Activation of the 43 kDa Inositol Polyphosphate 5-Phosphatase by 14-3-3ζ†

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ABSTRACT: The 43 kDa inositol polyphosphate 5-phosphatase (5-phosphatase) hydrolyzes and thereby inactivates the second messenger molecules inositol 1,4,5-trisphosphate [Ins(1,4,5)P_3] and inositol 1,3,4,5-tetrakisphosphate in a signal terminating reaction. Recent studies have shown that the platelet protein pleckstrin forms a complex with the 43 kDa 5-phosphatase and activates Ins(1,4,5)P_3 hydrolysis 2-fold [Auethavekiat, V., Abrams, C. S., & Majerus, P. W. (1997) *J. Biol. Chem. 272*, 1786−1790]. We now show that another platelet protein, 14-3-3ζ, forms a complex with the 43 kDa 5-phosphatase and thereby activates the hydrolysis of Ins(1,4,5)P_3. Both pleckstrin and 14-3-3ζ contain one or more pleckstrin-homology domains, both are present in platelet cytosol, and both dimerize and form complexes with other signalling proteins. Purified platelet pleckstrin and 14-3-3ζ enhanced the rate of the hydrolysis of Ins(1,4,5)P_3 by the 43 kDa 5-phosphatase 1.9- and 3.8-fold, respectively, but did not activate the 75 kDa 5-phosphatase. We have demonstrated that the mechanism of 5-phosphatase activation by 14-3-3ζ results from specific complex formation between the 43 kDa 5-phosphatase and 14-3-3ζ. Recombinant 43 kDa 5-phosphatase bound to recombinant glutathione *S*-transferase (GST)/14-3-3ζ fusion protein, but not GST alone, immobilized on glutathione−Sepharose. A potential 14-3-3 binding motif was located in the 43 kDa, but not the 75 kDa, 5-phosphatase. The motif "[363]RSESEE" is present in close proximity to the proposed catalytic domain of the 43 kDa 5-phosphatase. A synthetic peptide corresponding to the putative 14-3-3 binding motif demonstrated specific, saturable binding to purified ^{125}I-14-3-3, with a K_d of 92 nM. In addition, platelet cytosolic 5-phosphatase bound to recombinant 14-3-3ζ immobilized on glutathione−Sepharose. Thus, 14-3-3ζ serves in human platelets to activate the 43 kDa 5-phosphatase and may thereby function to prevent generation of Ins(1,4,5)P_3 -mediated calcium release in unstimulated platelets.

Agonist-stimulated hydrolysis of phosphatidylinositol 4,5-bisphosphate [PtdIns(4,5)P_2][1] by phospholipase C results in the formation of the second messenger molecules inositol 1,4,5-trisphosphate [Ins(1,4,5)P_3] and diacylglycerol. The latter moiety activates protein kinase C (PKC). Ins(1,4,5)-P_3 is also a substrate for the enzyme Ins(1,4,5)P_3 3-kinase in a reaction which produces another second messenger, inositol 1,3,4,5-tetrakisphosphate [Ins(1,3,4,5)P_4]. Both Ins-(1,4,5)P_3 and Ins(1,3,4,5)P_4 are involved in the agonist-induced mobilization of intracellular calcium. Ins(1,4,5)P_3 binds to specific receptors and stimulates the transient release of calcium from intracellular stores, whereas Ins(1,3,4,5)P_4 is believed to facilitate calcium influx at the plasma membrane [reviewed in Berridge (1983, 1993) and Majerus 1992)].

The metabolism of Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 by the inositol polyphosphate 5-phosphatase (5-phosphatase) family of enzymes serves a signal terminating function via the production of the inactive metabolites Ins(1,4)P_2 and Ins-(1,3,4)P_3, respectively. The 5-phosphatases can be subdivided into several groups according to such characteristics as molecular weight, substrate specificity, and associations formed with other signalling molecules [reviewed in Majerus (1996) and Mitchell et al. (1996)].

Type I enzymes are small proteins and hydrolyze only Ins-(1,4,5)P_3 and Ins(1,3,4,5)P_4. Only one type I 5-phosphatase has been cloned, the 43 kDa 5-phosphatase (also called 5-phosphatase I) (Connolly et al., 1985; Laxminarayan et al., 1993, 1994; Verjans et al., 1994). Type II enzymes are larger (69−160 kDa) and hydrolyze the soluble substrates Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 as well as the phosphoinositides PtdIns(4,5)P_2 and phosphatidylinositol 3,4,5-trisphosphate [PtdIns(3,4,5)P_3] (Matzaris et al., 1994; Zhang et al., 1995; Jefferson & Majerus, 1995). These include the 75 kDa 5-phosphatase, the protein defective in Lowe's oculocerebrorenal syndrome and synaptojanin (Mitchell et al., 1989; Attree et al., 1992; McPherson et al., 1996). Type III 5-phosphatases, such as SHIP (for SH2-containing inositol

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[1] Abbreviations: BSA, bovine serum albumin; GST, glutathione *S*-transferase; 14-3-3ζ−GST, glutathione *S*-transferase−14-3-3ζ fusion protein; Ins(1,3,4,5)P_4, inositol 1,3,4,5-tetrakisphosphate; Ins(1,4,5)-P_3, inositol 1,4,5-triphosphate; IPTG, isopropyl β-D-thiogalactopyranoside; K_d, dissociation constant; kDa, kilodalton(s); LB/Amp, Luria broth supplemented with ampicillin; MBP, maltose binding protein; OD, optical density; PH, pleckstrin homology; PKC, protein kinase C; 5-phosphatase; inositol polyphosphate 5-phosphatase; 5-phosphatase I, platelet type I 5-phosphatase; PtdIns(3,4,5)P_3, phosphatidylinositol 3,4,5-triphosphate; PtdIns(4,5)P_2, phosphatidylinositol 4,5-bisphosphate; PtdIns 4-P, phosphatidylinositol 4-phosphate; r43k−MBP, recombinant 43 kDa 5-phosphatase fusion protein; SDS, sodium dodecyl sulfate; SDS−PAGE, SDS−polyacrylamide gel electrophoresis; SH, *Src* homology; SHIP, SH2-containing inositol phosphatase.

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phosphatase), are unique in that they hydrolyze only Ins(1,3,4,5)P_4 and PtdIns(3,4,5)P_3 (Damen et al., 1996; Lioubin et al., 1996.) Furthermore, they possess SH2 and proline-rich domains and are distinguished by their association with other signalling proteins such as Shc and Grb2.

The pleckstrin homology (PH) domain is a recently described motif, which has been identified in numerous signalling and cytoskeletal proteins (Haslam et al., 1993; Gibson et al., 1994). The domain binds both soluble and lipid inositol phosphates as well as several protein substrates (Lemmon et al., 1995). Several studies by Abrams et al., (1995a,b) have implicated the PH domains of the PKC substrate pleckstrin, in the negative regulation of agonist-induced hydrolysis of PtdIns(4,5)P_2 by PLCβ and PLCγ. This inhibition is believed to be mediated via the binding of the PH domains of pleckstrin to PtdIns(4,5)P_2 (Abrams et al., 1995a). Given that PH domains have been demonstrated to bind Ins(1,4,5)P_3, Ins(1,3,4,5)P_4, and PtdIns(4,5)P_2 (Harlan et al., 1994; Lemmon et al., 1995; Davis & Bennett, 1994; Lombardo et al., 1994), it is possible that such association alters the interaction of these substrates with the 5-phosphatases. Majerus and colleagues have recently demonstrated that pleckstrin forms a complex with and activates the 43 kDa 5-phosphatase 2-fold, following thrombin stimulation of platelets (Auethavekiat et al., 1997). We have examined the interaction between the 43 kDa 5-phosphatase and another PH domain containing platelet protein 14-3-3ζ and demonstrated this scaffolding protein specifically binds to the 43 kDa 5-phosphatase, resulting in a 4-fold increase in Ins(1,4,5)P_3 hydrolysis.

