Phosphopleckstrin Inhibits Gβγ-activable Platelet Phosphatidylinositol-4,5-bisphosphate 3-Kinase*

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Pleckstrin, the prototypic protein containing two copies of the pleckstrin homology domain, is a prominent substrate of protein kinase C in platelets and neutrophils. Both cell types have p85 subunit-containing phosphoinositide 3-kinase (PI3K) (p85/PI3K), and non-p85/PI3K. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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‡The abbreviations used are: PI3K, phosphoinositide 3-kinase; PH, pleckstrin homology; Gβγ, βγ subunits of heterotrimeric GTP-binding proteins; PI, phosphatidylinositol (locants of other phosphates on the inositol ring are shown in parentheses); PS, phosphatidylserine; GTPγS, guanosine 5′-O-(3-thiotriphosphate); βARK-PH, β-adrenergic receptor kinase fragment containing the PH domain; PLECkwt, wild-type recombinant pleckstrin; PLECkΔ, pleckstrin variant containing glycine residues in place of phosphorlylatable Ser113, Thr114, and Ser117; PLECkΔG, variant of PLECkΔ containing glutamate in place of glycine at three sites; PMMA, phorbol 12-myristate 13-acetate; CSKR, cytoskeletal fractions from resting platelets; HPLC, high pressure liquid chromatography; SH2, Src homology 2.

CHEMOTAXIS AND AGGREGATION TO PROLIFERATION AND TRANSFORMATION. The first and better known form (here designated p85/PI3K) (reviewed in Refs. 1 and 2) is a heterodimer composed of 85-kDa (p85) and 110-kDa (p110) subunits, functioning as adapter and catalytic entities, respectively. Some p85/PI3K is associated with the cytoskeleton of resting platelets. In stimulated platelets, however, more p85/PI3K is activated and recruited to the cytoskeleton and is partially dependent upon the small GTP-binding protein Rho (3–6). This isoform appears to contribute to the conversion of integrin αIIbβ3 to a fibrinogen-binding conformation (6). The second form (here designated PI3Kγ) has a 110-kDa catalytic entity that lacks a binding site for p85, but contains a potential pleckstrin homology (PH) domain (7). PI3Kγ is activated by βγ subunits of heterotrimeric G-proteins (Gβγ) (7–9) and also associates with the cytoskeletal fraction of thrombin-stimulated platelets (10).

The phosphorylation of pleckstrin, a cytoplasmic protein that contains two different PH domains separated by a 150-residue peptide, is a well known marker of platelet activation (11). The biological functions of pleckstrin are just beginning to be explored. PH domains have been reported to interact both with PI(4,5)P2 and Gβγ (12–15). We have shown that, when overexpressed in COS-1 cells, pleckstrin inhibits PI(4,5)P2 hydrolysis mediated by phospholipase C activated either by G-protein or growth factor receptors (16, 17), consistent with an interaction between pleckstrin and PI(4,5)P2. This inhibitory effect is dependent on the phosphorylation of Ser113, Thr114, and Ser117 within pleckstrin. Substitution of glycine residues at these sites creates a pleckstrin variant (PLECKΔG) that is relatively inactive, while insertion of charged residues (e.g. glutamate) creates a variant (PLECKG) that is constitutively active. Recently, we reported that a product of PI(4,5)P2-directed PI3K activity, PI(3,4,5)P3, promotes the phosphorylation of pleckstrin in platelets (18). We have investigated in this study whether pleckstrin affects p85/PI3K and/or PI3Kγ activities and whether any such effects are specific to phosphopleckstrin.

