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Translocation of pleckstrin requires its phosphorylation and newly formed ligands☆

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Abstract

Pleckstrin is the major substrate of protein kinase C (PKC) in platelets. We sought to determine whether pleckstrin phosphorylation is sufficient to target the soluble protein to binding sites. Permeabilization of platelets by streptolysin O (SLO) was used to separate bound and soluble pleckstrin. Platelets were incubated with phorbol 12-myristate 13-acetate (PMA) and/or guanosine 5′-[γ-thio]triphosphate (GTP[S]) in the presence of [γ-^{32}P]ATP and SLO. PMA stimulated pleckstrin phosphorylation, but this pleckstrin diffused from permeabilized platelets. Addition of GTP[S] with PMA caused up to 40–50% of pleckstrin to be retained within platelets and enhanced secretion of platelet 5-hydroxytryptamine. PKCα pseudosubstrate peptide inhibited pleckstrin phosphorylation, the binding of pleckstrin and secretion. After extraction of permeabilized platelets containing bound pleckstrin with Triton X-100, the protein was solubilized. Thus, phosphorylated pleckstrin was retained in platelets only after activation of GTP-binding proteins that stimulate the formation of membrane-bound pleckstrin ligands. Translocation of pleckstrin may facilitate the associated secretion.

Keywords: Pleckstrin; PH domain; Platelet; Translocation; Protein kinase C; Phosphorylation; GTP-binding proteins

Pleckstrin is the major substrate of protein kinase C (PKC) in blood platelets and, on platelet activation, is phosphorylated on serine and threonine residues [1–4]. Pleckstrin contains three major domains, namely N- and C-terminal PH domains (N-PH and C-PH) separated by a central DEP domain of uncertain function [5–7]. The PKC phosphorylation sites on pleckstrin lie between the N-PH and DEP domains. PH domains have been shown to bind phosphoinositides with varying specificity [9,10]. In addition, protein ligands of certain PH domains have been identified, including G protein βγ-subunits [11], various PKC isoforms [12–14] and F-actin [15]. Both the N- and C- terminal PH domains of pleckstrin have been reported to bind PtdIns[4,5] P_2 [16] and $G_{\beta\gamma}$ subunits [17,18]. Moreover, pleckstrin appears to form a ternary complex with $G_{\beta\gamma}$ and the γ-isoform of phosphatidylinositol 3-kinase (PI3-Kγ) [19]. Phosphorylated pleckstrin has also been shown to bind to inositol polyphosphate 5-phosphatase I [20]. Pleckstrin inhibited PtdIns [4,5] P_2 hydrolysis when over-expressed in COS-1 cells [21]; this effect was mediated by either PH domain. Inhibition of inositol phosphate formation was not phosphorylation-dependent but was enhanced when pleckstrin was phosphorylated [21]. Over-expression of pleckstrin in COS-1 cells led to the translocation of pleckstrin to the plasma membrane [21], the formation of membrane projections [22], and reorganization of the actin cytoskeleton [23]. These effects were mediated exclusively by the N-terminal PH domain of pleckstrin and were dependent on pleckstrin phosphorylation. Stimulation of neutrophils has been reported to induce a translocation of soluble pleckstrin to membranes and the cytoskeleton, which required pleckstrin phosphorylation [24]. Thus, several studies have suggested that translocation of pleckstrin from the cytosol to intra-

☆ *Abbreviations:* ECL, enhanced chemiluminescence; GST, glutathione *S*-transferase; GTP[S], guanosine 5′-[γ-thio]triphosphate; 5-HT, 5-hydroxytryptamine; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; PI 3-Kγ, γ-isoform of phosphatidylinositol 3-kinase; SLO, streptolysin O.

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cellular binding sites is attributable to phosphorylation of the protein. Although these studies suggest potential functions of pleckstrin and of its phosphorylation by PKC, they cannot be extrapolated to platelets without further study, particularly as the translocation of pleckstrin to platelet membranes or other potential binding sites in platelets in response to platelet activation has never been clearly demonstrated.

