EDTA, 10 μg/ml aprotinin and leupeptin, 50 mM Tris, pH 7.4. The paper was then divided into 16 pieces and incubated for 15–24 h in buffer containing 350 μg/ml PtdInsP, 800 μg/ml crude phosphatidylcholine, 0.2% Triton X-100, 1% glycerol, 1 mM EDTA, 1 mM DTT, 10 μg/ml aprotinin and leupeptin, 50 mM HEPES, pH 7.5. PtdInsP kinase activity was assayed by adding 10 μl of [γ-$^{32}$P]ATP to 0.15 mM (2 mCi/ml) and incubating at room temperature for 90 min. The reactions were terminated, extracted, and analyzed as described above. Preactivated molecular mass markers were used to delineate lane borders and to measure efficiency of transfer to nitrocellulose. Recovery of PtdInsP kinase activity was 0.01%. Control assays of gels sections containing no protein showed no activity (data not shown).

**Identification of Phosphorylation Position on the Inositol Ring of PtdInsP$_2$ Product**—The SP-Sephadex-purified PtdInsP kinase was used to catalyze the phosphorylation of PtdIns(4)P in the presence of $^{32}$P-labeled ATP as described above except that PtdInsP was present at 400 μg ml$^{-1}$. Analysis of the products by thin layer chromatography as described above showed the presence of $^{32}$P-labeled PtdInsP$_2$. The labeled PtdInsP$_2$ was treated with 5 μl of purified rat liver cytosolic phospholipase C (a gift from Dr. John Lowenstein, Brandeis University) for 30 min at 37 °C in buffer containing 50 mM Tris, 100 μM EDTA, 100 mM CaCl$_2$, 180 mM NaCl, and 0.1% deoxycholate, pH 7.5. The reaction was terminated and extracted with 0.4 m1 of 1:1 n HCl:methanol and 0.4 ml of chloroform. The aqueous phase containing inositol phosphates was lyophilized and analyzed by HPLC on a Whatman 5-μm partisphere SAX column along with $^{32}$H-labeled InsP$_3$ standards (33). Ins(1,3,4)$^{32}$P$_3$ labeled PtdInsP$_2$ alone. The labeled PtdInsP$_2$ was treated with 5 μl of purified rat liver cytosolic phospholipase C (a gift from Dr. John Lowenstein, Brandeis University) for 30 min at 37 °C in buffer containing 50 mM Tris, 100 μM EDTA, 100 mM CaCl$_2$, 180 mM NaCl, and 0.1% deoxycholate, pH 7.5. The reaction was terminated and extracted with 0.4 ml of 1:1 n HCl:methanol and 0.4 ml of chloroform. The aqueous phase containing inositol phosphates was lyophilized and analyzed by HPLC on a Whatman 5-μm partisphere SAX column along with $^{32}$H-labeled InsP$_3$ standards (33). Ins(1,3,4)$^{32}$P$_3$ labeled PtdInsP$_2$ was prepared by incubation of $^{32}$H-labeled PtdIns(4,5)P$_2$ with [γ-$^{32}$P]ATP (1 μCi/ml) at 37 °C for 10 min in buffer containing 50 mM HEPES, 1 mM EDTA, 4 μM CaCl$_2$, 5 mM MgCl$_2$, 120 mM KCl, 30 mM NaCl, 1 mM DTT, pH 7.5.

The labeled PtdInsP$_2$ product was also analyzed by deacylation and HPLC. The labeled PtdInsP$_2$ was treated for 50 min at 53 °C with 1.8 ml of methylamine reagent containing 5.77 ml of 25% methylamine in water, 6.16 ml of methanol, and 1.54 ml of 1-butanol. After lyophilization, the sample was resuspended in 1.8 ml of water. To separate the water-soluble GroPtdIns(4,5)P$_2$ products from the insoluble GroPtdIns(4,5)P$_2$ products, the resuspended sample was extracted twice with 2 ml of a mixture of 1-butanollight petroleum (boiling point 40–60 °C) and ethyl formate (20:4:1). The extracted aqueous phase was lyophilized and analyzed by HPLC as described above (17). $^{32}$H-GroPtdIns(4,5)P$_2$ prepared by

