Phosphorylation of Platelet Pleckstrin Activates Inositol Polyphosphate 5-Phosphatase I*

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Pleckstrin is the major substrate phosphorylated on serine and threonine in response to stimulation of human platelets by thrombin (Abrams, C. S., Zhao, W., Belmonte, E., and Brass, L. F. (1995) J. Biol. Chem. 270, 23317–23321). We now show that pleckstrin in platelets is in a complex with inositol polyphosphate 5-phosphatase I (5-phosphatase I). This enzyme hydrolyzes the 5-phosphate from inositol 1,4,5-trisphosphate and inositol 1,3,4,5-tetraakisphosphate and thus serves as a calcium signal-terminating enzyme, since the substrates but not the products mobilize intracellular calcium. Pleckstrin co-immunoprecipitates with 5-phosphatase I in homogenates of platelets. Platelet homogenates fractionated by anion exchange chromatography show co-elution of pleckstrin and 5-phosphatase I. Fractions containing phosphorylated pleckstrin have 7-fold greater 5-phosphatase activity than those containing unphosphorylated pleckstrin. Mixing experiments with recombinant 5-phosphatase I and pleckstrin in vitro show that they form a stoichiometric complex. A mutant form of pleckstrin, in which the serine and threonine residues that are phosphorylated by protein kinase C are substituted with glutamic acid (pseudophosphorylated pleckstrin), activates recombinant 5-phosphatase I 2–3-fold while native unphosphorylated pleckstrin does not stimulate the enzyme. Thus pleckstrin functions to terminate calcium signaling in platelets when it is phosphorylated by binding to and activating 5-phosphatase I.

Inositol polyphosphate 5-phosphatases comprise a large family of enzymes that share two short amino acid motifs that define the family (2). They show varying substrate specificities toward the 5-phosphate containing inositol phosphates and phospholipids. One of these enzymes, inositol polyphosphate 5-phosphatase I (5-phosphatase I),1 that was originally identified in platelets (3) hydrolyzes only the inositol phosphate substrates, inositol 1,4,5-trisphosphate (Ins(1,4,5)P3) and inositol 1,3,4,5-tetraakisphosphate (Ins(1,3,4,5)P4) (4). This enzyme is most likely to function as a signal-terminating enzyme since its substrates function to mobilize calcium ions while its products do not. A previous study suggested that 5-phosphatase I was phosphorylated in response to thrombin and thereby activated. 5-Phosphatase I purified from platelets was phosphorylated in vitro by protein kinase C and activated 4-fold under this condition (5). This would provide one mechanism for terminating Ins(1,4,5)P3-stimulated calcium mobilization. Subsequent to this study, cDNA clones encoding 5-phosphatase I were isolated, and the recombinant protein was expressed in heterologous systems (6–8). The specific activity of the recombinant enzyme is about 50 times greater than that purified from platelets suggesting that the platelet enzyme was not homogeneous. We now report that 5-phosphatase I exists as a complex with another protein, pleckstrin, in platelets. When pleckstrin is phosphorylated in the complex, 5-phosphatase I is activated. In retrospect the previous results must have been obtained from a preparation that included both 5-phosphatase I and pleckstrin.

Pleckstrin is a 40-kDa protein of human platelets that was originally identified as the major substrate for phosphorylation in platelets upon stimulation by thrombin (9, 10). The protein was found to contain 100 amino acid repeats at either end that are approximately 30% identical. Since this discovery, similar motifs have been found in over 70 proteins, including a number of proteins involved in intracellular signaling reactions (11–17). These proteins include phospholipase C enzymes, small guanine nucleotide binding proteins, β-adrenergic receptor kinases, β-spectrin, and dynamin. The function of pleckstrin homology (PH) motifs is uncertain although elucidation of their three-dimensional structures indicates that they share a fold and therefore constitute a common motif despite limited amino acid similarity. Several of these motifs have been shown to bind to acidic phospholipids, especially phosphatidylinositol 4,5-bisphosphate (13, 15, 17). PH motifs have also been shown to bind to the βγ subunits of trimeric G proteins and thereby presumably inhibit further signaling from an agonist-activated receptor (12–14). Platelet pleckstrin has been transfected into COS-1 cells and shown to diminish agonist-induced phosphatidylinositol turnover (1, 18). This effect was observed in response to both G protein-linked and tyrosine kinase-linked receptors and thus cannot be explained solely by binding to βγ subunits. We now report a function for platelet pleckstrin. The protein forms a 1:1 complex with 5-phosphatase I, and upon phosphorylation of the former, the latter is activated.