MATERIALS AND METHODS

Assay Components—Human platelets were isolated, and cytoskeletal fractions (CSKα and CSKβ) were prepared from resting or thrombin-activated (2 units/ml, 60 s) washed platelets, respectively, as described (6, 10), for assay of p85/PI3K and PI3Kγ activities. We have reported (10) that cytoskeletal preparations from resting platelets (CSKα) contain p85/PI3K activity that can be stimulated (as is true for immunoprecipitated p55/PI3K by GTPγS, but no PI3K activity; however, thrombin-activated platelet cytoskeletal fractions (CSKβ) contain both p85/PI3K and PI3Kγ. Approximately 60% of the PI3K activity of CSKβ, is inhibitable by removing endogenous Gγ with βARK-PH, i.e. is due to activated PI3Kγ (6, 10). Antibodies to α- and β-p85 isoforms were used to immunoprecipitate p85/PI3K from Triton-soluble fractions or cytosol of resting platelets (6, 10) for studies of p85/PI3K activity. p85/PI3K had...
FIG. 1. Inhibitory effects of PI(4,5)P2 on phosphorylation of PI or PI(4)P by purified PI3Kγ. Purified PI3Kγ was incubated in the presence (hatched bars) or absence (open bars) of Gβγ (1 μM) and varied amounts of phosphoinositide substrates (indicated at the bottom of the figure) according to the procedure described under "Materials and Methods." PI(3)P, PI(4)P, and PI(3,4,5)P3. The 3-phosphorylated phosphoinositide products (indicated at the top of the figure) were separated and quantitated. Results are the average of one experiment performed in duplicate. Similar results were obtained in two other experiments when PI, PI(4)P, and PI(4,5)P2 were present simultaneously.

a lower apparent Kₜₐₜₜ for PI(4,5)P2 than did recombinant PI3Kγ (7). In some experiments, platelets were incubated for 60 s at 37°C with 200 nM α-PMA or β-PMA or buffer prior to lysis with ice-cold Triton buffer and removal from the Triton-soluble fractions of p85/PI3K (98% by immunoprecipitation) and of Triton and FMA (by Extraktilg) (6). The eluates obtained were used in PI kinase assays. PI3Kγ was purified >5000-fold from fresh pig platelet cytosol by sequentially using polyethylene glycol precipitation, Q-Sepharose, gel filtration, and heparin-Sepharose column chromatography. Separation of PI3Kγ from p85/PI3K was achieved using immunosorbent resin containing coupled anti-p85 antibodies. Gu subunits had only minor effects on PI3Kγ in comparison with Gβγ, which was purified as published (19). The native size of Gβγ-responsive PI3K (PI3Kγ) was estimated to be 210 kDa following gel filtration. No p85 protein was present, as monitored by Western blotting, but p110y immunoreactivity, detected using an anti-peptide antibody (the kind gift of Dr. R. Wetzker), copurified with Gβγ-stimulated PI3Kγ activity.

The cDNAs encoding PLECKs (i.e., PLECKWT, PLECKG, and PLECKE) have been described (16, 17). The cDNA coding the interdomain region of PLECKWT was prepared by reverse transcription-polymerase chain reaction, and its sequence was confirmed. These cDNAs were subcloned into a derivative of pET-11b, a plasmid vector that expresses proteins utilizing T7 polymerase, modified to insert a six-

FIG. 2. Effect of PI(4,5)P2 substrate + PS concentration on inhibition of PI3Kγ by PLECKs. Purified PI3Kγ was assayed as described under "Materials and Methods" in the presence of 0.5 μM Gβγ ± 2 μM PLECKG (squares), PLECKG (circles), or PLECKE (triangles) and varied [PI(4,5)P2/PS] (1:1), up to 1 μM PI(4,5)P2 and 1 mM PS. Results are the means ± S.D. of two experiments performed in duplicate, presented as the percent of control activity at a given [PI(4,5)P2/PS], where control values ranged from 3 × 10⁴ to 10⁶ dpm.