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**FIG. 2. SP-Sephadex separation of DEAE peak**. A, the peak fractions from DEAE-Sepharcel chromatography were pooled, dialyzed into pH 5.5 SP-Sephadex buffer to remove salt, and loaded onto SP-Sephadex. The column was eluted with a linear NaCl gradient from 0 to 0.8 M NaCl (solid line). 1-ml fractions were collected and assayed for PtdInsP kinase activity using method A (open squares). The protein content (expressed as relative units; solid diamonds) of each fraction was determined by laser densitometry of the silver-stained gel presented in B. B, 25 μl of each SP-Sephadex fraction was analyzed on 10% SDS-PAGE. The proteins were visualized by silver stain. The 55,000-dalton band is indicated by the large arrow. Small arrows show molecular masses. Lanes were loaded as follows: lane A, pooled DEAE peak fractions; lane B, flow-through eluate after loading pooled DEAE peak fractions; lane C, flow-through eluate after wash with SP-Sephadex loading buffer; lanes 1–20, fractionated NaCl gradient eluate; lane M, molecular mass markers. C, fractions 11–20 were pooled and 10% of the total volume was analyzed by 8.5% SDS-PAGE (lane 1). Lane 2 shows molecular mass markers. The proteins were stained with Coomassie Blue.
was separated by SDS-PAGE and either copurified with membranes. Between 80 and 90% of the membrane-bound activity was extracted by incubating the membranes briefly with 1 M NaCl. Thus, PtdInsP kinase was easily separated from the majority of the PtdIns kinase in this extraction step.

Preparation of antibodies against the 53,000-Dalton Protein—The 53,000-dalton protein was excised from an SDS-PAGE of pooled fractions from the second half of the SP-Sephadex peak of activity (see Fig. 2C). The protein was electroeluted from the gel, precipitated with 5 volumes of acetone, and resuspended in PBS. Approximately 50-100 µg of purified 53,000-dalton protein in 1:1 PBS-Freund's incomplete adjuvant was injected into the popliteal node of a New Zealand White rabbit. The rabbit was given booster injections subcutaneously of 20-100 µg of the purified protein in PBS and incomplete adjuvant (1:1) every 1-2 weeks. Serum was obtained by bleeding from the ear vein 1-2 weeks after each injection. Prior to immunization, preimmune serum was obtained from the same rabbit.

Immunoprecipitation of PtdInsP Kinase and 53,000-Dalton Protein—Indicated amounts of preimmune or immune serum were added to solubilized red blood cell membranes in 1% Nonidet P-40 and phosphate-buffered saline (with or without added tracer amounts of the same material labeled with 3H-phosphatidylinositol 4,5-bisphosphate) (see Fig. 4). The mixtures were brought to a total volume of 50 µl with phosphate-buffered saline, and incubated for 18 h at 4 °C. Protein A linked to Sepharose CL-4B was then added and the mixture was incubated for 1-3 h at 4 °C with occasional mixing. Beads were pelleted by brief centrifugation, and the supernatant was removed for later analysis. After 2 washes with 1% Nonidet P-40 in PBS followed by 3 washes in PBS, the beads were either assayed directly for PtdInsP kinase activity or incubated with SDS sample buffer and analyzed by gel electrophoresis and autoradiography. 20 µl of the remaining supernatants were also assayed for PtdInsP kinase activity. The 53,000-dalton band was excised and quantitated by scintillation counting.

Protein Determination—Protein was quantitated by the method of Lowry (26). Relative protein content of SDS-PAGE separated proteins was determined by absorbance at 280 nm or by absorbance on an LKB 2202 Ultrascan laser densitometer of Coomassie Blue-stained proteins.

Data Analysis—In figures where more than two data sets are presented, the data is expressed as the mean ± S.E. When two experiments are presented, the bars indicate the range. The apparent K_m for ATP and apparent K_m values for divalent cations were determined by nonlinear least squares fit of a simple hyperbolic model to the data.