Platelets are small enucleated cells and are not amenable to investigation using molecular biological techniques. In the present study, the translocation of pleckstrin in platelets was investigated using streptolysin O (SLO)-permeabilized platelets. Previous work using this system showed that permeabilization of resting platelets by SLO led to the rapid loss of pleckstrin from the cells [25]. This observation suggested a simple method for separating soluble cytosolic pleckstrin from any pleckstrin that is translocated to intracellular binding sites. In SLO-permeabilized platelets, activation of PKC by phorbol 12-myristate 13-acetate (PMA), and of GTP-binding proteins by guanosine 5′-[γ-thio]triphosphphate (GTP[S]) caused a synergistic stimulation of the Ca^{2+}-independent secretion of dense-granule 5-hydroxytryptamine (5-HT) [25]. By using these stimuli, we found that binding of pleckstrin within platelets requires not only phosphorylation of the protein by PKC but also the generation of new membrane ligands.

Materials and methods

Materials. [γ-^{32}P]ATP was obtained from NEN Life Sciences Products. 5-Hydroxy-[*side chain*-2-^{14}C]tryptamine ([^{14}C]5-HT) and horseradish peroxidase-linked donkey anti-rabbit IgG were from Amersham Pharmacia Biotech. Enhanced chemiluminescence (ECL) reagents and GTP[S] were from Roche Diagnostics. Recombinant SLO was supplied by Dr. S. Bakdi (Johannes-Gutenberg-Universität, Mainz, Germany). PMA, heparin, and glutathione–Sepharose 4B were from Sigma and PVDF membrane (Immobilon-P) from Millipore (Canada). The pseudosubstrate inhibitor peptide for PKC was RFAR KGALRQKNV($CONH_2$), corresponding to amino acid residues 19–31 of PKCα, and was synthesized by MOBIX at McMaster University. Other materials were from sources listed previously [25,26].

Recombinant GST–pleckstrin. Pleckstrin cDNA was amplified by PCR from a platelet cDNA library prepared using a Clontech Marathon cDNA amplification kit. The 5′-primer used was 5′-C**AGGATCC** GAACCAAAGCGGATCAGAGAGGGC-3′, which contained a *Bam*H1 site (bold) and nucleotides corresponding to amino acid residues 2–9 of human pleckstrin. The 3′-primer was 5′-ATG**CTCGAG**T CTCTTTACTTCCGAGTTCG-3′, which contained an *Xho*I site (bold) and nucleotides complementary to those encoding amino acid residues 347–350 and the stop codon of pleckstrin. The amplified product was cloned into the *Bam*H1 and *Xho*I sites of the GST-fusion protein vector, pGEX-4T-1, to permit expression of GST–pleckstrin in which the initiating Met residue was replaced by Gly–Ser residues. The protein was expressed in *Escherichia coli* BL21 [DE3] cells. An overnight culture (2 ml of LB medium containing ampicillin) was grown at 37 °C and expanded by incubating at 30 °C for 4 h with 300 ml of LB medium containing ampicillin but no isopropyl thio-β-D-galactoside. This was necessary to avoid proteolysis of GST–pleckstrin, which was then isolated on glutathione–Sepharose.

Isolation of human platelets. Human platelets were isolated and washed as described previously [25]. Briefly, blood was first collected into acid–citrate–dextrose (ACD) anticoagulant. Platelets were labelled with [^{14}C]5-HT in platelet-rich plasma and subsequently washed three times in Pipes-buffered Ca^{2+}-free Tyrode's solution (pH 6.5) containing 30 μg of apyrase/ml and 50 units of heparin/ml. The platelets were finally resuspended into buffer A (120 mM Na glutamate, 5 mM K glutamate, 20 mM Hepes, pH 7.4, 2.5 mM EDTA, 2.5 mM EGTA, 3.15 mM $MgCl_2$) at a concentration of 8×10^8 platelets/ml and incubated at 25 °C until used (within 3 h).