EXPERIMENTAL PROCEDURES

Materials

42P)Orthophosphate was from ICN and 32P-labeled Ins(1,4,5)P3 was prepared as described (19). Horseradish peroxidase-linked anti-rabbit IgG and Western blot detection reagents were purchased from Amer sham Life Sciences. The PVL 1392 baculoviral transfer vector and BaculoGold transfection kit were from PharMingen. pBluescriptSK and competent Echerichia coli were obtained from Stratagene. Anti-pleckstrin antibody was made as described (18). Mono Q column and pGEX-
5x-1 were from Pharmacia Biotech Inc. T4 DNA ligase, monoclonal HA antibody, and restriction enzymes were purchased from Boehringer Mannheim. Polymerase chain reaction reagents and Taq polymerase were from Perkin-Elmer. All other chemicals were obtained from Sigma.

**Methods**

**Construction of Different Forms of Recombinant Pleckstrin**—cDNA molecules encoding pleckstrin and various forms of pleckstrin were constructed as described previously (1, 18). These cDNA were then subcloned into a derivative of the plasmid vector pET-III that had been modified to contain sequences encoding six histidine residues at the amino terminus of the expressed protein. Proteins were expressed in E. coli as described previously (20) and purified using nickel-agarose (Qiagen). Recombinant proteins were eluted from nickel-agarose using 20% glycerol, 20 mM Tris, pH 7.9, 100 mM potassium chloride, 5 mM DTT, 0.5 mM PMSF, and 80 mM imidazole.

**Expression of Recombinant 5-Phosphatase I in Sf9 Cells**—cDNA encoding a truncated version of 5-phosphatase I in pCMV2 plasmid was provided by C. A. Mitchell (6). A full-length cDNA was obtained by polymerase chain reaction using a human umbilical vein endothelial cell cDNA library as template. The sense primer included an EcoRI site followed by nucleotide –3 to nucleotide 16 of human 5-phosphatase I cDNA (5'-cttaagatccatggcggggaagggcgg-3'); the antisense primer contained the complement of nucleotides 107–128 (5'-gttgcagcttctgatgactaat-3'). The polymerase chain reaction products were cleaved with EcoRI and PstI. The digested proteins containing nucleotides –3 to 88 were subcloned into pBluescriptSK. Nucleotides 89–1784, obtained by PstI digestion of the truncated 5-phosphatase I in pCMV2 plasmid, were subcloned into pBluescriptSK. The full-length cDNA was subsequently engineered into the baculovirus transfer vector pVL 1392 between the EcoRI and XhoI restriction sites. Sf9 cells grown at 27°C in TNM-FH insect medium (PharMingen) supplemented with 10% heat-inactivated fetal bovine serum and 100 μl TNM-FH insect medium (PharMingen) supplemented with 10% heat-inactivated fetal bovine serum and 100 μM deoxyribonuclease (10 μg/ml) were infected with the baculovirus construct containing 5-phosphatase I. Sf9 cells were cultured either in monolayer or in suspension (2 × 10⁵ cells/ml at the start of infection), and recombinant protein was harvested at 72 h after infection.

**Construction and Isolation of GST Fusion Protein**—Full-length 5-phosphatase I cDNA was isolated from pVL 1392 by EcoRI and NcoI restriction and transferred into pGEX-5x-1 plasmid between EcoRI and XhoI restriction sites. The plasmid was used to transform XL2 Blue E. coli (Stratagene). E. coli were grown in LB medium containing 20 μg of ampicillin, 50 μg of chloramphenicol, 1-β-D-galactopyranoside (1 mm) was added, and 4 h later E. coli were harvested by centrifugation and the cells were resuspended in 20 mM Tris, pH 7.5, 5 mM EDTA, 1 mM sodium azide, 1 mM sodium molybdate, and 4 mM sodium pyrophosphate. The extract from 1 × 10⁷ labeled platelets was plated with modified pET-IIb plasmid vectors contains encoding six histidine residues at the amino terminus of the expressed protein. Proteins were expressed in E. coli as described previously (20) and purified using nickel-agarose (Qiagen). Recombinant proteins were eluted from nickel-agarose using 20% glycerol, 20 mM Tris, pH 7.9, 100 mM potassium chloride, 5 mM DTT, 0.5 mM PMSF, and 80 mM imidazole.