from platelet cytosol (2 μg), were assayed for 5 min at 37°C, as described (6), in 50–100 μl with [32P]γATP (0.4 mM, 0.2–0.4 μCi/ml; ICN), 0.1–1 mM [PI(4,5)P2/PS, 0–4 μM Gβγ ± 2 μM PLECKWT, PLECKG, PLECKE, expressed interdomain peptide, or interdomain peptide fragments (PEPT² and PEPT⁴, 4 μM). After extraction of lipids for quantitation of HPLC-resolved [32P]PI(3,4,5)P3 aqueous portions were resolved by SDS-polyacrylamide gel electrophoresis, and 32P in pleckstrin was counted. In other studies, CSKφ was assayed for PI3Kγ activity in the absence or presence of PLECKWT or PLECKG (2 μM) or PAKR-PH (5 μM), using PI/PS, PI(4)P/PS, or PI(4,5)P2/PS (0.1 mM). Phosphorylation of added PLECKWT was also measured after resolution of proteins. In studies to examine the effect of PI(4,5)P2 on phosphorylation of PI or PI(4)P by purified PI3Kγ, assays contained 2 μg of PI3Kγ ± Gβγ (1 μM), 10 μM ATP, 10 μCi of [32P]γATP, and varied substrate: 25 or 250 μM [PI(4,5)P2, 25 μM PI, and 100 μM PS; or 25 or 250 μM PI(4,5)P2, 25 μM PI and 100 μM PS. In some experiments, PI(4,5)P2, PI(4)P, PI, and PS were presented to the enzyme together, in equimolar concentrations, versus phosphoinositide species/PS presented individually. Finally, PI kinase activities in Triton-soluble p85/PI3K-free fractions from α-PMA-, β-PMA-, or dimethyl sulfoxide/buffer-treated platelets (6) were assayed with 0–2 μM added Gβγ and PI/PS, PI(4)P/PS, or PI(4,5)P/PS (1 mM each) following removal of Triton and FMA with Extraktilg (6). The formation of radiolabeled PI(3)P, PI(4)P, PI(3,4)P2, PI(4,5)P2, and PI(4,5)P2 products resulting from incubations with the various substrates was monitored after HPLC (4). The presence of pleckstrin in Triton-soluble fractions was detected by Western blotting after resolution of proteins on 12.5% SDS gels (18).

RESULTS AND DISCUSSION

Phosphopleckstrin Selectively Inhibits PI3Kγ Activity—PI3Kγ, purified from platelets, was able to phosphorylate PI and PI(4)P as well as PI(4,5)P2 in a Gβγ-activated manner, but showed preferential activity with PI(4,5)P2 (Fig. 1). Gβγ was also more stimulatory for PI3Kγ acting on PI(4,5)P2 than on the other substrates. Of especial interest, PI(4,5)P2 inhibited the activity of purified PI3Kγ for PI or PI(4)P, implying that the same PI3Kγ utilizes all three substrates (Fig. 1). These studies were confirmed in experiments in which equimolar amounts of all three phosphoinositide substrates were present in the same assay mixture, in comparison with individually presented substrates (data not shown).

Pleckstrin greatly inhibited PI3Kγ activity. Under the standard assay conditions (substrate vesicles containing 100 μM each PI(4,5)P2 and PS), PLECKG (the constitutively activated pseudo-phosphorylated variant), and PLECKE (the phosphorylation-deficient variant) all were equally effective inhibitors of Gβγ-stimulated purified PI3Kγ (Fig. 2) and PI3Kγ in CSKφ (data not shown). Increasing the PI(4,5)P2 concentra-
tion to 1 mM (a concentration roughly comparable with that estimated to be present at the plasma membrane of cells) (2) by increasing the total substrate vesicle concentration and maintaining the PI(4,5)P2/PS ratio at 1:1 overcame inhibition of purified PI3K by PLECKWT and PLECKEG, but not inhibition by PLECKE. This diminished inhibition may have resulted in part from the decreased effective concentration of PLECK, assuming that it is bound to PI(4,5)P2, thereby decreasing the encounters between PLECKs and PI3K, and/or from competition by PI(4,5)P2 for a PLECK-binding site on PI3K. In this scenario, PLECKEG would compete most efficiently for PI3K, as it appears likely (based on studies with other PH domains; see below) that PLECKs bind to PI(4,5)P2 more efficiently when Gβγ is present. In any event, these results suggest that all three variants have at least weak inhibitory effects, but that PLECKEG, and hence phosphopleckstrin, has a greater affinity for Gβγ/PI(4,5)P2 than does PLECKWT or PLECKE, or that

**Fig. 4. Gβγ overcomes the inhibitory effects of PLECKE on Gβγ-stimulated PI3K.** Purified PI3K was assayed with 0.1 mM (circles) or 1 mM (triangles) PI(4,5)P2/PS, with solid symbols or without (open symbols) PLECKE (2μM) and varied [Gβγ]. Results of one experiment representative of two performed in duplicate are shown. As for PLECKEG, none of the other PLECKs (not shown) inhibited “basal” PI3K activity (i.e., in the absence of Gβγ).