Permeabilization of platelets with SLO and assay of [^{14}C]5-HT secretion. Platelets were permeabilized by mixing 50 μl of platelet suspension with 50 μl of additions including SLO (0.4 U/ml final), sufficient EGTA and EDTA to give a final pCa > 9, ATP (4 mM final) and PMA and/or GTP[S], as described [25]. PKCα pseudosubstrate peptide was included with these additions when required. Incubations were for 5 or 10 min at 25 °C. For measurement of dense-granule secretion, incubations contained [^{14}C]5-HT-labelled platelets and were terminated by the addition of 500 μl of ice-cold solution containing 1.8% (w/v) paraformaldehyde, 6 mM EDTA, and 0.15 M KCl, pH 7.4. The platelets were then pelleted by centrifugation and a sample of the supernatant was counted to determine the percent of [^{14}C]5-HT secreted [25].

Assay of pleckstrin phosphorylation. To measure pleckstrin phosphorylation, incubations of permeabilized platelets were carried out as above but with the addition of 10 μCi of [γ-^{32}P]ATP. These incubations were terminated by addition of 500 μl of ice-cold 10% (w/v) trichloroacetic acid. The precipitated proteins were analysed by SDS/PAGE and ^{32}P-labelled proteins were located by autoradiography. The region of the gels containing pleckstrin was cut out and the strips were exposed to storage phosphor-coated plates. The plates were scanned using a phosphorimager and the digital images analysed using ImageQuant software (Molecular Dynamics).

Measurement of pleckstrin translocation. Incubations, carried out as above, were terminated by centrifugation at 14,000*g* for 1 min and the supernatant was separated from the pelleted material. Proteins in each fraction were precipitated by the addition of ice-cold trichloroacetic acid at a final concentration of 8% (w/v) and were subsequently redissolved in electrophoresis sample buffer [2] for analysis of pleckstrin by SDS/PAGE and immunoblotting (see below).

SDS/PAGE, autoradiography, and quantitative immunoblotting. Acid-precipitated proteins were analysed by SDS/PAGE using 13% (w/v) acrylamide in the separating gel [2]. The gels were either dried for autoradiography on Kodak X-Omat AR film at −70 °C or the proteins were electroblotted onto Immobilon-P membranes. In the latter case, pleckstrin was visualized using a rabbit polyclonal antibody raised against purified pleckstrin. The immunoreactive protein was detected by ECL, using horseradish peroxidase-linked donkey anti-rabbit IgG. Quantitative assessment of the immunoblot signals was accomplished by mixing various amounts of GST–pleckstrin with the experimental samples prior to SDS/PAGE. This permitted simultaneous electrophoresis, electroblotting, and ECL detection of standards and experimental samples. The blots were exposed to X-ray film and the images digitized using a Umax Astra 1200s flatbed scanner with a transparency adapter. The bands corresponding to pleckstrin and GST–pleckstrin standards were quantified using 1D software (Eastman Kodak Company). After quantitation of pellet and supernatant pleckstrin, the amount found in the pellet was expressed as a percentage of the total.

Triton X-100 extraction of permeabilized platelets. Buffer B (50 mM Tris–HCl, pH 7.4, 10 mM EGTA, 4 mM Na_3VO_4, 5 mM $Na_4P_2O_7$, 0.2 μM calyculin A, 2 μg of aprotinin/ml, 200 μM leupeptin, 1 mM PMSF, 2 mM benzamidine), with or without 1% Triton X-100, was added to platelet pellets isolated by centrifugation, as above. The pellets were resuspended and incubated at room temperature for 5 min and at 4 °C for 10 min. Platelet cytoskeletons, which are sedimented by low *g* forces provided proteolysis is prevented [27], were then isolated

by centrifugation at 12,000*g* for 10 min at 4 °C. The pellets were washed once with buffer B and then resuspended in buffer B. Trichloroacetic acid (10% w/v) was added to the supernatants and resuspended pellets to give a final concentration of 8% (w/v). Acid-precipitated proteins were dissolved in electrophoresis sample buffer and analysed by SDS/PAGE.