RESULTS

Purification of PtdInsP Kinase from Red Blood Cells—PtdInsP kinase activity was found in both the soluble and particulate fractions of lysed fresh human red blood cells. Approximately 40% of the total PtdInsP kinase activity in these lysates copurified with membranes. Between 80 and 100% of the membrane-bound activity was extracted by incubating the membranes briefly with 1 M NaCl in lysis buffer (Table I). The activity of PtdIns kinase was also monitored during purification procedures. In contrast to PtdIns kinase, essentially all of the PtdIns kinase present in cell lysates remained with the membranes. Only 5% of the PtdIns kinase activity partitioned into the supernatant upon extraction with 1 M NaCl. Thus, PtdInsP kinase was easily separated from the majority of the PtdIns kinase in this extraction step.

The 53,000-dalton protein was further purified by ion exchange chromatography. The fractions obtained from these columns were assayed routinely by method A ("Experimental Procedures") which detects PtdInsP kinase activity as well as any possible contaminating PtdIns kinase activity. The high salt extract was first chromatographed on a DEAE-Sephaol column as shown in Fig. 1. At pH 7.5, PtdInsP kinase activity binds quantitatively to DEAE-Sephaol. The activity eluted with a linear gradient of NaCl as a single peak at approximately 250 mM NaCl and was purified 3-fold from the extract (Table I). The peak of activity from the DEAE-Sephaol was further purified 8-9 fold on an SP-Sephadex column (Fig. 2). All of the activity was bound to the column at pH 5.5 and eluted as a broad peak at 400 mM NaCl in a linear salt gradient. The bulk of protein eluted before the peak of activity (Fig. 2, A and B). Specific activities were determined for both the entire pooled peak represented by fractions 9-20 and the more purified second half of the peak represented by fractions 11-20. When assayed using method A, these two pools exhibited specific activities of 9 and 15 nmol/min/mg, respectively, which correspond to 2500- and 4300-fold purifications from the cell lysate (Table I). These fractions contained virtually no PtdIns kinase activity. The purified PtdInsP kinase peak was at least 100-fold more active in phosphorylating PtdInsP than PtdIns. Purified PtdInsP kinase activity in fractions 11-20 was also assayed using method B which has conditions optimized for PtdInsP kinase activity and does not measure PtdIns kinase activity. The specific activity obtained with method B was 117 nmol/min/mg.

SDS-PAGE analysis of the column fractions shows that a 53,000-dalton protein coeluted with PtdInsP kinase activity (Fig. 2B). The gel in Fig. 2B, which was overdeveloped for the 53,000-dalton protein, shows a number of bands of higher and lower molecular mass which do not coelute with activity and are thus likely to be contaminants. When the pooled fractions 11-20 which gave the highest specific activity were stained with Coomassie Blue in the linear range for the
Characterization and Purification of Membrane-associated PtdInsP

FIG. 4. Immunoprecipitation of PtdInsP kinase and 53,000-dalton protein. Trace amounts of 125I-labeled membranes (10⁶ cpm) were mixed with 12 μg of unlabeled membranes and the mixture was immunoprecipitated and assayed as described under “Experimental Procedures.” A, the amount of 125I-labeled 53,000-dalton protein precipitated by immune (solid diamonds) and preimmune (open squares) serum. B, PtdInsP kinase activity precipitated by immune (solid diamonds) and preimmune (open squares) serum; C, PtdInsP kinase activity remaining in the supernatant after precipitation with immune (solid diamonds) and preimmune (open squares) serum. D, the amount of 53,000-dalton protein found in the immunoprecipitates from A plotted against the activity in immunoprecipitates from B. E, 0.2 μg of a 1:1 mixture of NaCl extract of membranes and the PtdInsP kinase activity peak from Fig. 1 were mixed with trace amounts of the same material labeled with 125I and then incubated with the indicated amounts of serum followed by precipitation with protein A-Sepharose (see “Experimental Procedures”). The precipitates were analyzed by SDS-PAGE. Lanes 1–5 correspond to precipitates with 0.2, 0.5, 1.5, 3, and 5 μl of immune serum.

53,000-dalton protein the higher and lower molecular mass bands were not apparent, suggesting that the contaminant proteins are present only in trace amounts (Fig. 2C). Thus, the 53,000-dalton protein was the major protein in these peak fractions and was purified to near homogeneity. PtdInsP kinase activity in this most purified preparation was stable upon storage in concentrated form at −70 °C. Storage at 4 °C caused the 53,000-dalton band to be slowly degraded to a 45,000-dalton peptide.

