Protein Kinase C Regulates Pleckstrin by Phosphorylation of Sites Adjacent to the N-terminal Pleckstrin Homology Domain

(Received for publication, June 8, 1995, and in revised form, July 31, 1995)

Charles S. Abrams, Wei Zhao, Elizabeth Belmonte, and Lawrence F. Brass

From the Departments of Medicine and Pathology, the University of Pennsylvania, Philadelphia, Pennsylvania 19104 and the Philadelphia Veterans Administration Medical Center, Philadelphia, Pennsylvania 19104

Pleckstrin is a substrate for protein kinase C in activated platelets that contains at its N and C termini two of the pleckstrin homology (PH) domains that have been proposed to mediate protein-protein and protein-lipid interactions. We have recently shown that pleckstrin can inhibit agonist-induced phosphoinositide hydrolysis and that this inhibition requires an intact N-terminal PH domain (residues 6 to 99). In the present studies, we have identified the sites of phosphorylation in pleckstrin and examined their contribution to pleckstrin function. In human platelets activated with thrombin or phorbol esters, and in COS-1 cells expressing pleckstrin, a combination of phosphopeptide analysis and site-directed mutagenesis shows that three residues in the intervening sequence between the two pleckstrin PH domains become phosphorylated: Ser<sup>113</sup>, Thr<sup>134</sup>, and Ser<sup>137</sup>. Replacing all three of these sites with glycine decreased phosphorylation by >90% and reduced pleckstrin's ability to inhibit phosphoinositide hydrolysis by as much as 80%. Replacing the phosphorylation sites with alanine residues had a similar effect, while substitution with aspartate, glutamate, or lysine residues produced pleckstrin variants that were fully active even in the absence of phosphorylation. These results suggest that phosphorylation enhances pleckstrin's activity by introducing a cluster of charges into a region adjacent to, but not within, the N-terminal PH domain. This may have an allosteric effect on the N-terminal PH domain, regulating its interaction with other molecules necessary for the inhibition of phosphoinositide hydrolysis.

Proteins that are important in signal transduction often contain discrete domains that mediate protein-protein interactions. Examples of this include Src homology domains 2 and 3 (SH2 and SH3) which interact with specific tyrosine-phosphorylated and proline-rich amino acid sequences, respectively (1, 2). It has been proposed that the N and C termini of the hematopoietic protein, pleckstrin, are the prototypes for a new family of intermolecular interaction domains, referred to as pleckstrin homology or PH<sup>1</sup> domains (3, 4). Within the past year, similar three-dimensional structures have been reported for the PH domains from β-spectrin, dynamin, and the N terminus of pleckstrin, supporting their status as a bona fide structural motif, despite large differences in their primary sequences (5–10).

Although it is generally believed that PH domains will turn out to mediate intermolecular interactions and may be involved in membrane targeting, there is as yet no consensus on the specifics of this interaction. Both G<sub>α<sub>β</sub></sub>, (11) and phosphatidylinositol 4,5-bisphosphate (PIP<sub>2</sub>) (12) have been proposed as potential partners for PH domains, and several recent reports have suggested that the PH domain of the β-adrenergic receptor kinase may interact individually (13) or simultaneously with both (14, 15). Pleckstrin or p47<sup>2</sup>C is a 40-kDa protein present in platelets and leukocytes that becomes phosphorylated when platelets are activated by agonists that directly or indirectly activate protein kinase C (16). Phosphoamino acid analysis and two-dimensional electrophoresis suggest that pleckstrin is phosphorylated heterogeneously on one or more serine and threonine residues, but not tyrosine residues (17). The function of pleckstrin has not been fully characterized, but we have shown previously that when transfected into COS-1 or HEK-293 cells pleckstrin can inhibit agonist-induced phosphoinositide hydrolysis initiated by G-protein-coupled receptors and growth factor receptors (18). In that model system, pleckstrin also inhibited the increase in inositol phosphate formation caused by the expression of a constitutively active variant of G<sub>α<sub>q</sub></sub>, but had no effect on G<sub>α<sub>β</sub></sub> or G<sub>α<sub>β</sub></sub>-mediated regulation of adenylyl cyclase. The inhibition of phosphoinositide hydrolysis required an intact N-terminal PH domain and was additive with that observed when mock-transfected cells were preincubated briefly with PMA, suggesting that it is independent of the phosphorylation of receptors, G-proteins, and phospholipase C known to occur under the same conditions (19–22).