Results

We first investigated the binding of ^{32}P-labelled phosphorylated pleckstrin to intracellular structures by incubating platelets with increasing concentrations of PMA (10–100 nM) in the presence of [γ-^{32}P]ATP and SLO and absence of Ca^{2+} ions (pCa > 9). Rapid centrifugation of the platelets to yield pellet and supernatant fractions and analysis of these fractions by SDS/PAGE showed that PMA stimulated pleckstrin phosphorylation in a concentration-dependent manner, but that even at the highest concentration used (100 nM), the phosphorylated pleckstrin diffused almost completely into the supernatant fraction (Fig. 1, upper panel). As found previously [25], high concentrations of PMA (30–100 nM) caused secretion of [^{14}C]5-HT from platelet dense granules (Fig. 1, upper panel), indicating that binding of phosphorylated pleckstrin within platelets is not essential to PMA-induced secretion. Addition of a low concentration (10 μM) of GTP[S] alone caused, in this experiment, some phosphorylation of pleckstrin and, when added in combination with PMA, increased pleckstrin phosphorylation above the level seen with PMA alone, particularly with the lowest PMA concentration tested (10 nM). Under these conditions, GTP[S] and PMA acted synergistically to promote [^{14}C]5-HT secretion from the platelets, as found previously [25]. However, when GTP[S] alone or GTP[S] and PMA were present, much of the phosphorylated pleckstrin was found within the platelets in the pellet fraction (Fig. 1, lower panel). The dependence of this effect on the GTP[S] concentration was investigated (Fig. 2). The results showed that at all concentrations tested (10–300 μM), GTP[S] alone caused only small increases in ^{32}P-labelled phosphorylated pleckstrin, about half of which was retained within the platelets. However, in the presence of 100 μM PMA and GTP[S], much more ^{32}P-labelled phosphorylated pleckstrin was formed and up to 50% was found within the platelets. Maximal immobilization of phosphorylated pleckstrin was seen with 30 μM GTP[S] (Figs. 2a and b).

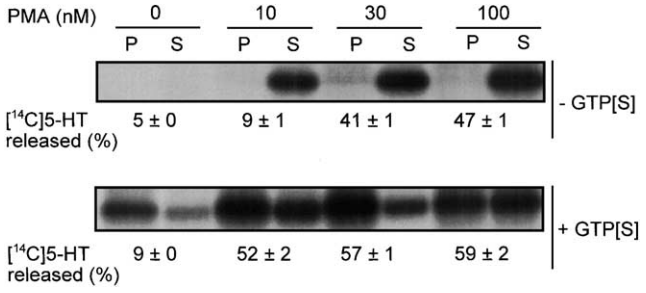

Fig. 1. Effects of different PMA concentrations on the translocation of ^{32}P-labelled pleckstrin to the pellet fraction of SLO-permeabilized platelets in the absence and presence of GTP[S]. Platelets labelled with [^{14}C]5-HT were permeabilized at pCa > 9 in the presence of [γ-^{32}P]ATP, the indicated PMA concentrations and supernatant (S) in the absence and presence of 10 μM GTP[S]. Incubations were for 10 min at 25 °C. Platelets were then sedimented, pellet (P) and supernatant (S) fractions were isolated. Proteins in each fraction were resolved by SDS/PAGE and ^{32}P-labelled proteins visualized by autoradiography. Parallel incubations were carried out to measure dense granule secretion; the release of [^{14}C]5-HT under each experimental condition is shown (mean ± SEM, $n = 3$).