53,000-Dalton Protein Contains PtdInsP Kinase Activity
Characterization and Purification of Membrane-associated PtdInsP

after Renaturation from SDS-PAGE—To determine the migration position of the PtdInsP kinase on SDS-gel electrophoresis. A renaturation procedure was utilized. The SP-Sephadex-purified preparation was run on SDS-PAGE, and the proteins were renatured by transferring to nitrocellulose and incubating overnight in phospholipid and detergent containing buffer. Fig. 3 shows the profile of protein (A) versus PtdInsP kinase activity (B). The peak of activity is coincident with the 53,000-dalton protein. No activity was obtained in the absence of renaturation. A small amount of activity was seen in the gel sections between 60,000 and 67,000 daltons. However, appearance of this more slowly migrating activity was variable and may have been due to the 53,000-dalton protein which had not completely denatured (the sample was not boiled in SDS sample buffer prior to electrophoresis). These results demonstrate that the 53,000-dalton protein contains PtdInsP kinase activity but do not rule out the possibility that other impurities also migrate at this position.

Immunoprecipitation of a PtdInsP Kinase Activity with Antibodies against the 53,000-Dalton Protein—To further verify that the 53,000-dalton protein contained PtdInsP kinase activity, a rabbit was immunized with SDS-PAGE-purified 53,000-dalton peptide. Immune serum obtained from this rabbit precipitated in a specific and dose-dependent manner both the 53,000-dalton protein (Fig. 4, A and E) and PtdInsP kinase activity (Fig. 4, B and C) from membranes as well as from NaCl extracts of membranes and from more purified fractions. Precipitation of the 53,000-dalton protein correlated very well with the precipitation of PtdInsP kinase activity (Fig. 4D). Preimmune serum precipitated neither the 53,000-dalton protein nor PtdInsP kinase activity (Fig. 4, A and B). Up to 90% of the total activity was removed from ghosts by the immune serum (Fig. 4C). This activity removed by immunoprecipitation from ghosts was not reflected in the immune pellets, suggesting that activity may be inhibited in these immune complexes (Fig. 4, B and C). In some preparations, a 45,000-dalton protein was detectable by protein staining; the immune serum also precipitated this peptide (Fig. 4E), consistent with the possibility that it is a cleavage product of the 53,000-dalton protein. This cleavage product is absent from fresh preparations, but is formed during 125I labeling and storage at 4°C (see "Purification of PtdInsP Kinase from Red Blood Cells" under "Results").

Gel Filtration and Size Determination of Purified PtdInsP Kinase—The size of the native PtdInsP kinase in the SP-Sephadex-purified preparation was determined by gel filtration on an S-400 Sephacryl column. Fig. 5 shows that the peak of PtdInsP kinase activity elutes near the 150,000-dalton...
Characterization and Purification of Membrane-associated PtdInsP

**Fig. 8. Divalent cation dependence.** SP-Sepharose-purified material was assayed for PtdInsP kinase activity in the absence of Triton X-100 and PtdIns as described in Fig. 7. Assays were performed with varying concentrations of free Mg$^{2+}$ (A) or free Mn$^{2+}$ (B). The pooled peak fractions were dialyzed to remove MgCl and EDTA and assayed in the absence of EDTA. Data are from a representative experiment.

**Fig. 9. PtdInsP dependence.** PtdInsP kinase activity was assayed at 25°C with SP-Sepharose-purified PtdInsP kinase in increasing amounts of PtdInsP alone in the presence (A) or absence (B) of 0.04% Triton X-100. Data in A are from four experiments and in B from a representative experiment. Curves were estimated and drawn by hand.

**Fig. 10. Effect of PtdSer.** The pooled peak fractions from the SP-Sepharose column were assayed for PtdInsP kinase activity using method A except that PtdIns was omitted. The ratio of PtdInsP to PtdSer was varied while total phospholipid concentration was kept constant at 200 μg ml$^{-1}$. Relative PtdInsP kinase activity at various PtdInsP to PtdSer ratios were assayed in the absence of Triton X-100 (open squares) and the presence of Triton X-100 (solid diamonds). Activity at the optimal PtdInsP to PtdSer ratios for assays under either condition were 2 pmol min$^{-1}$. Data in the absence of Triton X-100 are an average of two experiments; in the presence of Triton X-100, an average of three experiments.