EXPERIMENTAL PROCEDURES

The Q Sepharose, SP Sepharose, glutathione−Sepharose, and ATP were obtained from Pharmacia. The hydroxylapatite resin was from BioRad, and all radionucleotides were from Dupont−NEN. The rabbit IgG was from Silenus Laboratories, Australia. All other reagents were of analytical grade and were obtained from Sigma Chemical Co. St. Louis, MO.

Measurement of Ins(1,4,5)P_3 Hydrolysis. Hydrolysis of Ins(1,[^{32}P]4,[^{32}P]5)P_3 was determined as previously described (Downes et al., 1982; Connolly et al., 1985). Unless otherwise indicated, the concentration of Ins(1,4,5)P_3 in each assay was 30 μM.

Purification of 43 and 75 kDa 5-Phosphatases. The 43 kDa placental membrane-associated 5-phosphatase was purified to homogeneity from human placental extracts as described (Laxminarayan et al., 1993). The specific activity of purified enzyme was 250 μmol of Ins(1,4,5)P_3 hydrolyzed min^{-1} (mg of protein).$^{-1}$ Enzyme-containing fractions were concentrated before storage at −70 °C. The separation of cytosolic type II 5-phosphatase from the type I enzyme was performed as previously described (Mitchell et al., 1989; Ross et al., 1991; Matzaris et al., 1994).

Purification of Platelet 14-3-3ζ. Platelet 14-3-3ζ was purified from platelet cytosol to homogeneity as described (Du et al., 1994). The protein's identity was confirmed by amino acid sequence analysis of the purified protein (Ward et al., 1996).

Purification of Recombinant 43 kDa 5-Phosphatase. The cDNA encoding the murine 43 kDa 5-phosphatase was obtained from a mouse brain cDNA library and subcloned into the pMAL-c2 expression vector. Induction and purification of the recombinant 43 kDa 5-phosphatase fusion protein (r43k−MBP) were performed according to the method of Erneux et al. (1995).

Purification of Platelet Pleckstrin. The purification was a modification of a previously described method (Imaoka et al., 1983). Briefly, platelet cytosol (1.7 g) was dialyzed overnight against 15 mM MES, pH 6.5, 11 mM Tris, 2 mM EGTA, and 4 mM 2-mercaptoethanol and then applied to Q Sepharose (1.5 × 20 cm), and 15 mL fractions were eluted with a 700 mL linear gradient of 0−0.3 M NaCl in the dialysis buffer. Fractions were subjected to SDS−PAGE analysis, and those fractions eluting between 30 and 50 mM NaCl and containing a p47 band were pooled, made 40 mM with respect to potassium phosphate, pH 6.8, and applied to a 12 × 1.5 cm hydroxylapatite column. Proteins were eluted with a 400 mL gradient of 0.05−0.50 M potassium phosphate, pH 7.0, in 50 mM K_2HPO_4·KH_2PO_4, pH 7.0, containing 10% glycerol, and 10 mL fractions were collected. Those fractions from the hydroxylapatite chromatography which contained the strongest p47 band as demonstrated by SDS−PAGE were pooled and dialyzed overnight at 4 °C against 15 mM MES, 11 mM Tris, pH 6.5, containing 2 mM EGTA and 4 mM 2 mercaptoethanol and applied to a SP Sepharose (0.5 × 7 cm) column. Proteins were eluted in 4 mL fractions with a 50 mL gradient of 0−0.2 M NaCl in the dialysis buffer. During the purification, collected fractions were analyzed by 10% SDS−PAGE and phosphorylation by PKC and assayed for Ins(1,4,5)P_3 5-phosphatase activity.

Phosphorylation of Pleckstrin by Protein Kinase C. One hundred nanograms of purified pleckstrin and 15 ng of PKC were incubated at 30 °C in a reaction volume of 25 μL in the presence of 20 mM Tris-HCl, pH 7.4, 25 mM 2-mercaptoethanol, 40 μg/mL phosphatidylserine, 0.8 μg/mL 1,2-diolein, 0.5 mM $CaCl_2$, 5 mM $MgCl_2$, and 100 mM [γ-^{33}P]ATP (1 × 10^4 cpm/pmol) for 0−30 min. Reactions were stopped by the addition of 15 μL of SDS−PAGE sample buffer. The samples were then boiled for 2 min and subjected to 10% SDS−PAGE analysis and autoradiography.

Effect of Pleckstrin or 14-3-3ζ on the 43 and 75 kDa 5-Phosphatase Activity. Ins(1,[^{32}P]4,[^{32}P]5)P_3 hydrolysis by the 43 or 75 kDa Ins(1,4,5)P_3 5-phosphatase was measured in the presence of 0.1 μM pleckstrin or incremental amounts (0−2 μM) of purified platelet 14-3-3ζ. The total protein concentration was kept constant at 1.5 μg in all reactions by the addition of rabbit IgG to counter any nonspecific activation of the 5-phosphatase.

Effect of Phosphorylated Pleckstrin on 43 and 75 kDa 5-Phosphatase Activity. Pleckstrin (0.1 μM) was incubated in the presence or absence of PKC (25 ng) in a reaction volume of 40 μL with 50 mM MES, pH 6.5, 3 mM $MgCl_2$, 2 mM EGTA, and 100 μM ATP at 30 °C for 10 min. The reactions were stopped by placing on ice, and then 30 μM Ins(1,[^{32}P]4,[^{32}P]5)P_3 and either type I or type II 5-phosphatase were added to a total reaction volume of 50 μL. The reactions were kept at 37 °C for a further 15 min and then stopped. The phosphorylation of pleckstrin by PKC was confirmed by using 100 μM [γ-^{33}P]ATP (1 × 10^4 cpm/pmol) in a control reaction and subjecting the products of this reaction to SDS−PAGE analysis and autoradiography.

Effect of Pleckstrin and 14-3-3ζ on the Hydrolysis of Increasing Concentrations of Ins(1,4,5)P_3 by the 43 kDa 5-Phosphatase. The rate of hydrolysis of 1−15 μM Ins(1,-

[^{32}P]4,[^{32}P]5)P_3 by the 43 kDa placental 5-phosphatase was determined in the presence or absence of either 0.1 μM pleckstrin or 0.8 μM 14-3-3ζ. The reaction volume was increased to 50 μL.

Induction of a 14-3-3ζ Fusion Protein. The cDNA for bovine 14-3-3ζ was derived from PCR amplification of full-length 14-3-3ζ (clone FS62) using the primers FS62−*Bam*H1, 5′-GAA CAT GGA TCC ATG GAT AAA AAT GAG CTG GTT CAG AAG-3′, and FS62−*Eco*R1 5′-TTG GAG AAT TCG TTA ATT TTC CCC TCC TTC TCC TGC TTC AGC-3′. The resulting fragment was subcloned into the pGEX2 plasmid and transformed into competent *E. coli* DH5a. Induction of the fusion protein was essentially performed according to the method of Smith and Johnson, (1988).