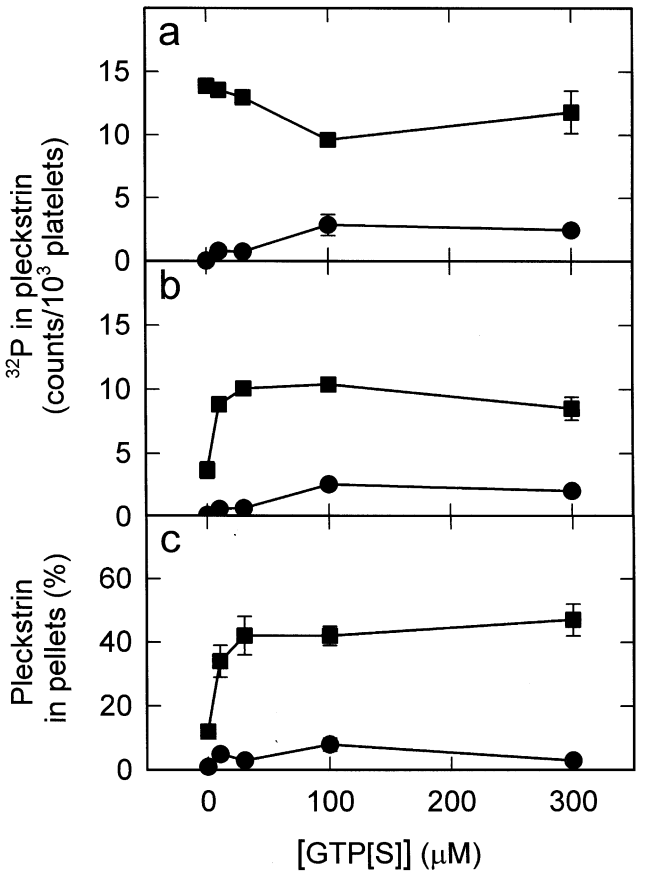

Fig. 2. Effects of different GTP[S] concentrations of GTP[S] on pleckstrin translocation in the absence and presence of PMA. Platelets were permeabilized at pCa > 9 and incubated at 25 °C for 5 min with [γ-^{32}P]ATP and the indicated concentrations of GTP[S] in the absence (●) and presence (■) of 100 nM PMA. At the end of the incubations supernatantand pellet fractions were isolated and analysed by SDS/PAGE. ^{32}P incorporation into pleckstrin was determined using a phosphorimager and the total pleckstrin in each fraction by quantitative immunoblotting, as described under Methods. (a) The ^{32}P in the pleckstrin found in supernatant fractions; (b) the ^{32}P in the pleckstrin found in pellet fractions; (c) the pleckstrin pellet fractions expressed as percentages of total platelet pleckstrin. All values are means ± SEM from three determinations in the same experiment.

Because equilibration of exogenous [γ-^{32}P]ATP with unlabelled platelet ATP after permeabilization with SLO is unlikely to be instantaneous, we considered the possibility that binding of ^{32}P-labelled pleckstrin within the platelets might not accurately reflect the binding of total phosphorylated pleckstrin. We therefore used quantitative immunoblotting, standardized by inclusion of known amounts of GST–pleckstrin in each sample of the electrophoresis sample buffer, to determine the percentages of the total pleckstrin bound in pellet fractions (Fig. 2c). The results closely paralleled those obtained by measurement of ^{32}P-labelled phosphorylated pleckstrin (Fig. 2b), in that a maximum binding of 40–50% of platelet pleckstrin was obtained with a GTP[S] concentration of 30 μM, provided PMA was also present. In contrast, GTP[S] alone had small and variable effects on pleckstrin phosphorylation and binding within platelets (compare Figs. 1 and 2). The results indicate that in addition to causing a partial activation of PKC, GTP[S] generates binding sites for phosphorylated pleckstrin within the permeabilized platelets.