**Purification of 5-Phosphatase I by MonoQ Chromatography**—Sf9 cell extracts were prepared by suspending Sf9 cells in 10 mM imidazole, pH 7.2, 3 mM MgCl₂, 1 mM EDTA, 0.3 μM sucrose, 5 mM 2-mercaptoethanol, 50 μM PMSF, and 1% Triton X-100. The column was eluted with 4 mM sodium pyrophosphate. The extract from 1 × 10⁷ labeled platelets was plated with modified pET-IIb plasmid vectors contains encoding six histidine residues at the amino terminus of the expressed protein. Proteins were expressed in E. coli as described previously (20) and purified using nickel-agarose (Qiagen). Recombinant proteins were eluted from nickel-agarose using 20% glycerol, 20 mM Tris, pH 7.9, 100 mM potassium chloride, 5 mM DTT, 0.5 mM PMSF, and 80 mM imidazole.

**5%-Phosphatase I from Platelet Extracts**—The extract from 2.5 × 10⁷ labeled platelets was incubated with 10 μg of anti-5-phosphatase I IgG at 4°C for 1 h in 20 μl containing 20 mM Tris, pH 7.5, 1 mM EDTA, 1 μM pepstatin A, and 1% Triton X-100. Protein A-Sepharose (40 μl of a 50% slurry in 50 mM Tris, pH 7.5, 150 mM NaCl, and 1% Triton X-100) was added, and the mixture was agitated at 4°C for 1 h. The protein A-Sepharose pellets were collected by centrifugation and washed once with 1 ml of the same buffer. The washed pellets were subjected to 10% SDS-PAGE and transferred to nitrocellulose membranes. The extract from 1 × 10⁷ labeled platelets was also run on the same gel to serve as a marker for phosphorylated proteins. In some experiments 1 mM N-ethylmaleimide was substituted for DTT in the gel loading buffer in order to visualize proteins migrating in the region near IgG when membranes were immobiloblotted.

**Immunoprecipitation of Recombinant Proteins by Monoclonal Anti-HA Antibody**—For immunoprecipitation with monoclonal anti-HA antibody, 2.5 μg of purified GST-5-phosphatase I fusion protein and 0.16 μg of HA-tagged pleckstrin were incubated with 0.3 μg of monoclonal anti-HA antibody (IgG2b) or irrelevant IgG2b in 30 μl of 80 mM imidazole, pH 7.5, 100 mM KCl, 50 μM PMSF, 20% glycerol at 4°C for 1 h. 30 μl of 50% protein A-Sepharose was added to each mixture, washed, and subjected to 10% SDS-PAGE and transferred to nitrocellulose membranes. The extract from 1 × 10⁷ labeled platelets was also run on the same gel to serve as a marker for phosphorylated proteins. In some experiments 1 mM N-ethylmaleimide was substituted for DTT in the gel loading buffer in order to visualize proteins migrating in the region near IgG when membranes were immobiloblotted.

**Binding of Pleckstrin to GST-5-Phosphatase I**—1.6 nmol of GST or GST-5-phosphatase I fusion protein were linked to 1 ml GST-agarose columns, equilibrated with 20 mM Tris, pH 7.5, 1 mM EDTA, 1 mM DTT, 100 mM NaCl, and 0.5% Triton X-100. 200 μg of platelet extract was applied to each column, and they were then washed with 10 column volumes of equilibrating buffer. Bound proteins were eluted with 5 column volumes of 20 mM Tris, pH 7.5, 20 mM GSH, 1 mM EDTA, and 1 mM DTT. Fractions (1 ml) were collected and 20 μl of each was subjected to 10% SDS-PAGE followed by immunoblotting with anti-pleckstrin antibody. The fraction of pleckstrin in the eluates was estimated by comparison of the band intensity of the immunoblots to those of known amounts of the recombinant proteins.

**Activation of 5-Phosphatase I by Various Forms of Pleckstrin**—Pleckstrin, pseudophosphorylated pleckstrin, or ΔPH pleckstrin, in which both pleckstrin domains were deleted, were added to 0.4 μg of purified GST-5-phosphatase I in 10 μl containing 50 mM Tris, pH 7.5, and 3 mM MgCl₂. The mixtures were immediately diluted with 50 mM Tris, pH 7.5, and 5 mM MgCl₂, and assayed for 5-phosphatase activity. The same procedures were used with GST-5-phosphatase I fusion protein as a source of 5-phosphatase.