14-3-3ζ−GST and Recombinant 43 kDa 5-Phosphatase Binding Studies. All steps were performed at 4 °C. The 14-3-3ζ−GST fusion protein was induced as described above. Detergent-extracted bacterial lysate containing recombinant 14-3-3ζ−GST (approximately 600 μg) was mixed with glutathione−Sepharose (2 mL) beads for 4−16 h, and then washed 3 times with 10 bed volumes of 20 mM Tris, pH 7.4, containing 150 mM NaCl. Purified recombinant 43 kDa 5-phosphatase, expressed as a pMAL fusion protein (40 μg), was mixed overnight with the washed 14-3-3ζ−GST bound to glutathione−Sepharose beads. The next day, the glutathione Sepharose was poured into a 2 mL column and was washed with 100 mL of 20 mM Tris, pH 7.4, containing 150 mM NaCl, prior to the elution of the immobilized 14-3-3ζ−GST with 10 mL of 10 mM reduced glutathione in 50 mM Tris, pH 8.0. Fractions (0.5 mL) were collected and analyzed by SDS−PAGE and determination of Ins(1,4,5)P_3 5-phosphatase activity.

14-3-3ζ and Platelet 5-Phosphatase I Binding Studies. The platelet cytosol was repeatedly reapplied to the immobilized 14-3-3ζ−GST (600 μg) on glutathione−Sepharose for 5 h by means of a peristaltic pump, and then washed with 25 column volumes of 20 mM Tris, pH 7.2, containing 150 mM NaCl. The fusion protein was eluted by the application of 10 mL of 10 mM reduced glutathione in 50 mM Tris, pH 8.0. Fractions (0.5 mL) were then collected for determination of Ins(1,4,5)P_3 5-phosphatase activity and analysis by SDS−PAGE.

Conjugation of a Peptide Derived from the Sequence of the 43 kDa 5-Phosphatase to Affigel Resin. A synthetic peptide corresponding to the putative 14-3-3ζ binding domain of the 43 kDa 5-phosphatase (ELVLRSESEEKVV), containing an N-terminal cysteine to facilitate coupling, was purified by reverse-phase HPLC and characterized by mass spectrometry (Chiron Mimitopes, Australia). The peptide was coupled to bovine serum albumin (BSA) (1 mg of peptide/10 mg of BSA) with *m*-maleimidobenzoyl-*N*-hydroxysuccinimide as previously described (Lerner et al., 1981). The peptide−BSA conjugates or the peptide alone were coupled to a 1:1 mixture of Affigel 10 and 15 (10 mg of peptide−BSA/5 mL of resin, 1 mg of peptide/5 mL of resin) according to the manufacturer's instructions (BioRad Laboratories, USA). The resins coupled to the peptide and the corresponding peptide−BSA conjugate were pooled, washed extensively in 20 mM Tris, pH 7.4, and 150 mM NaCl, and resuspended as a 1:1 suspension in the same buffer.

Binding of 14-3-3ζ to Affigel Coated with a Synthetic Peptide Derived from the 43 kDa 5-Phosphatase. The binding of 14-3-3ζ to the peptide-coated resin was measured by incubating 25% (v/v) peptide-conjugated resin, 0.5 μg/mL ^{125}I-labeled 14-3-3ζ, and unlabeled 14-3-3ζ (0−100 μg/mL) in a final volume of 100 μL of Tris−saline supplemented with 0.1% BSA. The samples were incubated at 22 °C for 30 min, and then the peptide-coated resin was pelleted by centrifugation at 8750*g*. The amount of unbound ^{125}I-14-3-3ζ was quantitated by counting aliquot's of the supernatant in a γ-counter. Nonspecific binding was estimated in a parallel assay, by incubation with a 50-fold excess of unlabeled 14-3-3ζ.

RESULTS

The platelet protein pleckstrin forms a 1:1 complex with the 43 kDa 5-phosphatase, and following phosphorylation of pleckstrin, the 5-phosphatase is activated 2-fold (Auethavekiat et al., 1997). We chose another platelet protein, 14-3-3ζ, to compare its effect on 43 kDa Ins(1,4,5)P_3 5-phosphatase activity with that of pleckstrin. These proteins share a number of features in that they are both present abundantly in platelet cytosol, and contain one or more PH domains (Imaoka et al., 1983; Haslam et al., 1993; Dellambra et al., 1995; Aitken, 1996; Wheeler-Jones et al., 1996). In addition, both pleckstrin and 14-3-3ζ have been observed to form dimers (Jones et al., 1995a; McDermott & Haslam, 1996). In the latter case, the hetero- and homodimerization of 14-3-3 isoforms functions as the basis of their proposed role as scaffolding proteins (Jones et al., 1995b).

We measured the hydrolysis of Ins(1,4,5)P_3 by both the 43 kDa and 75 kDa 5-phosphatases in the presence of increasing amounts of 14-3-3ζ. Although 14-3-3ζ has been reported to interact with PKC, unlike pleckstrin, it is not a substrate for this enzyme and would appear to mediate its activity rather than be regulated by PKC itself (Aitken, 1996). We therefore used unmodified 14-3-3ζ, purified to homogeneity from human platelet cytosol, for these experiments. In the presence of incremental concentrations of 14-3-3ζ, the hydrolysis of Ins(1,4,5)P_3 by the purified 43 kDa 5-phosphatase was increased, up to a maximum of 3.8-fold at 2 μM 14-3-3ζ (Figure 1A). In contrast, the activity of the 75 kDa platelet Ins(1,4,5)P_3 5-phosphatase was unaffected by the addition of 14-3-3ζ (Figure 1B).

In control studies, platelet pleckstrin was purified to homogeneity using a modified method of that described by Imaoka et al., (1983) (Figure 2A). Pleckstrin was separated from platelet 5-phosphatase I by chromatography on SP-Sepharose (Figure 2B) in contrast to other studies where the two proteins copurified and pleckstrin was incorrectly identified as 5-phosphatase I (Connolly et al., 1986). Ins(1,4,5)P_3 hydrolysis was measured in the presence of nonphosphorylated pleckstrin and pleckstrin which had been phosphorylated *in vitro* by incubation with PKC (Figure 2C). Hydrolysis of Ins(1,4,5)P_3 by the purified 43 kDa 5-phosphatase was increased 1.9-fold in the presence of nonphosphorylated pleckstrin. The fold activation of the enzyme was consistent with the maximum activation observed by Auethavekiat et al. (1997) at any concentration of pleckstrin; however, in contrast to their findings, the effect was unchanged by phosphorylation of the pleckstrin, prior to its addition to the assay. Phosphorylation of purified pleckstrin was confirmed by trace-labeling the reaction with [γ-^{33}P]-

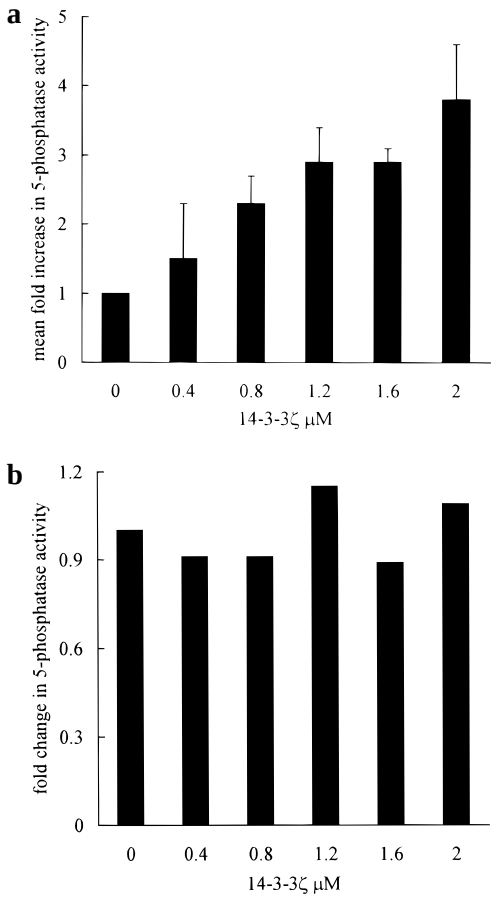

FIGURE 1: Effect of 14-3-3ζ on 43 and 75 kDa Ins(1,4,5)P_3 5-phosphatase activity. The hydrolysis of 30 μM Ins(1,4,5)P_3 by purified 43 kDa (A) [6.6 nmol of Ins(1,4,5)P_3 hydrolyzed min^{-1} mL^{-1}] or 75 kDa platelet 5-phosphatase (B) [0.16 nmol of Ins(1,4,5)P_3 hydrolyzed min^{-1} mL^{-1}] was determined in the presence of 0−2 μM purified platelet 14-3-3ζ. The results shown are the mean of three experiments and are expressed as the fold increase in enzyme activity, compared to control reactions performed in the absence of 14-3-3ζ.