Given the ability of pleckstrin's PH domains to bind to lipid micelles containing PIP<sub>2</sub> (12), one possible explanation for these results is that pleckstrin inhibits phosphoinositide hydrolysis by binding to PIP<sub>2</sub> and that phosphorylation of pleckstrin by protein kinase C promotes this interaction. In this context, pleckstrin could play a role in the feedback regulation of phosphoinositide hydrolysis following activation of protein kinase C, limiting the duration of inositol 1,4,5-trisphosphate formation. Conceivably, pleckstrin might also interact with G<sub>α<sub>q</sub></sub>, such an interaction has been demonstrated for GST fusion proteins containing pleckstrin's PH domains (23, 2) but this...
alone would not readily account for the observed ability of pleckstrin to inhibit phosphoinositide hydrolysis initiated by TrkA receptors and constitutively active Gq11.

In the present studies, we have identified the sites of phosphorylation within pleckstrin and examined their relationship to pleckstrin’s ability to inhibit agonist-induced phosphoinositide hydrolysis. The results show that: pleckstrin is variably phosphorylated on a cluster of residues located near, but not within, the N-terminal PH domain, 2) elimination of any one of these sites does not alter the overall phosphorylation of the molecule, presumably because of compensatory increases in phosphorylation at the other sites, 3) phosphorylation of pleckstrin is required for maximal inhibition of phosphoinositide hydrolysis, and 4) the effects of phosphorylation may be due largely to the introduction of charged residues into the region between the two PH domains.

**EXPERIMENTAL PROCEDURES**

Construction of Pleckstrin Expression Vectors—The expression plasmid encoding for full-length human pleckstrin was described previously (18). Pleckstrin variants containing mutations at Ser113, Thr114, and Ser117 were generated using the technique of Landt et al. (24). All of the pleckstrin variants were cloned into pCMV5. The cDNA sequences of the cloned wild type pleckstrin and variants were all confirmed and in agreement with the published sequence of pleckstrin (25).

Mapping of Phosphorylation Sites—Human platelets isolated by differential centrifugation from blood anticoagulated with ACD were washed in HEPES/Tyrode’s buffer (129 mM NaCl, 8.9 mM NaHCO3, 2.8 mM KCl, 0.8 mM KH2PO4, 56 mM dextrose, 10 mM HEPES, pH 7.4, 0.02 mM MgCl2) with 1 mM prostaglandin E1. A total of 1 × 108 platelets were resuspended in 1 ml of HEPES/Tyrode’s buffer with 250 μCi of orthophosphate. After incubation at 30°C for 1 h, 10 ml of HEPES/Tyrode’s buffer with 1 μg prostaglandin E1 was added, and the platelet suspension was centrifuged at 500 g for 15 min. The platelets were then resuspended in HEPES/Tyrode’s buffer, stimulated with 50 mM PMA for 5 min, and then lysed with ice-cold lysis buffer (1 × lysis buffer contains 1% Triton, 10 mM Tris, pH 7.6, 50 mM NaCl, 30 mM sodium pyrophosphate, 50 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 0.1% aprotinin, 1 mM vanadate, and 25 μg/ml leupeptin). After lysis of the platelets, the pleckstrin was immunoprecipitated with rabbit anti-pleckstrin sera 354 raised against a recombinant protein corresponding to residues Glu414–Asp153 of human pleckstrin (18). In the studies with COS-1 cells transiently expressing pleckstrin, the cells were incubated in phosphate-free media with 1 mM l-glutamine, 5 μCi/ml 32P, for 3.5 h, stimulated with 50 mM PMA at 37°C for 10 min, and then mixed with 2 × lysis buffer before the pleckstrin was immunoprecipitated.

CNBr mapping was performed on immunoprecipitated pleckstrin which was gel-purified by SDS-polyacrylamide gel electrophoresis. A gel slice containing the phosphopleckstrin was mixed with 50 mM ammonium carbonate (pH 8.5), 0.1% SDS, 1% β-mercaptoethanol. Pleckstrin contained in the eluate was passed through glass wool, trichloroacetic acid-accentuated, washed twice with a 50:50 mixture of cold ethanol and ether, and then vacuum-dried. The sample was resuspended in 30 μl of 50 mM MgCl2 in 70% formic acid and incubated at room temperature for 1 h. At this point, the digest was lyophilized with 1 ml of distilled water and again vacuum-dried. When noted, the CNBr fragments were further digested by incubating them overnight in the dark at room temperature in 30 μl of 10 mg/ml iodoacetobenzoxime (IBZO) in 50% acetic acid with 4 μg guanidine HCl (26). All samples were then lyophilized with 1 ml of distilled water, vacuum-dried an additional three times, and fractionated on a Tricine gel (27).

