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PHOSPHATIDYLINOSITOL 4-PHOSPHATE KINASE IS ASSOCIATED WITH THE MEMBRANE SKELETON IN HUMAN ERYTHROCYTES

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Phosphatidylinositol 4-phosphate kinase was eluted from human erythrocyte stroma by three separate and distinct techniques which are known to disrupt the membrane skeleton. In addition, this kinase was found to be associated with the intact skeletons prepared by Triton X-100 extraction of stroma. Phosphatidylinositol 4-phosphate kinase which has been extracted from the membrane is a freely soluble protein with poor enzymatic activity toward added phosphatidylinositol-4-phosphate; however, the enzyme was shown to reassociate with skeleton-depleted stroma and then regain full enzymatic activity toward stromal bound substrate.

Phosphatidylinositol is present on the cytoplasmic surface of the erythrocyte membrane and is enzymatically phosphorylated to give phosphatidylinositol 4-phosphate and phosphatidylinositol 4,5-bisphosphate, collectively known as phosphoinositides (1). This phosphorylation/dephosphorylation cycle for phosphoinositides is very active in human erythrocytes and has been proposed to play a role in maintenance of the cell's normal biconcave disc shape (2-4). This hypothesis is based on the observation that in vitro manipulations of the erythrocyte which cause a decrease in phosphoinositide levels also result in a coincident change in the cell's shape from a biconcave disc to an echinocyte. Regeneration of phosphoinositide levels results in a shape change back to the biconcave disc.

The phosphoinositide cycle in the erythrocyte includes two kinases (1), phosphatidylinositol kinase (E.C. 2.7.1.67) and phosphatidylinositol 4-

ABBREVIATIONS: PI, phosphatidylinositol; PI-P, phosphatidylinositol 4-phosphate; PI-P_2, phosphatidylinositol 4,5-bisphosphate; EGTA, ethylene glycol-bis-(amino ethyl ether) tetra acetic acid.

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phosphate kinase (E.C. 2.7.1.68); both activities are known to be associated with the membrane. An earlier report indicated that PI kinase is an integral membrane protein which requires detergents to be solubilized (5); however, the nature of the PI-P kinase was unknown. The data presented here demonstrate that PI-P kinase is, in fact, a peripheral membrane protein associated with the erythrocyte skeleton. This observation lends further credibility to the argument that the phosphoinositide cycle is an important component of the membrane/skeleton system and may be involved in the maintenance of the erythrocyte shape.

METHODS

Phosphatidylinositol from soybean, phosphatidylinositol 4-phosphate from bovine brain, p-mercuriphenylsulfonate, lithium 3,5-diiodosalicylate, and Triton X-100 were obtained from Sigma Chemical Co., St. Louis, MO. Gamma [^{32}P]ATP was synthesized by the method of Beutler et al. (6) using [^{32}P] phosphate from ICN Radiochemicals, Irvine, CA. Silica gel 60 thin-layer plates, 0.25 mm thickness, with preabsorbent spotting area were purchased from Merck, Darmstadt, F.R.G.

Blood from normal human donors was defibrinated by shaking with glass beads; leukocytes were removed by cellulose filtration (7). Erythrocytes were sedimented at 1100 xg for 10 minutes and washed three times with saline. Stroma were prepared by lysing erythrocytes in 20 volumes of 0° 0.5 mM EGTA, 10 mM Tris, pH 7.4 (lysis buffer) and centrifuging at 25,000 xg for 10 minutes. The stroma were washed repeatedly with this buffer until white.

The membrane skeleton was released from the stroma by three procedures: 1) One ml of packed stroma was added to 7 ml of 5 mM p-mercuriphenylsulfonate in 10 mM Tris, pH 7.4 and incubated at 37° for 10 minutes (8,9). The stripped membranes were then washed two times with cold lysis buffer and left in a final volume of 1 ml. 2) One ml of packed stroma was added to 7 ml of 10 mM lithium 3,5- diiodosalicylate in 10 mM Tris, pH 7.4 and incubated at 0° for 30 minutes (8). The stripped stroma were washed four times with lysis buffer since diiodosalicylate was found to be a potent inhibitor of PI kinase (G.L. Dale, unpublished observation). 3) One ml of stroma was added to 10 ml of 0.5 mM EDTA and the pH adjusted to 8.5. After incubation at 37° for 30 minutes, the stroma were washed 2 times with 0.5 mM EDTA, pH 8.5 to remove spectrin and actin (10). The stroma were then added to 3 volumes of 1.33 M KCl, 5 mM Na phosphate, pH 7.6 to obtain a final concentration of 1.0 M KCl. After incubation at 37° for 30 minutes (10), the preparation was centrifuged at 25,000 xg for 10 minutes; the supernatant was dialyzed at 4° against lysis buffer and the pelleted membranes were washed two times with lysis buffer. The validity of each skeleton extraction procedure was verified by SDS-PAGE electrophoresis of the stripped membranes (data not shown).