ATP and analysis of the phosphorylated protein by SDS−PAGE and autoradiography (Figure 2D). It has been reported that native unphosphorylated pleckstrin can stimulate 5-phosphatase enzyme activity when added in a 10:1 pleckstrin:phosphatase molar ratio, consistent with our assay conditions (Auethavekiat et al., 1997).

In contrast to the results observed with the 43 kDa 5-phosphatase, pleckstrin did not stimulate Ins(1,4,5)P_3 hydrolysis by the 75 kDa 5-phosphatase (Figure 2C). Collectively these studies demonstrate the 14-3-3ζ activates the 43 kDa 5-phosphatase, but not the 75 kDa 5-phosphatase, at least 2-fold greater than that reported for platelet pleckstrin.

The effect of varying the concentration of Ins(1,4,5)P_3 on the activity of the 43 kDa 5-phosphatase in the presence or absence of pleckstrin or 14-3-3ζ was determined and is represented in Figure 3. A dramatic increase in 5-phosphatase activity in the reactions containing 0.1 μM pleckstrin or 0.8 μM 14-3-3ζ was apparent at all concentrations of Ins(1,4,5)P_3 used. The V_{max} of the enzyme was determined by Lineweaver−Burk plot analysis and increased by 1.8- and 3.5-fold in the presence of pleckstrin or 14-3-3ζ, respectively, whereas the apparent K_m of the 43 kDa 5-phosphatase for Ins(1,4,5)P_3 in these experiments was unchanged (data not shown). The enhancement of Ins(1,4,5)P_3 hydrolysis in the presence of 14-3-3ζ is at least 2-fold higher than that

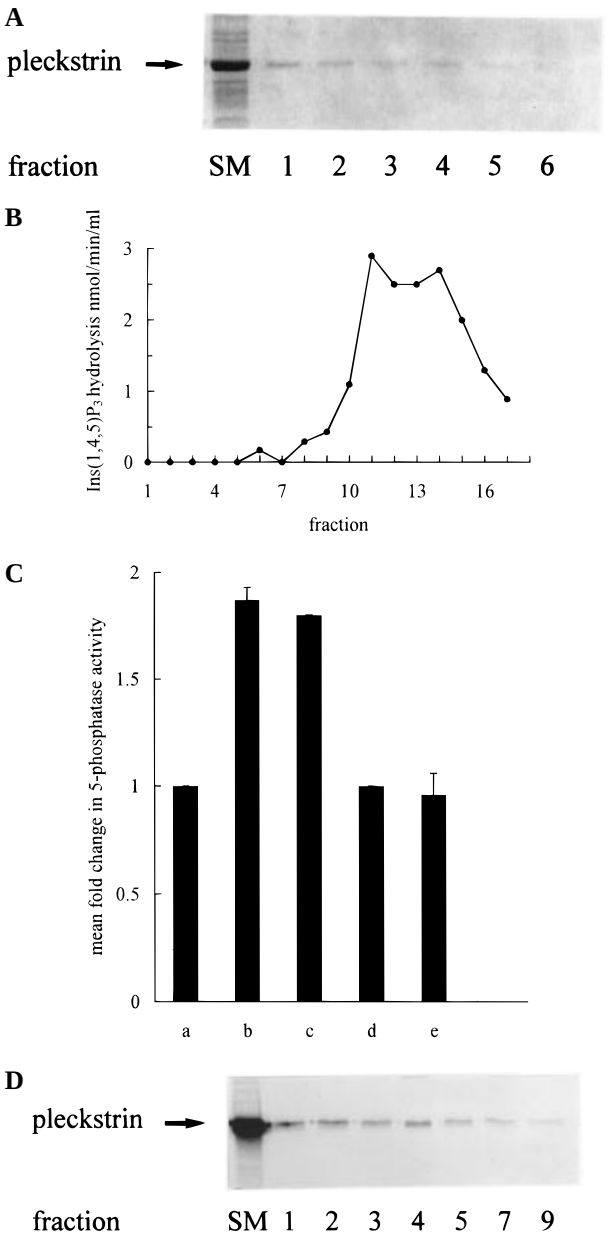

FIGURE 2: Activation of the 43 kDa 5-phosphatase by pleckstrin. (A) Pleckstrin was purified from platelet cytosol (1.2 g of starting material) as described under Experimental Procedures. Eluting fractions from SP-Sepharose (fractions 1−6) were analyzed by SDS−PAGE and Coomassie staining SM = starting material SP-Sepharose. (B) Fractions eluting from SP-Sepharose were assessed for Ins(1,4,5)P_3 5-phosphatase activity as described under Experimental Procedures. (C) The hydrolysis of Ins(1,4,5)P_3 by the 43 kDa (a−c) or 75 kDa 5-phosphatase (d, e) was determined in the presence of phosphorylated (c) or nonphosphorylated pleckstrin (b, e) (0.1 μM) as described under Experimental Procedures and expressed as the fold change in enzyme activity compared to control reactions containing no pleckstrin and represents the mean of three experiments. (D) Phosphorylation of pleckstrin by PKC was confirmed by incubation of purified fractions (1−9) with 15 ng of PKC and 100 μM [γ-^{33}P]ATP for 10 min at 30 °C. The reactions were analyzed by SDS−PAGE and autoradiography.

observed in the presence of any concentration of pleckstrin, either phosphorylated or nonphosphorylated.

Identification of a Putative 14-3-3 Binding Site in the 43 kDa 5-Phosphatase. The finding that two platelet PH domain-containing proteins, pleckstrin and 14-3-3ζ, activate the hydrolysis of Ins(1,4,5)P_3 by the 43 kDa 5-phosphatase suggests the binding of the enzyme substrate, Ins(1,4,5)P_3 by the PH domain, may play a role in enzyme activation.

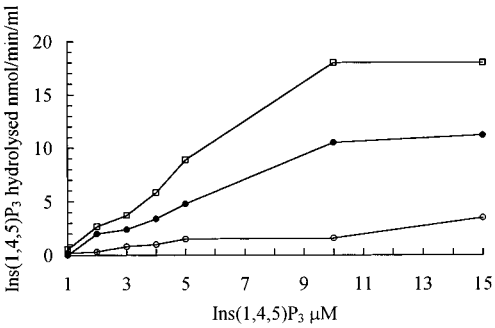

FIGURE 3: Effect of pleckstrin and 14-3-3ζ on the hydrolysis of increasing concentrations of Ins(1,4,5)P_3 by the 43 kDa 5-phosphatase. The hydrolysis of 1−15 μM Ins(1,4,5)P_3 by the 43 kDa 5-phosphatase alone (○) or in the presence of 0.1 μM pleckstrin (●) or 0.8 μM 14-3-3ζ (□) was determined by incubation with the indicated concentration of Ins(1,4,5)P_3 in 50 mM MES, pH 6.8, and 3 mM $MgCl_2$ for 15 min at 37 °C. The assay was stopped by the addition of perchloric acid and the hydrolysis of Ins(1,4,5)P_3 determined, as described under Experimental Procedures.