*marker. A logarithmic plot of molecular mass versus elution volume/void volume revealed an apparent molecular mass between 125,000 and 175,000 daltons for the native, SP-Sepharose-purified PtdInsP kinase particle.

**PtdInsP Kinase Activity Phosphorylates PtdIns(4)P at the 5' Position**—The characterization of PtdInsP kinase by Brockerhoff and Ballou (14) showed that PtdInsP kinase activity in brain tissue phosphorylated the inositol ring at the 5'-hydroxyl. Analysis of the PtdInsP$_2$ formed by the red blood cell PtdInsP kinase is also consistent with phosphorylation of PtdIns(4)P at this position. Phospholipase C treatment of the PtdInsP$_2$ product yielded inositol triphosphate which was then analyzed by HPLC. The analysis showed a single peak comigrating with Ins(1,4,5)P$_3$ and distinct from inositol 1,3,4-trisphosphate (Fig. 6A). The PtdInsP$_2$ product was also analyzed by deacylation to GroPtdInsP$_2$. The deacylation product comigrated exactly with GroPtdIns(4,5)P$_2$ (Fig. 6B). This method of analysis has been used to resolve the 4,5-isomer from another PtdInsP$_2$ isomer produced in vitro using immunoprecipitates of polyoma middle T. 2 Both of these results suggest that the purified PtdInsP kinase phosphorylates PtdIns(4)P specifically at the 5' position of the inositol ring.

**ATP Dependence and Divalent Cation Dependence**—The

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ATP concentration dependence and Mg$^{2+}$ and Mn$^{2+}$ concentration dependence of the SP-Sephadex-purified PtdInsP kinase are presented in Figs. 7 and 8. The apparent $K_a$ for ATP was 2 $\mu$M, and optimal activity was observed at 0.1 mM (Fig. 7). Mg$^{2+}$ was three times more effective than Mn$^{2+}$ in supporting PtdInsP kinase activity (Fig. 8). However, the apparent $K_a$ for Mg$^{2+}$ was approximately 2 mM, whereas that for Mn$^{2+}$ was approximately 0.2 mM.

**PtdInsP Dependence and Phospholipid Effectors**—The SP-Sephadex-purified PtdInsP kinase showed different PtdInsP dependences in the presence and absence of 0.04% Triton X-100 (Fig. 9). Half-maximal activity in the presence of detergent occurs at 0.7 mg ml$^{-1}$ (Fig. 9A) and in the absence of detergent at 0.25 mg ml$^{-1}$ (Fig. 9B). The optimal activity obtained in Triton X-100 was 6-fold higher than that obtained in the absence of Triton X-100.

Previous results with PtdInsP kinase from Friend murine erythroleukemia cells (27) and rat brain (22) suggest that PtdSer enhances activity. Fig. 10 illustrates the effect of PtdSer on the purified red blood cell PtdInsP kinase. This experiment was done both with and without 0.04% Triton X-100 with the total phospholipid concentration held constant at 200 $\mu$g ml$^{-1}$. In the absence of detergent, optimal PtdInsP kinase activity was obtained at a PtdInsP:PtdSer ratio of 25:75 (Fig. 10A). PtdInsP kinase activity at this ratio was 2-fold higher than with 100% PtdInsP. In the presence of 0.04% Triton X-100, maximal activity was obtained at a higher PtdInsP to PtdSer ratio, 70:30 (Fig. 10B). Although Triton X-100 decreases the PtdSer required for maximal activity, the activities at the optimal PtdSer concentrations were approximately the same with and without detergent.

Under these conditions, PtdInsP$_2$, the product of PtdInsP phosphorylation, was found to inhibit PtdInsP kinase activity both in the presence and absence of detergent. Fig. 11 demonstrates that addition of increasing amounts of PtdInsP$_2$ to the optimal PtdInsP:PtdSer concentrations inhibited PtdInsP kinase activity to a greater extent than the addition of PtdIns. The slight inhibition seen with PtdIns at high PtdIns to PtdInsP ratios probably reflects a dilution of the substrate PtdInsP. PtdInsP$_2$ clearly shows an additional inhibitory effect. Half-maximal inhibition by PtdInsP$_2$ occurred at PtdInsP$_2$ to PtdInsP ratios of 1.5:1 in the absence of Triton X-100 and 0:4:1 in the presence of Triton X-100.