**RESULTS**

It was previously reported that a human platelet inositol polyphosphate 5-phosphatase (designated 5-phosphatase I)
was phosphorylated and activated by protein kinase C (5). Since this protein is approximately the same size as the major protein phosphorylated in platelets in response to thrombin, pleckstrin was assumed to be that protein. Recently cDNA molecules encoding 5-phosphatase I have been isolated (6, 7), and the predicted sequence bears no primary sequence similarity to pleckstrin (10). There are now tools including recombinant proteins and antibodies that allow a re-examination of the relationship between pleckstrin and 5-phosphatase I. Initially we carried out SDS-PAGE of platelet proteins and performed Western blots using antibodies against 5-phosphatase I and pleckstrin to identify these proteins. While the proteins have approximately the same mass, they are readily separated on SDS-PAGE with 5-phosphatase I migrating at an apparent molecular weight slightly greater than that of pleckstrin as shown in Fig. 1A (lanes 1 and 2). The platelets in this experiment were labeled with $^{32}$P orthophosphate to identify the proteins phosphorylated in response to thrombin as shown in Fig. 1B (compare Western blot in lanes 3 and 4 to autoradiography in B). It is clear that pleckstrin is the major phosphoprotein in this experiment. Since the amount of pleckstrin in platelets (0.1% of platelet protein$^2$) is approximately 25 times that of

2 C. S. Abrams and S. E. Rittenhouse, unpublished observations.
Phosphorylation of pleckstrin increases the Vmax of different amounts of recombinant GST-5-phosphatase I: 0.083, 0.138, 0.208, 0.6, and 1.2 μg in lanes 1, 2, 3, 4, and 5, respectively.

FIG. 4. SDS-PAGE and Western blot analysis of co-immunoprecipitation of recombinant pleckstrin and recombinant GST-5-phosphatase I fusion protein. A, GST-5-phosphatase I (2.5 μg) was mixed with 0.16 μg of HA-tagged pleckstrin followed by immunoprecipitation using monoclonal IgG2b anti-HA antibody (lane 1) or irrelevant monoclonal IgG2b (lane 2). Western blots were developed using both anti-5-phosphatase I and anti-pleckstrin. B, Western blot analysis of different amounts of recombinant GST-5-phosphatase I: 0.083, 0.138, 0.208, 0.6, and 1.2 μg in lanes 1, 2, 3, 4, and 5, respectively.

We next determined the effect of the association between pleckstrin and 5-phosphatase I on 5-phosphatase catalytic activity. In this experiment we mixed pleckstrin, pseudophosphorylated pleckstrin, and a pleckstrin peptide where the PH domains are deleted with purified recombinant 5-phosphatase I and immediately assayed for 5-phosphatase activity using Ins(1,4,5)P3. The activity of 5-phosphatase I was 120 μmol of Ins(1,4,5)P3 hydrolyzed per min per mg of protein in the absence of pleckstrin.

The pseudophosphorylated form of pleckstrin, and pseudophosphorylated pleckstrin and 5-phosphatase I on 5-phosphatase catalytic activity were assayed for 5-phosphatase activity using Ins(1,4,5)P3. The activity of 5-phosphatase I was 120 μmol of Ins(1,4,5)P3 hydrolyzed per min per mg of protein.

Native pleckstrin, pseudophosphorylated pleckstrin, and PH pleckstrin (in which both PH domains are deleted) with purified recombinant 5-phosphatase I (specific activity, 120 mU/mg of protein). Only the pseudophosphorylated form of pleckstrin affected enzyme activity using Ins(1,4,5)P3. The activity of 5-phosphatase I was 121 μmol of Ins(1,4,5)P3 hydrolyzed per min per mg of protein. Another sample of pseudophosphorylated form of pleckstrin-5-phosphatase I complex was calculated. It is possible that pleckstrin also binds to other proteins in platelets and found no effect on enzyme activity (data not shown).