However, the lack of activation of the 75 kDa 5-phosphatase by either pleckstrin or 14-3-3 implies this mechanism of activation is unlikely. In addition, Auethavekiat et al. (1997) propose activation of the 43 kDa 5-phosphatase by pleckstrin is mediated by direct protein−protein interaction, although a binding site for pleckstrin on the 43 kDa 5-phosphatase was not shown.

The interaction of 14-3-3 with a wide range of different proteins has been proposed to occur via a specific phosphoserine motif, RSXS*XP, where S* is a phosphoserine (Muslin et al., 1996; Zha et al., 1996). Although it is likely that not all interactions with 14-3-3 occur at this site, a growing number of proteins which associate with and/or are modulated by 14-3-3 possess similar motifs (Aitken, 1996). Because of the discrepancy in the response of the 43 and 75 kDa 5-phosphatases to 14-3-3ζ, the amino acid sequence of each of these enzymes was examined for putative 14-3-3 binding motifs. No such motif was found in the 75 kDa 5-phosphatase, consistent with the lack of response to 14-3-3ζ *in vitro*. However, a potential 14-3-3 binding motif, ^{363}RSESEE, was identified in the C-terminal portion of the 43 kDa 5-phosphatase, in close proximity to the putative 5-phosphatase catalytic domain (De Smedt et al., 1994; Jefferson & Majerus, 1995; Communi et al., 1996; Communi & Erneux, 1996; Mitchell et al., 1996) (Table 1). Although this sequence does not contain a proline at +2, we consider it significant since replacement of this residue by other amino acids in synthetic peptides has only a minimal effect on 14-3-3 recognition (Muslin et al., 1996). Furthermore, although this motif is not known to be phosphorylated *in vivo*, Aitken (1996) has identified similar unphosphorylated sequences in several proteins as likely sites of interaction with 14-3-3.

The demonstration of 14-3-3ζ-mediated activation of the 43 kDa 5-phosphatase and the identification of a potential binding site for 14-3-3 proteins within the 43 kDa 5-phosphatase sequence are suggestive of complex formation between the two proteins. To investigate this possibility, recombinant 43 kDa 5-phosphatase (r43k−MBP) (40 μg) was applied to a glutathione *S*-transferase−14-3-3ζ fusion protein (14-3-3ζ−GST) (600 μg) which had been immobilized on a glutathione−Sepharose column, as described under Experimental Procedures. In control studies, r43k−MBP (40 μg) was applied to glutathione *S*-transferase alone (GST) (600 μg) immobilized on glutathione−Sepharose.

Table 1: Comparison of RSXSXP-Related Sequences in 14-3-3 Binding Proteins

protein	amino acid sequence[a]
43-kDa 5-phosphatase:	.... L V L R S E 366 S E E K V V ....
Raf-1:	.... Q R Q R S T 259 S T P N V H ....
Raf-1:	.... K I N R S A 621 S E P S L H ....
polyoma middle T:	.... S V M R S H 271 S Y P P T R ....
cdc25b:	.... R L F R S P 304 S M P C S V ....
PKCε:	.... E E D R S K 346 S A P T S P ....
bcr-1:	.... D K S R S P 371 S Q N S Q Q ....
B-Raf:	.... G R D R S S 364 S A P N V H ....
BAD:	.... R G R S R 136 S A P P N L ....
BAD:	.... R S R H S 112 S Y P A G T ....

[a] The consensus RSXSXP-related sequence that binds 14-3-3 in Raf-1 (Muslin et al., 1996), BAD (Zha et al., 1996), and 43-kDa inositol polyphosphate 5-phosphatase (this study) is boxed.

After application of the recombinant 43 kDa 5-phosphatase, the column was washed extensively, and elution of 14-3-3ζ−GST or GST alone was achieved with reduced glutathione. Coelution of Ins(1,4,5)P_3 5-phosphatase activity was observed with the 14-3-3ζ−GST, but not GST alone (Figure 4A,B). These results strongly suggest that the 43 kDa 5-phosphatase and 14-3-3ζ form a specific complex *in vitro*.

To further investigate the mechanism of complex formation between 14-3-3ζ and the 43 kDa 5-phosphatase, the ability of ^{125}I-labeled 14-3-3ζ to bind to a peptide corresponding to the putative 43 kDa 5-phosphatase 14-3-3 binding motif was examined. A synthetic peptide corresponding to the 43 kDa 5-phosphatase 14-3-3 binding motif was left unmodified, or coupled to BSA and then applied to Affigel resin as described under Experimental Procedures. The two resins containing peptide−BSA−Affigel or peptide−Affigel conjugates were mixed 1:1 and incubated for 30 min at room temperature in the presence of ^{125}I-14-3-3ζ (0.5 μg/mL) and unlabeled 14-3-3ζ (0−100 μg/mL). The degree of 14-3-3ζ binding was determined by centrifugation of the samples, followed by determination of the amount of unbound ^{125}I-14-3-3ζ by γ counting of the supernatants. By this method, specific binding of ^{125}I-14-3-3 to the 43 kDa 5-phosphatase peptide was demonstrated (Figure 5A), with the K_d measured at 92 nM (Figure 5B). The apparent affinity is comparable to that reported for the synthetic peptide sequences from Raf-1 (Muslin et al., 1996).

The 43 kDa 5-phosphatase peptide tested was not phosphorylated and did not correspond exactly to the motif as proposed by Muslin et al. (1996) (Table 1). The observation of specific and high-affinity binding of 14-3-3ζ to this sequence is therefore in keeping with the contention that not all 14-3-3 binding motifs are phosphorylated *in vivo* (Aitken, 1996). Furthermore, the requirement for proline at +2 in the 14-3-3 binding motif is also challenged by these findings.

Binding of 5-Phosphatase I to a 14-3-3ζ−GST. The relationship between the 43 kDa 5-phosphatase cloned from both brain and placenta (De Smedt et al., 1994; Laxmi-