PLECKE (phosphopleckstrin) has a greater affinity for PI3K than does PLECKWT or PLECKE. Given the molar excess of PI(4,5)P2 over PLECKs under all conditions (25:1 to 250:1), it seems unlikely that PLECKs (2μM) are acting simply as competitors for PI(4,5)P2, somehow sequestering this substrate. In contrast, Gβγ (0.5μM) would be stoichiometrically limited. At the 0.1 mM substrate concentration noted above, PLECKE and PLECKWT, but not PLECKE, were effective inhibitors of PI3K in CSKA (Fig. 3A), whereas only PLECKEG inhibited purified PI3K (Figs. 2 and 3A). The apparent discrepancy between the inhibitory activity of PLECKWT on CSKA versus purified PI3K is readily attributable to the ability of CSKA to phosphorylate PLECKWT (see Fig. 6), whereas the purified preparation lacks protein kinase activity capable of phosphorylating pleckstrin (data not shown). In contrast, neither PLECKWT nor any of the variants affected basal or GTPγS-stimulated p85/PI3K activity. These results were observed using either cytoskeleton from unstimulated platelets (CSKA) or immunoprecipitated p85/PI3K (Fig. 3B).

**Inhibition of PI3K by Phosphopleckstrin Is Specific for Gβγ-stimulated PI3K** by Phosphopleckstrin Is Overcome by Increasing the Concentration of Gβγ.—To evaluate further the role of pleckstrin in regulating PI(3,4,5)P3 synthesis by PI3K, we tested whether varying the concentration of Gβγ would affect the inhibitory activity of PLECKE. Inhibition by PLECKEG decreased with increasing [Gβγ] at low or high [PI(4,5)P2/PS] (Fig. 4). This implies that phosphopleckstrin might be inhibiting PI3K activity by binding to Gβγ, thereby making it unavailable to activate PI3K. Alternatively, phosphopleckstrin, while binding PI(4,5)P2 and Gβγ near PI3K, might impair the activity of PI3K by a more direct route, e.g., one dependent upon a phosphopleckstrin-susceptible site on PI3K, and overcome by Gβγ. Consistent with either of these possibilities is the finding that the basal activity of purified PI3K, i.e., in the absence of Gβγ, was not inhibited by PLECKEG (Fig. 4) or PLECKWT (data not shown) at 0.1 or 1 mM PI(4,5)P2. The elucidation in detail of the inhibitory mechanism that is at work is obviously impaired by the fact that three PH domains (two on pleckstrin and one on PI3K), each potentially vying for Gβγ/PI(4,5)P2, are present in this system.

To rule out a direct inhibitory interaction between the phosphorylated interdomain region and PI(4,5)P2, Gβγ, or PI3K, two peptides and the expressed interdomain region of PLECKWT were utilized in some assays. None of these affected PI3K activity (purified or in CSKA) (data not shown).