To assess the importance of pleckstrin phosphorylation in the intracellular binding of the protein more directly, experiments were conducted using the PKC pseudosubstrate peptide, PKCα$_{19–31}$. Addition of 30 μM peptide, sufficient to inhibit the secretion of platelet [^{14}C]5-HT caused by 30 nM PMA or by 30 nM PMA and 100 μM GTP[S], also markedly inhibited the immobilization of pleckstrin in the pellet fractions, when this was measured by quantitative immunoblotting (Table 1). These results also demonstrate a correlation between the effects of GTP[S] on pleckstrin immobilization and secretion of [^{14}C]5-HT. Fig. 3 shows the relationship between pleckstrin phosphorylation and immobilization when higher concentrations of PMA and PKCα$_{19–31}$ were used. In this experiment, 100 μM GTP[S] alone was almost without effect on pleckstrin phosphorylation and caused very little of the protein to be retained in the pellet fraction, whereas 100 nM PMA also caused marked phosphorylation of pleckstrin, little of which was bound within the platelet. However, in combination, PMA and GTP[S] again acted synergistically to promote the immobilization of phosphorylated pleckstrin in the pellet fraction (Fig. 3). With these high stimulus concentrations, the large effect of GTP[S] on the retention of phosphorylated pleckstrin in the presence of PMA occurred with only a small increase in total pleckstrin phosphorylation. Addition of 100 μM PKC α$_{19–31}$ almost abolished both pleckstrin phosphorylation and the binding of pleckstrin within the platelets (Fig. 3). In three identical experiments, 100 μM PKCα$_{19–31}$ decreased the immobilization of pleckstrin within SLO-permeabilized platelets stimulated with 100 μM GTP[S] and 100 nM PMA from 45 ± 3% to 7 ± 3% (means ± SE). These observations strongly suggest that phosphorylation of pleckstrin is necessary but not sufficient for retention of the protein within permeabilized platelets.

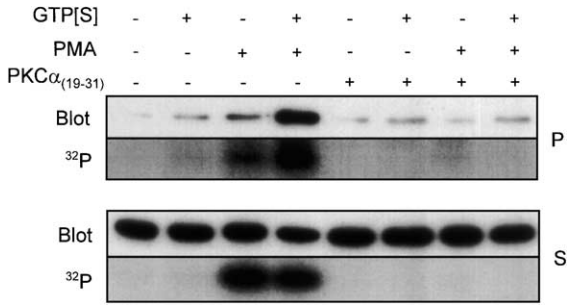

Fig. 3. Effects of inhibition of PKC activity on pleckstrin phosphorylation and translocation in SLO-permeabilized platelets. Platelets were permeabilized at pCa > 9 and incubated at 25 °C for 5 min with [γ-^{32}P] ATP in the absence or presence of 100 μM PKC pseudosubstrate peptide (PKCα$_{19–32}$) and no other additions, 100 μM GTP[S], 100 nM PMA, or both as indicated. At the end of the incubations, supernatant and pellet fractions were separated, and the proteins in each fraction analysed SDS/PAGE. Pleckstrin was detected by immunoblotting (Blot) and ^{32}P-labelled phosphorylated pleckstrin by autoradiography [^{32}P].

Table 1
Effects of inhibition of PKC activity on Ca^{2+}-independent [^{14}C]5-HT secretion and pleckstrin translocation in SLO-permeabilized platelets

Additions	Secretion of [^{14}C]5-HT (% of total)		Pleckstrin translocation (% of total)	
	Without PKCα$_{19–31}$	With PKCα$_{19–31}$	Without PKCα$_{19–31}$	With PKCα$_{19–31}$
Control	6 ± 0	6 ± 0	7 ± 3	4 ± 0
GTP[S]	7 ± 0	7 ± 0	9 ± 2	10 ± 3
PMA	30 ± 2	7 ± 0	12 ± 1	9 ± 2
PMA + GTP[S]	77 ± 1	10 ± 1	43 ± 2	9 ± 2