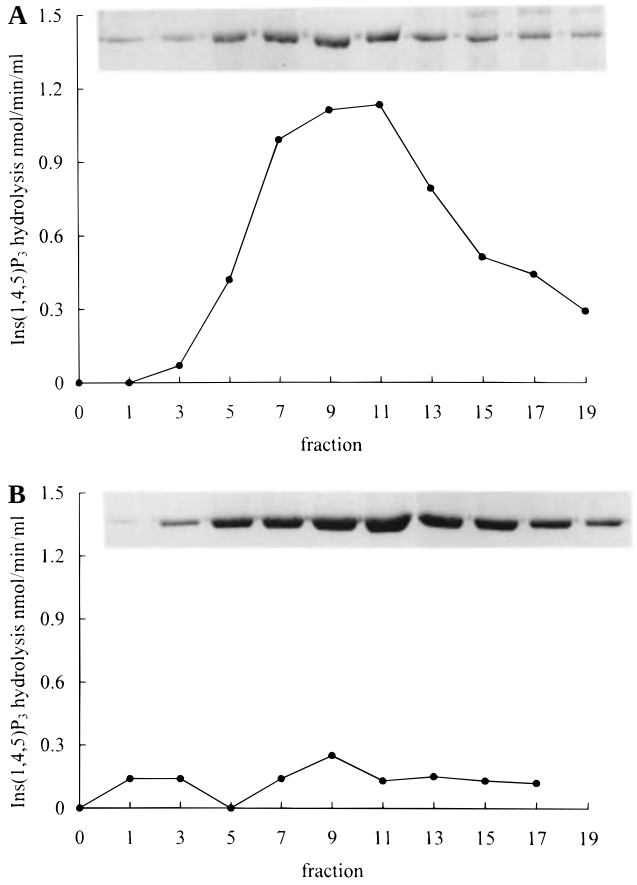

FIGURE 4: Complex formation between recombinant 43 kDa 5-phosphatase and 14-3-3ζ. Recombinant 43 kDa 5-phosphatase [40 μg, 51 nmol of Ins(1,4,5)P_3 hydrolyzed min^{-1} mL^{-1}] was applied to either GST (670 μg)-glutathione−Sepharose or 14-3-3ζ−GST (600 μg)−glutathione−Sepharose. The GST proteins were eluted with 10 mM reduced glutathione and 0.5 mL fractions collected for analysis by SDS−PAGE and measurement of Ins-(1,4,5)P_3 5-phosphatase activity. (A) Elution of 14-3-3ζ−GST. Ins-(1,4,5)P_3 activity [(●) nmol of Ins(1,4,5)P_3 hydrolyzed min^{-1} mL^{-1}] was determined for the fractions shown. The elution of 14-3-3ζ−GST in the corresponding fractions was analyzed by SDS−PAGE followed by Coomassie staining of the gel, and is shown at the top of the figure (B) Elution of GST. As for (A), except that 14-3-3ζ−GST is replaced by the same concentration of GST alone.

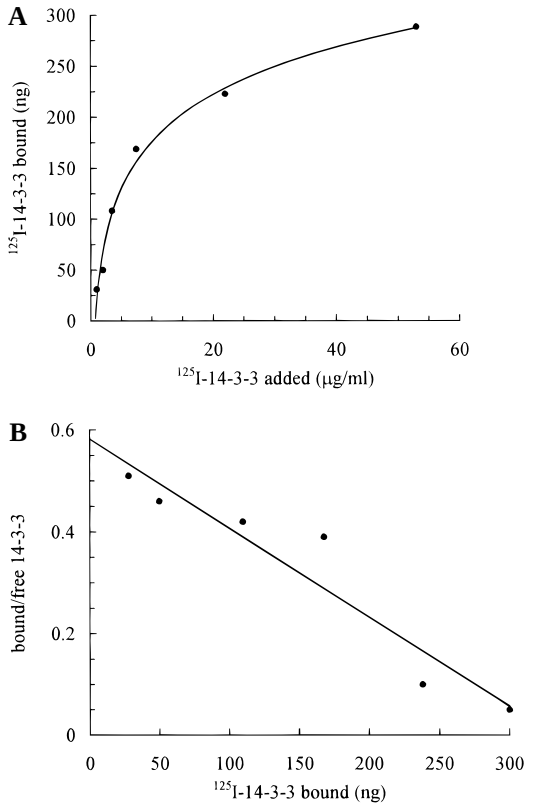

FIGURE 5: Binding of ^{125}I-14-3-3ζ to a peptide corresponding to the putative 14-3-3 binding motif of the 43 kDa 5-phosphatase. A synthetic peptide corresponding to the putative 14-3-3 binding motif of the 43 kDa 5−phosphatase (1 mg/5 mL of resin) was coupled to Affigel resin and incubated with ^{125}I-14-3-3ζ (0.5 μg/mL) and 0−100 μg/mL 14-3-3ζ for 30 min at 22 °C as described under Experimental Procedures. The amount of ^{125}I-14-3-3ζ bound to the peptide-coated resin was determined by subtraction of unbound radiolabeled 14-3-3ζ in the supernatant of each sample from the total ^{125}I-14-3-3ζ added. (A) The proportion of ^{125}I-14-3-3ζ bound was expressed as a function of ^{125}I-14-3-3ζ added. (B) Scatchard plot of data in (A).

narayan et al., 1994) and the platelet type I 5-phosphatase (5-phosphatase I) is contentious. The platelet enzyme has not been cloned or sequenced. However, these two 5-phosphatases share identical chromatographic characteristics and have the same molecular weight (Laxminarayan et al., 1993), and antibodies raised against the 43 kDa 5-phosphatase recognized the platelet enzyme in two separate studies (Verjans et al., 1990; Laxminarayan et al., 1993). Furthermore Auethavekiat and colleagues have shown complex formation between 5-phosphatase I and pleckstrin using antibodies to the recombinant 43 kDa 5-phosphatase (Auethavekiat et al., 1997). It is probable, therefore, that if the 5-phosphatase I is not identical to the 43 kDa 5-phosphatase, that they share substantial homology. For this reason, we used concentrated platelet cytosol as a relatively abundant *in vivo* source of the 5-phosphatase I, to examine its binding to recombinant 14-3-3ζ.

Recombinant 14-3-3ζ−GST (600 μg) was expressed as a GST fusion protein and immobilized on a glutathione−Sepharose column as described under Experimental Procedures. Concentrated platelet cytosol (1.2 g) was repeatedly passed over the immobilized protein and the column extensively washed before the 14-3-3ζ−GST was eluted with 10 mM reduced glutathione. As determined by SDS−PAGE analysis, 14-3-3ζ−GST was detected in the eluate at fraction 9 and eluted at a constant rate in subsequent fractions (Figure 6). Ins(1,4,5)P_3 5-phosphatase activity was detected in the same fractions and correlated with the elution of 14-3-3ζ−GST from the column, strongly suggestive of the ability of these platelet proteins to complex.

DISCUSSION

We have shown two PH domain-containing platelet proteins, pleckstrin and 14-3-3ζ, can enhance the rate of Ins-(1,4,5)P_3-mediated hydrolysis by the 43 kDa, but not the 75 kDa, 5-phosphatase. Of all the 14-3-3 isoforms, only 14-3-3ζ and 14-3-3σ possess a PH domain (Dellambra et al., 1995). The specific function of this domain has not been determined for 14-3-3ζ. However, numerous studies of the PH domains of other proteins have demonstrated their ability to bind Ins(1,4,5)P_3, PtdIns 4-P, and PtdIns(4,5)P_2. The possibility exists that the effect of 14-3-3ζ or pleckstrin on the 43 kDa 5-phosphatase, following complex formation, is mediated via an interaction between the PH domain(s) of these scaffolding proteins and Ins(1,4,5)P_3. Dimerization of 14-3-3ζ molecules may entrap Ins(1,4,5)P_3 in a low-affinity

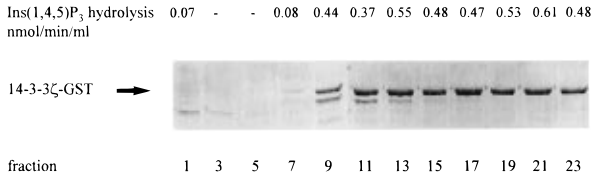

FIGURE 6: Binding of platelet 5-phosphatase I to immobilized 14-3-3ζ−GST fusion protein. Platelet cytosol (1.2 g of starting protein) was dialyzed overnight against 20 mM Tris, pH 7.2, 150 mM NaCl, and then repeatedly applied to 600 μg of 14-3-3ζ−GST immobilized on a 2 mL glutathione−Sepharose column. After extensive washing, the 14-3-3ζ−GST fusion protein was eluted with 10 mM reduced glutathione in 50 mM Tris, pH 8.0, and 0.5 mL fractions were collected. Each fraction was assayed for Ins(1,4,5)P_3 5-phosphatase activity and subjected to 10% SDS−PAGE analysis and staining with Coomassie Brilliant Blue. Lanes 1−12 = fractions 1, 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, and 23 eluted from glutathione−Sepharose. The Ins(1,4,5)P_3 5-phosphatase activity [nmol of Ins(1,4,5)P_3 hydrolyzed min^{-1} mL^{-1}] for each fraction is noted above the figure. The arrow indicates the migration of the eluted 14-3-3ζ−GST fusion protein.