**Inhibition of PI3K by Phosphopleckstrin Is Specific for**
PI(4,5)P₂ Phosphorylation—Since PI3K can phosphorylate all three phosphoinositides (Fig. 1), we determined whether the inhibitory effects of pleckstrin were restricted to PI(4,5)P₂. In contrast to their effects on the phosphorylation of PI(4,5)P₂, neither PLEC Ki nor PLECK WT altered the phosphorylation at the 3-OH position of PI or PI(4)P by PI3Kγ in CSK A (Fig. 5). Yet, the fragment of β-adrenergic receptor kinase that contains a PH domain and binds Gβγ (βARK-PH) (10) was inhibitory for all three substrates (Fig. 5). Clearly, PLEC Ki do not act, like βARK-PH, to “chelate” Gβγ in the absence of PI(4,5)P₂. Notably also, the phosphorylation of PLECK WT that occurred in the presence of PI(4,5)P₂ was more than three times that observed with PI or PI(4)P (Fig. 6). Thus, PI(4,5)P₂ strongly augments pleckstrin phosphorylation by activated cytoskeletal fractions, which may be related to the ability of the PI(4,5)P₂ 3-kinase product, PI(3,4,5)P₃, to stimulate local protein kinase activity and thereby phosphorylate pleckstrin (18). The absence of pleckstrin phosphorylation was not the sole reason for the lack of effect of PLECK WT on PI3Kγ acting on PI or PI(4)P, however, since the pseudo-phosphorylated variant, PLEC K, also did not inhibit PI3Kγ when PI or PI(4)P was employed. It appears, therefore, that inhibitory effects of pleckstrin are specific for PI(4,5)P₂ substrate, consistent with a recent report that the bisphosphate group is necessary for binding of the inositol ring to PH domains (20). The data also imply that binding of PI(4,5)P₂ by phosphopleckstrin may be necessary either for phosphopleckstrin to bind Gβγ optimally or to hinder binding of Gβγ by PI3Kγ, or otherwise to allow an inhibitory interaction between phosphopleckstrin and PI3Kγ. The first interpretation would be in keeping with the findings of Lefkowitz and co-workers (14, 15) that suggest a cooperativity between PI(4,5)P₂ and Gβγ for binding to PH domains.

Phosphorylation of Endogenous Pleckstrin in Vivo Mimics the Inhibitory Effect of PLEC K in Vitro—To begin to ascertain the relevance of pleckstrin phosphorylation by intact platelets...
specifically with respect to PI3K activity, we assessed the effects of prior treatment of platelets with protein kinase C-activating or -nonactivating isomers of PMA (β-PMA and α-PMA, respectively) or dimethyl sulfoxide (vehicle)/buffer on several PI kinase activities. All Triton-soluble fractions contained traces of Gβγ; as detected by Western blotting (data not shown). As illustrated in Fig. 7, although all three PI3K activities were enhanced by additional exogenous Gβγ, only PI(4,5)P₂-directed PI3K activity was inhibited significantly by prior treatment of platelets with β-PMA to stimulate pleckstrin phosphorylation in vivo. α-PMA was without effect (data not shown). Consistent with the data shown in Fig. 4, the inhibitory effect was overcome by inclusion of increasing concentrations of Gβγ, thereby decreasing inhibition from 55% (without exogenous Gβγ) to 15% (with 2 μM exogenous Gβγ). The selectivity of the β-PMA activity is also consistent with the formation of phosphopeckstrin in response to β-PMA, but not α-PMA (Fig. 8) (6, 10), and the inhibitory effect with respect to PI(4,5)P₂ substrate is in keeping with our observation that inhibition by phosphopleckstrin is restricted to PI3Kγ acting on PI(4,5)P₂ (Fig. 5).

These findings have implications for the generation of PI(3,4)P₂ as well as PI(3,4,5)P₃ in intact cells. PI(3,4,5)P₃ accumulates transiently in stimulated cells, whereas PI(3,4)P₂ increases in a more sustained manner, after a slight delay (21–23). One would thus predict that if PI(3,4)P₂ is formed in vivo primarily as a result of phosphorylation of PI(4)P by PI3Kγ, as opposed to 5-phosphatase-mediated hydrolysis of PI(3,4,5)P₃, phosphoseckstrin should have relatively little effect on accumulations of PI(3,4)P₂. Of further interest, neither PMA nor Gβγ affects PI(3,4)P₂ or PI(4)P accumulation. This implies that neither phosphopleckstrin nor Gβγ regulates PI(4)P β-kinase or PI 4-kinase activities. Based upon our findings, we would predict that, under conditions of maximum pleckstrin phosphorylation, the activity of p85/PI3K in agonist-activated intact platelets would be favored over PI(4,5)P₂-directed PI3Kγ. Preliminary studies support this expectation in that brief exposure of platelets to β-PMA prior to thrombin receptor-directed agonists decreases the production of PI(3,4,5)P₃ formed by PI3Kγ.