Transfection of COS-1 Cells and Thrombin-induced Inositol Phosphate Production—This was performed as described previously (18). In brief, two 100-mm tissue culture plates of COS-1 cells were transfected using DEAE-dextran (28) with the human thrombin receptor cDNA (a gift from Mark Stinski, University of Iowa) plus either wild type pleckstrin or constitutively active Gq11. The other three are located in the intervening sequence between the two PH domains (Ser113, Thr114, and Ser117) in a region (570QKFKARSTTRPSASRL 120) that closely resembles the protein kinase C pseudosubstrate site, RARFKGSLQ (see Fig. 1) (25, 30). To determine which of these potential sites actually becomes phosphorylated, pleckstrin was immunoprecipitated from platelets that had been labeled with [32P]Pi and stimulated with PMA. The immunoprecipitate was digested with CNBr, which degrades methionine residues to produce abundant CNBr fragments that were fractionated on a gel electrophoresis and autoradiography (Fig. 1). The predominant radioactive fragment had an apparent molecular mass of approximately 8 kDa that corresponds to residues Phe67–Met133. In some experiments, two additional phosphorylated fragments were also observed. When present, these bands always contained <10% of the total incorporated [32P]. The first was a 19-kDa fragment corresponding to residues Ser197, Ala242. The second was a 10-kDa fragment corresponding to residues Ile18, Met133 that was produced by incomplete cleavage at Met66.

Of the six potential sites for phosphorylation, four are located in the 8-kDa Phe67–Met133 CNBr fragment: Thr73, Ser113, Thr114, and Ser117 (Fig. 1). To determine which of these four sites becomes phosphorylated, the [32P]-labeled CNBr fragments were incubated with IBZO, which degrades after tryptic digestion (26). IBZO is predicted to liberate two fragments of 3 kDa (Phe67–Trp92) and 5 kDa (Val93–Met133) from the 8-kDa CNBr fragment. As shown in the right lane of Fig. 1A, only the 5-kDa fragment contained [32P], effectively limiting the potential phosphorylation site(s) to residues Ser113, Thr114, and Ser117. Although not formally excluded by this part of the analysis, the only other serine and threonine residues within pleckstrin's ability to inhibit agonist-induced phosphoinositidase.
Phosphorylation and Function—Previous studies have shown that phosphorylated pleckstrin is comprised of multiple isoforms that can be resolved by two-dimensional electrophoresis (17). Together with the present results, this suggests that pleckstrin normally becomes phosphorylated on residues Ser113, Thr114, and Ser117 near, but not within, the N-terminal PH domain and that no one site is predominant. Since elimination of any one of these sites has no effect on overall phosphorylation, there is apparently a compensatory increase in phosphorylation at the other sites, which is also consistent with the observation that the stoichiometry of phosphorylation of recombinant pleckstrin is 2:1 rather than 3:1. An alternative interpretation of the data, which is that phosphorylation actually occurs at unidentified sites elsewhere in the molecule and is indirectly affected by mutagenesis of Ser113, Thr114, and/or Ser117 is less likely when the results of the mutagenesis studies are combined with those from the CNBr/IBZO double digest of 32P-labeled platelet pleckstrin. The minor CNBr fragment that at times becomes phosphorylated, Ser197-Ala344, accounts for <10% of total phosphorylation and does not show increased phosphorylation when Ser113, Thr114, and Ser117 are individually mutated.

Phosphorylation and Function—Previous reports have shown that a brief exposure to the phorbol ester, PMA, will inhibit agonist-induced phosphoinositide hydrolysis in a variety of cells, including platelets (31–33). This effect is seen in cells that express pleckstrin as well as those that do not and has been attributed variously to the phosphorylation of receptors, G proteins, and/or phospholipase C (19–22). We have shown previously that when expressed in COS-1 or HEK-293 cells pleckstrin will inhibit phosphoinositide hydrolysis initiated by thrombin receptors, angiotensin-II AT1A receptors, muscarinic M1 receptors, and α2A receptors, as well as by TrkA receptors.
and a constitutively active variant of Gq, while having no effect on the regulation of cAMP formation (18). Since agonists that stimulate phosphoinositide hydrolysis cause pleckstrin to become phosphorylated, we next examined the impact of phosphorylation on pleckstrin’s ability to inhibit inositol phosphate formation.