interaction with the 14-3-3ζ PH domains, in effect stabilizing and localizing the Ins(1,4,5)P_3 molecules for hydrolysis by the bound 43 kDa 5-phosphatase. However, as evidenced by the lack of activation of the 75 kDa 5-phosphatase, an interaction between 14-3-3 and Ins(1,4,5)P_3 alone is insufficient to explain the enhanced 5-phosphatase activity. An intriguing possibility exists, however, that the 14-3-3ζ-PH domain and the 43 kDa 5-phosphatase−14-3-3 binding motif act together to increase Ins(1,4,5)P_3 hydrolysis. In this model, 14-3-3ζ dimers form a scaffold and tether the Ins(1,4,5)P_3. Binding of the 43 kDa 5-phosphatase to 14-3-3ζ would then stabilize the enzyme and improve its access to Ins(1,4,5)P_3. Empirical data to support this model are provided by the demonstration that 14-3-3, Ras, and Raf-1 (a substrate for Ras) form a stable ternary complex (Suen et al., 1995). The 14-3-3ζ−43 kDa 5-phosphatase association could also improve 5-phosphatase activity, by inducing a conformational change in the enzyme to enhance Ins(1,4,5)P_3 hydrolysis.

Although PH domains can bind Ins(1,4,5)P_3, it would appear that the activation of the 43 kDa 5-phosphatase by both pleckstrin and 14-3-3ζ is dependent on specific protein−protein interaction. The results of the experiments we have performed argue strongly in favor of an association between 14-3-3ζ and the 43 kDa type I 5-phosphatase, which results in increased hydrolysis of Ins(1,4,5)P_3. The association between 14-3-3 and other signalling proteins has been shown to occur via a conserved phosphoserine motif, RSXS*XP. The motif RSXS*X also binds to 14-3-3, albeit with lower affinity (Muslin et al., 1996; Zha et al., 1996). Aitken (1996) has observed that this motif is not present in all proteins demonstrated to associate with 14-3-3 *in vivo*. Similarly, in some cases, *in vivo* phosphorylation of the putative motifs has not been shown. Nevertheless, the binding of 14-3-3 to proteins containing this motif accounts for the majority of the associations observed thus far. It is interesting to speculate that the ability of 14-3-3ζ to bind nonphosphorylated ^{363}RSESEE peptide may be conferred by the presence of these negatively-charge glutamate residues (Table 1). This putative 14-3-3 binding motif in the 43 kDa 5-phosphatase is located in the C-terminal region of the protein, in close proximity to the proposed catalytic domain (Jefferson & Majerus, 1996; Communi et al., 1996; Communi & Erneux., 1996).

We have demonstrated pleckstrin activates 43 kDa 5-phosphatase-mediated hydrolysis of Ins(1,4,5)P_3 by 2-fold, which is in agreement with the maximum activation of the 43 kDa 5-phosphatase observed by Auethavekiat et al. (1997) with any concentration of pleckstrin. However, in contrast to the latter studies, we repetitively showed no further enhancement in 43 kDa 5-phosphatase enzyme activity following phosphorylation of pleckstrin. We used pleckstrin purified from human platelets, while Auethavekiat and colleagues used the recombinant protein. We cannot exclude the possibility that the platelets used to purify pleckstrin were partially activated. However, as we obtained purified pleckstrin from three different preparations, with essentially the same result on all occasions, and the purified pleckstrin demonstrated significant phosphorylation by PKC, this appears unlikely. Also from the same preparations we could clearly separate 5-phosphatase I activity from pleckstrin, which should not be achievable if the platelets were activated and the pleckstrin phosphorylated. It appears more likely that the reported variation in activation of the 5-phosphatase by nonphosphorylated pleckstrin results from differences in the 5-phosphatase enzyme preparation. In our studies, purified native 43 kDa 5-phosphatase with a specific activity of 250 μmol of Ins(1,4,5)P_3 hydrolyzed min^{-1} mg^{-1} was utilized, rather than recombinant enzyme, which is less active [120 μmol of Ins(1,4,5)P_3 hydrolyzed min^{-1} mg^{-1}]. We routinely required a 10-fold molar excess of pleckstrin or 14-3-3ζ to demonstrate activation of the native 5-phosphatase. In constrast, Majerus and colleagues used recombinant 5-phosphatase I which had been produced either in Sf9 cells or as a GST/5-phosphatase fusion protein. The former recombinant protein was only activated when incubated with phosphorylated pleckstrin, while the GST/5-phosphatase recombinant enzyme was activated by nonphosphorylated pleckstrin when the molar ratio of pleckstrin to 5-phosphatase was 10:1 (Auethavekiat et al., 1997). These latter results are consistent with our findings and imply that conformational changes in the 43 kDa 5-phosphatase when produced as a recombinant protein may result in alteration of the enzyme's activation by nonphosphorylated pleckstrin.

The association between the 43 kDa 5-phosphatase and 14-3-3ζ can occur in unstimulated platelets as shown by these studies. Several authors have noted that the signalling protein Raf-1 constitutively associates with 14-3-3 in unstimulated cells (Freed et al., 1994; Fu et al., 1994; Li et al., 1995). The effect of this association on Raf-kinase activity is contentious, with both activation and no change in activity both described. Negative regulation of signal transduction has also been reported as a consequence of 14-3-3τ association with p85/p110 PI 3-kinase in activated T cells, thereby preventing the generation of the messenger molecules PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3 (Bonnefoy-Bernard et al., 1995). Recent studies by Wheeler-Jones et al. (1996) have shown synthetic peptides derived from the 14-3-3 amino acid sequence inhibit PKC activity in human platelets. We have provided further evidence that 14-3-3ζ acts in platelets as a negative regulator of signals that lead to platelet activation. We propose the binding of 14-3-3ζ to the 43 kDa 5-phosphatase acts to up-regulate the 5-phosphatase enzyme activity, and by increasing the hydrolysis of Ins(1,4,5)P_3 in this way 14-3-3ζ may well act as a negative mediator of Ins(1,4,5)P_3-derived calcium release and thereby platelet activation. This negative regulation by 14-3-3ζ in unstimulated

platelets appears more profound than that mediated by pleckstrin, following platelet activation. Finally, both the 43 kDa 5-phosphatase and 14-3-3 have a widespread tissue and cellular distribution. In contrast, pleckstrin is only expressed in platelets and leukocytes. Therefore, it is likely that the activation of the 43 kDa 5-phosphatase by 14-3-3ζ is a more widespread mechanism for regulating 5-phosphatase enzyme activity than that achieved by pleckstrin.