Intact skeletons were prepared (11) by adding 1 ml of stroma to 4 ml of 2.5% (w/v) Triton X-100, 5 mM Na phosphate, pH 7.2, and incubating at 0° for 30 minutes. The skeleton fraction was sedimented through 15 ml of 35% (w/v) sucrose, 100 mM NaCl, 5 mM Na phosphate, pH 7.2, by centrifuging at 23,000xg for 40 minutes in a swinging bucket rotor; the skeletons were then washed one time with lysis buffer and left in 1 ml of this buffer.

Assay 1: To measure PI kinase and PI-P kinase activities present in membranes, 100 μl of membrane (containing approximately 450 μg protein) was added to 100 μl of 2 mM γ[^{32}P]ATP, 10 mM $MgCl_2$, 1 mM EGTA, 20 mM 2-

mercaptoethanol, 50 mM imidazole, pH 7.4 and incubated at 37° for 60 minutes. The reaction was stopped with 1.5 ml of $CHCl_3/CH_3OH$/conc. HCl (20:40:1) followed by 0.5 ml each of water and $CHCl_3$. Seven hundred fifty microliters of the lower phase was removed for thin-layer chromatography and quantitation as described (12).

Assay 2: PI-P kinase activity present in the isolated skeletons was assayed by adding 100 µl of the preparation to 100 µl of 0.18 mM PI-P, 2 mM $\gamma[^{32}P]$ATP, 10 mM MgCl, 1 mM EGTA, 20 mM 2-mercaptoethanol, 0.2% Triton X-100, 50 mM imidazole, pH 7.4 and incubating at 37° for 60 minutes. Product analysis was performed as detailed above. PI kinase activity was measured similarly but substituting PI for PI-P.

RESULTS AND DISCUSSION

Erythrocyte stroma were depleted of their major skeletal proteins by three separate and distinct techniques (8-10). The skeleton-depleted membranes were then analyzed for the presence of PI-P kinase. The results shown in Figure 1 clearly demonstrate that the three skeleton depletion techniques removed the majority of PI-P kinase from the membrane as shown by the inability of the stroma to synthesize PI-P_2. Specifically, treatment with p-mercuriphenylsulfonate removes 89-93% of the enzyme from the membrane; treatment with 10 mM lithium 3,5-diiodosalicylate releases 85-88%; and incubation of the stroma with low ionic strength EDTA at pH 8.5 followed by

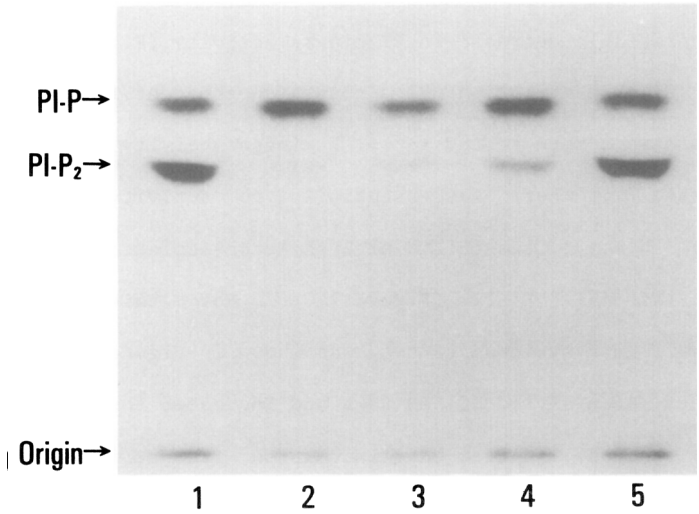

Figure 1 PI-P Kinase Activity of Skeleton-Depleted Stroma. Stroma were pretreated as detailed in the Methods section and were then incubated with $\gamma[^{32}P]$ATP (Assay 1). The radioactive lipids were separated by thin layer chromatography and the phosphoinositides localized by autoradiography. Lanes 1 and 5 are control stroma; Lane 2 is p-mercuriphenylsulfonate treated stroma; Lane 3 is after extraction with 3,5-diiodosalicylate; Lane 4 is stroma after sequential extractions with low ionic strength EDTA and 1 M KCl.

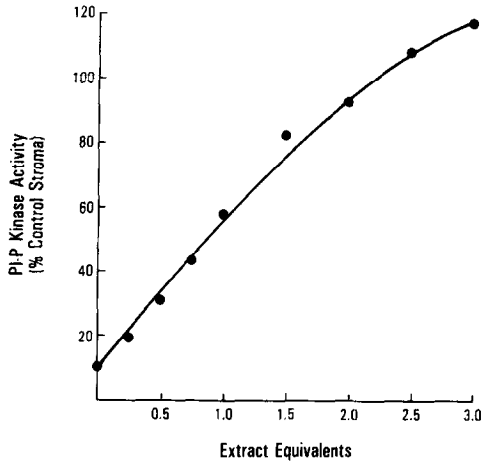

Figure 2 Reassociation of PI-P Kinase with Skeleton-Depleted Stroma. Para-mercuriphenylsulfonate stripped stroma (100 ml; 110 mg protein) were incubated with varying amounts of dialyzed KCl extract as detailed in text. One extract equivalent represents the KCl extract from a quantity of stroma equal to that used in this assay. After the reassociation incubation, the stroma were washed and analyzed with Assay 1. PI-P kinase activity is quantitated by the [^{32}P] incorporated into PI-P_2 and is presented as percent of control stroma.