A scheme depicting the “feedback” inhibitory mode for PI3Kγ is presented in Fig. 9. We propose that the rapid phosphorylation of pleckstrin in activated cells should have important inhibitory consequences for Gβγ and PI(4,5)P₂-requiring signals. In the platelet, PI(3,4,5)P₂ and diacylglycerol-stimulated protein kinase(s) may well target Gβγ-activated and PI(4,5)P₂-directed PI3Kγ and phospholipase Cβ by phosphorylating pleckstrin at intracellular locales near these lipid-metabolizing signaling enzymes. Potentially, phospholeckstrin may be generated by kinase(s) stimulated by three different routes (i.e. phospholipase C→diacylglycerol, p85/PI3K→PI(3,4,5)P₃ and PI3Kγ→PI(3,4,5)P₃). It is possible that each pathway participates in inhibiting the synthesis of PI(3,4,5)P₃ by PI3Kγ.

One would expect that there should be a negative regulatory mechanism for p85/PI3K as well, even though pleckstrin/pleckstrin is not involved directly. A potential route for inhibition of p85/PI3K in vivo might involve the ability of PI(3,4,5)P₃ to compete with phosphorytrosine for binding to SH2 domains of p85 (24), thereby perhaps removing platelet p85/PI3K from an important membrane locale. Binding of PI(3,4,5)P₃ to SH2 domains may even be directly inhibitory for p85/PI3K activity, as well as for localization, although it has not been demonstrated that such occupancy by PI(3,4,5)P₃ is not as stimulatory for p85/PI3K as is occupancy by phosphoryrosines. It is also noteworthy that PI(3,4)P₂ is significantly less effective than PI(3,4,5)P₃ in binding to SH2 domains (24). This would gradually favor PI(3,4)P₂ generation by p85/PI3K acting on PI(4)P, leading to a delayed increase in PI(3,4)P₂, as observed (21–23). PI(3,4,5)P₃, formed by PI3Kγ, may also compete with phosphorytrosines for p85/PI3K, and in this setting, inhibition of PI3Kγ by phospholeckstrin could relieve the

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**Fig. 8. Effect of treatment of platelets with PMA on accumulation of phospholeckstrin in Triton-soluble fractions.** Proteins in Triton-soluble fractions utilized for the experiments in Fig. 7 were resolved on SDS gels as described under “Materials and Methods” and immunoblotted for pleckstrin (18). Lane 1, dimethyl sulfoxide/buffer; lane 2, α-PMA; lane 3, β-PMA. The upper band comigrated with ³²P-labeled pleckstrin in phosphorylation assays.

**Fig. 9. Scheme showing the inhibition of PI3Kγ, PI(4,5)P₂, PI(3,4,5)P₃, PI(3,4)P₂, PI(4)P, IP₃, inositol 1,4,5-trisphosphate; DAG, diacylglycerol. Protein kinase Cε and -δ (PKC) are shown as putative, not proven, targets for PI(3,4,5)P₃ in vivo. The double bar indicates inhibition of the pathway catalyzed by PI3Kγ. PLC, phospholipase C.**

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PI3K-linked inhibition by PI(3,4,5)P3 of p85/PI3K, favoring p85/PI3K activity. Our preliminary studies indicate that this is likely. Based on the data that we have presented above, it appears that phosphopleckstrin, in amounts generated in vivo, may bind Gβγ avidly when associated with PI(4,5)P2 at the plasma membrane, more avidly than does pleckstrin, and may also interact locally with PI3K. The result is that the formation of PI(3,4,5)P3 by PI3K is inhibited. Studies are currently underway to define the roles of the two pleckstrin PH domains and the phosphorylation of the interdomain in achieving this inhibitory effect.

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