**DISCUSSION**

This characterization of the PtdInsP kinase from human red blood cell membranes elucidates several biochemical properties of the enzyme. The most purified preparation had a specific activity of 117 nmol min$^{-1}$ mg$^{-1}$ and contained a major 53,000-dalton protein. Antibodies made against the highly purified 53,000-dalton protein specifically precipitated both a 53,000-dalton protein and PtdInsP kinase activity. Finally, renaturation of activity from SDS-PAGE shows the peak of activity to be at 53,000 daltons. These results indicate that the major PtdInsP kinase in human red blood cell membranes has a peptide molecular mass of 53,000 daltons. The results of renaturation also show some activity at higher molecular masses, but renaturation of activity at these molecular masses was not consistent in several experiments. If higher molecular mass forms of PtdInsP kinase do exist in this preparation, they may be unrelated to the 53,000-dalton form since they are not recognized by the antiserum against the 53,000-dalton protein. Another caveat which can not be ruled out at present is that the 53,000-dalton protein may not consist entirely of PtdInsP kinase.

PtdInsP kinase activity in the most purified preparations specifically phosphorylated PtdIns(4)P at the 5' position. These results are consistent with the conclusions of Brockerhoff and Bailou (14). Unlike the recently purified 45,000-dalton brain myelin protein which contained both PtdIns and PtdInsP kinase activities (28), the red blood cell enzyme was specific for PtdInsP and exhibited less than 1% PtdIns kinase activity. These results support the hypothesis that PtdIns and PtdInsP kinase are distinct enzymes which sequentially phosphorylate PtdIns at the 4' position and the 5' position, respectively, to produce PtdInsP$_2$.

The membrane-associated erythrocyte PtdInsP kinase described here showed a number of similarities to a soluble PtdInsP kinase purified from rat brain (22, 23). The enzyme from red blood cells had a molecular mass of 53,000 daltons, and the native purified enzyme migrated between 125,000 and 175,000 daltons. The rat brain PtdInsP kinase was identified as a 45,000-dalton protein which migrated as a 110,000-dalton particle in the nondenatured form. Both enzymes required Mg$^{2+}$ as a cofactor with an optimum concentration of 10–20 mM (22–24). Mn$^{2+}$ was able to substitute for Mg$^{2+}$ but supported a much lower level of activity (22, 23). Red blood cell PtdInsP kinase, like the rat brain (22) and Friend murine erythroleukemia cell (27) enzymes, showed enhanced activity in the presence of PtdSer. PtdSer increased activity 3–4-fold both with and without detergent. Although the same maximal
activity was reached with or without detergent, the optimal ratio of PtdInsP to PtdSer was increased in detergent, a result which is most likely due to dilution of PtdInsP by detergent.

The red blood cell enzyme was inhibited by its product, PtdInsP. Inhibition of PtdInsP kinase activity by exogenously added PtdInsP has been observed previously in soluble fractions of retinal (20) and pituitary cell (21) homogenates. In contrast, PtdInsP kinase activity in particulate fractions of pituitary cell homogenates has been found to be activated by PtdInsP (21). The significance of these varied results is difficult to assess since the phospholipid environment (membranes versus vesicles and micelles) has not been characterized in these systems. However, these results are useful for optimizing activity assays under given conditions.

It has not been determined if there is heterogeneity in the enzyme itself which may have led to differences in the effect of PtdInsP. As discussed previously, PtdInsP kinase has been found in both soluble and particulate fractions in several different tissues (20-24). Hypotonically lysed red blood cells contained a roughly equal distribution of activity between the membrane and cytosolic fractions which is similar to the distribution found in rat brain (22). The membrane-associated form of PtdInsP kinase in red blood cells appeared to be a peripheral membrane protein since it was easily solubilized in the absence of detergent by high ionic strength. Given the loose association of red blood cell PtdInsP kinase with the membrane and its similarities to the soluble enzyme from rat brain, it will be of interest to determine if the soluble and membrane-bound forms are composed of two distinct isozymes or a single enzyme which distributes between these two locations.

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