extraction with 1 M KCl releases 75-79%. The EDTA extraction by itself in this last procedure does not release PI-P kinase from the membrane (data not shown). These stroma which have been depleted of their skeletal components do not have an appreciable decrease in PI kinase activity (Figure 1).

When the KCl or p-mercuriphenylsulfonate supernatant fractions which were extracted from stroma were tested for PI-P kinase activity by adding PI-P and γ[^{32}P]ATP (Assay 2), there was essentially no activity. However, these extracts were able to reassociate with skeleton-depleted stroma and then phosphorylate stromal PI-P (Figure 2). For this experiment, p-mercuriphenylsulfonate stripped stroma, as shown in Figure 1, were added to varying amounts of dialyzed KCl extract and incubated for 60 minutes at 0° (see Methods section). After this incubation, the stroma were washed and incubated with [^{32}P]ATP (Assay 1). Figure 2 demonstrates that PI-P kinase reassociates with the stripped stroma in a dose-dependent manner to re-establish an active, membrane-bound PI-P kinase enzyme. The reassociation is not efficient since approximately 2.5 times more extract is required than theoretically expected to obtain the original amount of PI-P kinase activity.

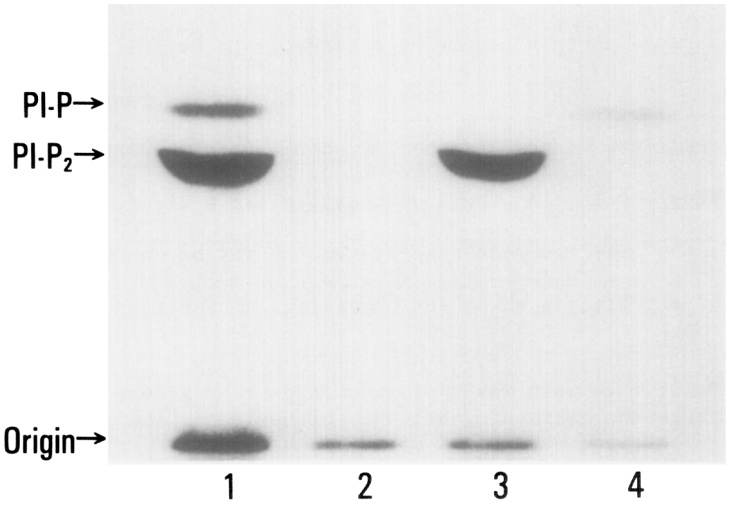

Figure 3 Association of PI-P Kinase with Isolated Erythrocyte Skeletons. Skeletons (Triton shells) were isolated as described. Lanes 1 and 2 are starting stroma and skeletons, respectively, assayed with [^{32}P]ATP (Assay 1). Lanes 3 and 4 are skeleton and skeleton supernatant, respectively, assayed with exogenous PI-P and [^{32}P]ATP (Assay 2).

An alternative procedure to verify that PI-P kinase is, in fact, associated with the membrane skeleton is to isolate intact skeletons or 'Triton shells' and assay for the kinase. A skeleton fraction and Triton supernatant were prepared by the method of Shen et al (11) and assayed for PI-P kinase as shown in Figure 3. The skeleton fraction clearly contains PI-P kinase as demonstrated in Lane 3 where exogenous PI-P and [^{32}P]ATP were added to the skeletons; the level of PI-P_2 synthesis was approximately 60% of that seen with the starting stroma. This PI-P kinase activity was totally dependent on the addition of PI-P as shown in Lane 2; presumably all of the enzymes's substrate (i.e., stromal PI-P) was solubilized by the Triton X-100 extraction. The Triton supernatant from the skeleton preparation does not have any observable PI-P kinase activity but does have PI kinase activity as shown by the faint band of PI-P in Lane 4; this was verified by independent assays of PI kinase activity (data not shown).

These data indicate that PI-P kinase in the erythrocyte is a peripheral membrane protein associated with the membrane skeleton and can be released in a freely water soluble form by procedures which disrupt the skeleton. In

addition, these data reaffirm previous observations that PI kinase is an integral membrane protein requiring detergents to be fully solubilized from the membrane (5). The fact that PI-P kinase is associated with the erythrocyte skeleton lends further credence to the argument that the phosphoinositide cycle in the erythrocyte plays a significant role in the dynamics of the skeleton/membrane system and may be a key component for maintenance of erythrocyte shape and physiological function (2-4).

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