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REFERENCES

Abrams, C. S., Wu, H., Zhao, W., Belmonte, E., White, E., White, D., & Brass, L. F. (1995a) *J. Biol. Chem. 270*, 14485−14492.
Abrams, C. S., Zhao, W., Belmonte, E., & Brass, L. F. (1995b) *J. Biol. Chem. 270*, 23317−23321.
Aitken, A. (1996) *Trends Cell Biol. 6*, 341−347.
Attree, O., Olivos, I., Okabe, L. C., Bailey, D. L., Nelson, R. A., Lewis, R. R., McInnes, R. R., & Nussbaum, R. L. (1992) *Nature 358*, 239−242.
Auethavekiat, V., Abrahms, C. S., & Majerus, P. W. (1996) *J. Biol. Chem. 272*, 1786−1790.
Berridge, M. J. (1993) *Nature 361*, 315−325.
Berridge, M. J., Dawson, R. M. C., Downes, C. P., Heslop, J. P., & Irvine, R. F. (1983) *Biochem. J. 212*, 473−482
Bonnefoy-Bernard, N., Liu, Y. C., von Willebrand, M., Sung, A., Elly, C., Mustelin, T., Yoshida, H., Ishizaka, K., & Altman, A. (1995) *Proc. Natl. Acad. Sci. U.S.A. 92*, 10142−10146.
Communi, D., & Erneux, C. (1996) *Biochem. J. 320*, 181−186.
Communi, D., Lecocq, R., & Erneux, C. (1996) *J. Biol. Chem. 271*, 11676−11683.
Connolly, T. M., Bross, T. R., & Majerus, P. W. (1985) *J. Biol. Chem. 260*, 7868−7874.
Connolly, T. M., Lawing, W. J., & Majerus, P. W. (1986) *Cell 46*, 951−958.
Damen, J. E., Liu, L., Rosten, P., Humphries, R. K., Jefferson, A. B., Majerus, P. W., & Krystal, G. (1996) *Proc. Natl. Acad. Sci. U.S.A. 93*, 1689−1693.
Davis, L. H., & Bennett, V. (1994) *J. Biol. Chem. 269*, 4409−4416.
Dellambra, E., Patrone, M., Sparatore, B., Negri, A., Ceciliani, F., Bondanza, S., Molina, F., Cancedda, F. D., & Deluca, M. (1995) *J. Cell Sci. 108*, 3569−3579.
De Smedt, F., Verjans, B., Mailleux, P., & Erneux, C. (1994) *FEBS Lett. 347*, 69−72.
Downes, C. P., Mussat, M. C., & Michell, R. H. (1982) *Biochem. J. 203*, 169−177.
Du, X., Harris, S. J., Tetaz, T. J., Ginsberg, M. H., & Berndt, M. C. (1994) *J. Biol. Chem. 269*, 23776−23783.
Erneux, C., De Smedt, F., Moreau, C., Rider, M., & Communi, D. (1995) *Eur. J. Biochem. 234*, 598−602.
Freed, E., Symons, M., Macdonald, S. G., McCormick, F., & Ruggieri, R. (1994) *Science 265*, 1713−1715.
Fu, H., Xia, K., Pallas, D. C., Cui, C., Conroy, K., Narsimhan, R. P., Mamon, H., Collier, J., & Roberts, T. (1994) *Science 266*, 126−128.
Gibson, T. J., Hyvonen, M., Musacchio, A., Saraste, M., & Birney, E. (1994) *Trends Biochem. Sci. 19*, 349−353.
Harlan, J. E., Hajduk, P. J., Yoon, H. S., & Fesik, S. W. (1994) *Nature 371*, 168−170.
Haslam, R. J., Kolde, B. J., & Hemmings, B. A. (1993) *Nature 363*, 309−310.
Imaoka, T., Lynham, J. A., & Haslam, R. J. (1983) *J. Biol. Chem. 264*, 6070−6074.
Jefferson, A. B., & Majerus, P. W. (1995) *J. Biol. Chem. 270*, 9370−9377.
Jefferson, A. B., & Majerus, P. W. (1996) *Biochemistry 35*, 7890−7894.
Jones, D. H. A., Ley, S., & Aitken, A. (1995a) *FEBS Lett. 368*, 55−58.
Jones, D. H. A., Martin, H., Madrazo, J., Robinson, K. A., Nielson, P., Roaseboom, P. H., Patel, Y., Howell, S. A., Aitken, A. (1995b) *J. Mol. Biol. 245*, 375−384.
Laxminarayan, K. M., Matzaris, M., Speed, C. J., & Mitchell, C. A. (1993) *J. Biol. Chem. 268*, 4968−4974.
Laxminarayan, K. M., Chan, B. K., Tetaz, T., Bird, P. I., & Mitchell, C. A. (1994) *J. Biol. Chem. 269*, 17305−17310.
Lemmon, M. A., Fergurson, K. M., O'Brien, R., Sigler, P. B., & Schlessinger, J. (1995) *Proc. Natl. Acad. Sci. U.S.A. 92*, 10472−10476.
Lerner, R. A., Green, N., Alexander, H., Liu, F., Sutcliffe, J. G., & Shinnick, T. M. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 3403−3407.
Li, S., Janosch, P., Tanji, M., Rosenfeld, G. C., Waymire, J. C., Mischak, H., Kolch, W., & Sedivy, J. M. (1995) *EMBO J. 14*, 685−696.
Lioubin, M., Algate, P. A., Schickwann, T., Carlberg, K., Aebersold, R., & Rohrschneider, L. R. (1996) *Genes Dev. 10*, 1084−1095.
Lombardo, C. R., Weed, S. A., Kennedy, S. P., Forget, B. G., & Marrow, J. S. (1994) *J. Biol. Chem. 269*, 29212−29219.
Majerus, P. W. (1992) *Annu. Rev. Biochem. 61*, 225−250.
Majerus, P. W. (1996) *Genes Dev. 10*, 1051−1052.
Matzaris, M., Jackson, S. P., Laxminarayan, K. M., Speed, C. J., & Mitchell, C. A. (1994) *J. Biol. Chem. 269*, 3397−3402.
McDermott, A. M., & Haslam, R. J. (1996) *Biochem. J. 317*, 119−124.
McPherson, P. S., Garcia, E. P., Slepnev, V. I., David, C., Zhang, X. M., Grabs, D., Sossin, W. S., Bauerfeind, R., Nemoto, Y., & Decamilli, P. (1996) *Nature 379*, 353−357.
Mitchell, C. A., Connolly, T. M., & Majerus, P. W. (1989) *J. Biol. Chem. 264*, 8873−8877.
Mitchell, C. A., Brown, S., Campbell, J. K., Munday, A. D., & Speed, C. J. (1996) *Biochem. Soc. Trans. 24*, 994−1000.
Muslin, A. J., Tanner, J. W., Allen, P. M., & Shaw, A. S. (1996) *Cell 84*, 882−897.
Ross, T. S., Jefferson, A. B., Mitchell, C. A., & Majerus, P. W. (1991) *J. Biol. Chem. 266*, 20283−20289.
Smith, D. E., & Johnson, K. (1988) *Gene 67*, 31−35.
Suen, K. L., Bustelo, X. R., & Baracid, M. (1995) *Oncogene 11*, 825−831.
Verjans, B., Hollande, F., Moreau, C., Lejeune, C., & Erneux, C. (1990) *Cell Signalling 2*, 595−599.
Verjans, B., De Smedt, F., Lecocq, R., Vaneyenberg, V., Moreau, C., & Erneux, C. (1994) *Biochem. J. 300*, 85−90.
Ward, C. M., Andrews, R. K., Smith, A. I., & Berndt, M. C. (1996) *Biochemistry 35*, 4929−4938.
Wheeler-Jones, C. P. D., Learmouth, M. P., Martin, H., & Aitken, A. (1996) *Biochem. J. 315*, 41−47.
Zha, J., Harada, H., Yang, E., Jockel, J., & Korsmeyer, S. J. (1996) *Cell 87*, 619−628.
Zhang, X., Jefferson, A. B., Auethavekiat, V., & Majerus, P. W. (1995) *Proc. Natl. Acad. Sci. U.S.A. 92*, 4853−4856.

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