Note. Platelets were permeabilized a pCa > 9 and incubated at 25 °C for 5 min with [γ-^{32}P]ATP in the presence of no stimulus (control), 30 nM PMA, 100 μM GTP[S], or both. Incubations were carried out in both the absence and presence of 30 μM PKC pseudosubstrate peptide (PKCα$_{19–31}$). The platelets were sedimented, supernatant and pellet fractions were separated and the proteins in each fraction were resolved by SDS/PAGE. Dense granule ([^{14}C]5-HT) secretion and pleckstrin translocation were measured as described in the Methods section. Values for secretion are means ± range from two separate experiments, whereas values for pleckstrin translocation are means ± SE from three separate experiments.

The subcellular structures to which pleckstrin binds could be membranous or cytoskeletal. Permeabilized platelets containing bound pleckstrin were therefore separated into Triton X-100 soluble and insoluble fractions. Fig. 4 shows that Triton X-100 extracted all the pleckstrin found in pellets from permeabilized platelets that had been incubated with GTP[S] and PMA, whereas much of the pleckstrin originally present was retained in control pellets extracted with buffer lacking Triton X-100. Some pleckstrin was lost from the pellets during the blank extraction procedure, perhaps as a

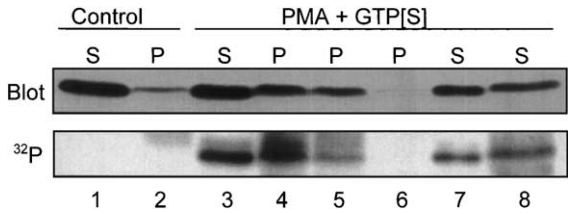

Fig. 4. Triton X-100 extraction of pleckstrin from GTP[S]-stimulated permeabilized platelets. Platelets were permeabilized at pCa > 9 in the presence of [γ-^{32}P]ATP and incubated at 25 °C for 10 min. Lanes 1–4: platelets incubated with no other addition (lanes 1 and 2) or with both 100 μM GTP[S] and 100 nM PMA (lanes 3 and 4) were separated into supernatant (S) and pellet (P) fractions and the proteins in each fraction analysed by SDS/PAGE. Lanes 5–8: pellet fractions from stimulated platelets were re-suspended in buffer B (lanes 5 and 7) or in buffer B containing 1% (v/v) Triton X-100 (lanes 6 and 8). After extraction, the resuspended material was again separated into pellet (lanes 5 and 6) and supernatant (lanes 7 and 8) fractions and the proteins in each fraction were analysed by SDS/PAGE. Pleckstrin was detected by immunoblotting (Blot) and ^{32}P-labelled phosphorylated pleckstrin by autoradiography [^{32}P].

result of partial dephosphorylation of the protein. The same results were obtained in two other identical experiments. This indicates that most of the pleckstrin retained in permeabilized platelets stimulated by GTP[S] and PMA are membrane-bound and does not form a stable association with the cytoskeleton.

Discussion

The results presented here confirm previous evidence [25] that pleckstrin is cytosolic in resting platelets, since permeabilization by SLO released essentially all of the pleckstrin into the surrounding medium. However, upon stimulation of permeabilized platelets with both PMA and GTP[S], up to 40–50% of total platelet pleckstrin translocated to membranes. Examination of the role of pleckstrin phosphorylation showed that this is necessary but not sufficient for translocation of pleckstrin to platelet binding sites. Thus, PMA alone potently stimulates pleckstrin phosphorylation but does not cause translocation of pleckstrin. The importance of pleckstrin phosphorylation was further demonstrated when platelets were incubated with the PKC pseudosubstrate peptide inhibitor, PKCα_{19-31}, which abolished both the pleckstrin phosphorylation and translocation induced by incubating platelets with both PMA and GTP[S]. These results indicate that incubation of permeabilized platelets with GTP[S] must generate intracellular binding sites for pleckstrin.