In order to evaluate whether phosphorylated pleckstrin also activates 5-phosphatase I in platelets, we fractionated extracts from platelets on a Mono Q column and subjected each fraction to Western blotting with both anti-pleckstrin and anti-5-phosphatase I antibodies and assayed enzyme activity. Another sample of 32P-labeled platelets was also fractionated on Mono Q and subjected to autoradiography to locate phosphorylated pleckstrin as shown in Fig. 6. Western blotting indicated that both proteins were eluted together in fractions 21–27 with pleckstrin continuing to fraction 31 as shown in Fig. 7. The phosphorylated pleckstrin eluted almost entirely in fraction 21 as shown in Fig. 6. The 5-phosphatase activity was also highest in this fraction indicating that pleckstrin also activates the enzyme in platelets when it is phosphorylated. By comparing the intensity of the 5-phosphatase I immunoblots, we estimate that phosphorylation increases enzyme activity by at least 7-fold (compare fractions 21 and 23 in Fig. 7, A and B). The specific activity is also high in fraction 20, which contains some phosphorylated pleckstrin (Fig. 6); however, the amount of 5-phosphatase I in this fraction is below the detection limit of Western blotting (Fig. 7A) so that specific activity cannot be calculated. It is possible that pleckstrin also binds to other proteins in platelets since some phosphorylated pleckstrin is in fraction 27. The 5-phosphatase activity in this fraction includes 5-phosphatase II, which is another major inositol polyphos-
phate-5-phosphatase in platelets, that eluted in fraction 27, and 5-phosphatase I in this fraction cannot be estimated (21).

DISSCUSSION

We have demonstrated that pleckstrin plays a role in phosphatidylinositol-mediated calcium signaling. When pleckstrin is phosphorylated on serine and threonine residues in response to thrombin, it binds to and activates 5-phosphatase I thereby accelerating the degradation of the calcium ion-mobilizing messenger molecule Ins(1,4,5)P3. Since the amount of pleckstrin in platelets is approximately 25 times that of 5-phosphatase I and the complex between the two is stoichiometric, it is clear that most of the platelet pleckstrin must have another function. Additionally, since most of the pleckstrin phosphorylated after thrombin treatment of platelets is immunoprecipitated by 5-phosphatase I, it is clear that most of pleckstrin is not phosphorylated in response to thrombin. It has been suggested that pleckstrin also leads to inhibition of phosphatidylinositol turnover in response to agonists (18). This would also serve to diminish calcium mobilization and thus the molecule may have a concerted action in terminating agonist responses. The effect on 5-phosphatase I is direct since pseudophosphorylated pleckstrin activates recombinant 5-phosphatase I in vitro. These results can explain our previous report that protein kinase C phosphorylates and activates platelet 5-phosphatase I. That preparation of enzyme had an activity of 4 \( \mu \text{mol} \) of Ins(1,4,5)P3 hydrolyzed per min per mg of protein compared with 120 \( \mu \text{mol} \) of Ins(1,4,5)P3 hydrolyzed per min per mg of protein for recombinant 5-phosphatase I purified from baculovirus-infected Sf9 cells. Since pleckstrin forms complexes with 5-phosphatase I and activates it, the earlier preparation undoubtedly contained pleckstrin as the major Coomassie Blue staining protein. Thus in the presence of protein kinase C, pleckstrin, which was the major protein in the partially purified enzyme preparation, was phosphorylated, and the phosphorylation of pleckstrin that bound to 5-phosphatase activated the enzyme. We cannot exclude the possibility that 5-phosphatase I is also phosphorylated. In two-dimensional gels in which one dimension is SDS-PAGE and the other is isoelectric focusing, 5-phosphatase I appears as a series of discrete spots (after Western blotting) along the pH dimension characteristic of phosphorylated proteins such as pleckstrin (data not shown). It is possible that this finding does not reflect phosphorylation but some other modification since at least in Sf9 cells labeled with [\( ^{32} \text{P} \)]orthophosphate, 5-phosphatase I appears not to be labeled and recombinant 5-phosphatase I purified from E. coli does not appear to be a substrate for protein kinase C (8). However, it has been reported that Sf9 cells are devoid of protein kinase C activity (22).

The function of pleckstrin domains remains uncertain in most cases. Several studies have suggested that proteins containing pleckstrin domains bind phospholipids in order to anchor proteins to membranes (13, 23, 24). In the studies reported here there is a clear action of pleckstrin that involves a direct protein-protein interaction without any role for lipids.

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