The diversity of known PH-domain ligands suggests that phosphorylated pleckstrin could bind to one or more inositol phospholipids or to proteins located on membranes or associated with the actin cytoskeleton. The PH domains of pleckstrin have been reported to bind to F-actin [15] and might therefore bind to the actin cytoskeleton directly. To investigate this possibility, platelets were separated into pellet and supernatant fractions after permeabilization with SLO and the pellet fraction was subsequently separated into Triton X-100 soluble and insoluble fractions. The results indicated that phosphorylated pleckstrin was associated with the membrane fraction rather than the actin cytoskeleton. Our results differ in certain respects from those obtained by others [21,24]. In one study [21], a fraction of the total pleckstrin was detected in membrane fractions but not cytoskeletal fractions from unstimulated platelets, suggesting a stimulation-independent association of pleckstrin with membranes. In a second study with fMLP- or PMA-stimulated neutrophils [24], pleckstrin was isolated with both membranes and the cytoskeleton. This was dependent on PKC activity since it was inhibited by bisindolylmaleimide [24]. In neither study was a role for new membrane binding sites evident, though this was not excluded.

Our results suggest that the translocation of pleckstrin to membrane-binding sites is highly regulated. In studies with a chemical cross-linking reagent [28], we have shown that non-phosphorylated pleckstrin is oligomeric and that phosphorylation of the protein by PKC causes its dissociation into monomers. It is likely that, after activation of PKC, non-phosphorylated oligomeric pleckstrin associates weakly with the plasma membrane and is phosphorylated. Phosphorylated monomers then become anchored to membranes at high-affinity binding sites formed after activation of one or more GTP-binding proteins [29]. Although it is clear that phosphorylation of pleckstrin is necessary in order for the protein to be retained inside permeabilized platelets, the role phosphorylation plays is not clear. The simplest explanation may be that phosphorylation causes a conformational change in pleckstrin that simultaneously prevents oligomerization of the protein and enables pleckstrin monomers to bind to sites created by the actions of GTP-binding proteins. Since no more than 50% of the phosphorylated pleckstrin was found on membranes, it is likely that the membrane ligands to which pleckstrin binds become saturated. Although the isolated N- and C-terminal PH domains of pleckstrin have been reported to bind to phosphatidylinositol 4,5-bisphosphate [16], which is present in unstimulated platelets, it seems unlikely that this is a physiological ligand, since neither non-phosphorylated pleckstrin nor pleckstrin phosphorylated after treatment of platelets with PMA are immobilized in permeabilized platelets. Rather, activation of GTP-binding proteins by GTP[S] most likely induces the formation of phospholipids that are ligands for the PH domains of pleckstrin. In this context, a recent study in this laboratory [30] has shown that phosphorylated pleckstrin binds selectively, and more strongly than non-phosphorylated pleckstrin, to

phosphatidic acid, phosphatidylinositol 3,4-bisphosphate, and phosphatidylinositol 3,4,5-trisphosphate. It is therefore of considerable interest that GTP[S] and PMA stimulate phosphatidic acid formation by phospholipase D in electropermeabilized platelets even in the absence of Ca^{2+} [31] and that platelets contain the γ-isoform of P I3-K which is activated by $G_{\beta\gamma}$ [32]. In this context, pleckstrin can be co-immunoprecipitated with $G_{\beta\gamma}$ and P I3-Kγ [19]. Cooperative actions of lipid and protein ligands could well participate in the translocation of pleckstrin to membranes, just as $G_{\beta\gamma}$ and phosphatidylinositol 4,5-bisphosphate interact synergistically to promote binding of the GRK2 PH domain to membranes [33]. Also, binding of some proteins to membranes has been reported to depend on the cooperative involvement of a PH domain and a second domain of the same protein [34–36]. In the case of pleckstrin, both PH domains, and even the DEP domain, could be required.

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