To accomplish this, COS-1 cells were transfected with thrombin receptors and either wild type pleckstrin or the pleckstrin variant described above in which all three sites of phosphorylation were mutated to glycine. In the absence of PMA, wild type pleckstrin inhibited thrombin-induced [3H]inositol phosphate formation by 46% (Fig. 3). As we have shown previously, PMA in the absence of pleckstrin, or pleckstrin in the absence of PMA, each inhibited thrombin-induced phosphoinositide hydrolysis by 40–50%. The combination of pleckstrin plus PMA was additive, inhibiting thrombin-induced [3H]inositol phosphate formation by 91%. Keeping in mind that thrombin, like PMA, causes the phosphorylation of pleckstrin, we found that replacing residues Ser113, Thr114, and Ser117 with glycine (3 Gly) reduced pleckstrin’s ability to inhibit phosphoinositide hydrolysis by at least two-thirds in either the presence or absence of PMA (Fig. 3B). This was not due to a decrease in the level of pleckstrin expression, which was the same for the glycine variant as it was for wild type pleckstrin (Fig. 3C).

Replacing the sites of phosphorylation with another neutral amino acid, alanine, had a similar effect (data not shown). These results suggest that phosphorylation is required for maximal inhibition of thrombin-induced phosphoinositide hydrolysis by pleckstrin, but also show that some inhibition can occur even in the absence of phosphorylation.

Finally, to assess the importance of any one of the phosphorylation sites, COS-1 cells were transfected with the pleckstrin variants in which the phosphorylation sites were mutated to glycine individually or in pairs. The results that were obtained mirrored the effects on phosphorylation: the single-site mutants behaved like wild type pleckstrin and the double-site mutants had an intermediate effect (data not shown). These results suggest that the phosphorylation of at least two of the sites is required for maximal pleckstrin activity and that this phosphorylation can occur on any two of the three sites.

Replacement of Phosphorylation Sites with Charged Residues—Since the pleckstrin expressed in the transfected COS-1 cells becomes phosphorylated when the cells are stimulated with thrombin, we next examined whether replacing the sites of phosphorylation with charged, rather than neutral, amino acids would mimic the effects of phosphorylation. Pleckstrin variants in which all three sites were mutated to either positively charged lysine residues or negatively charged glutamate residues were expressed in COS-1 cells and compared with wild type pleckstrin and the glycine-mutated pleckstrin for their ability to inhibit thrombin-induced phosphoinositide hydrolysis. In contrast to the “uncharged” glycine variants, the “charged” variants were at least as active as wild type pleckstrin (compare Figs. 3 and 4), showing that the effects of phosphorylation can be mimicked by introducing positive or negative charges into the regulatory region adjacent to the N-terminal PH domain. Similar results were obtained when these sites were replaced with aspartate residues (data not shown). Since pleckstrin expressed in COS-1 cells is almost maximally phosphorylated after thrombin stimulation, the charged variants, unlike the glycine and alanine variants, possess activity roughly comparable to wild type phosphopleckstrin.

Conclusion—In summary, these observations demonstrate that pleckstrin is principally phosphorylated by protein kinase.
C on a cluster of two serine and one threonine residues located adjacent to, but not within, the N-terminal PH domain. The stoichiometry of phosphorylation approximates 2:1, and elimination of any one of the three potential sites appears to result in a compensatory increase in phosphorylation of the other two. The various combinations of phosphorylation at two out of a total of three potential sites accounts in part for the multiplicity of phosphorylated pleckstrin isoforms previously demonstrated by Haslam and co-workers (17) using isoelectric focusing. Inhibiting pleckstrin’s ability to undergo phosphorylation by replacing the target serine and threonine residues with neutral glycine or alanine residues greatly limits pleckstrin’s ability to undergo phosphorylation by replacing the target serine and threonine residues with neutral glycine or alanine residues greatly limits pleckstrin’s ability to inhibit thrombin-induced phosphoinositide hydrolysis, while replacing the phosphorylation sites with charged lysine, glutamate, or aspartate residues mimics the effects of phosphorylation. Although the present studies focused on thrombin receptors, previous results obtained with other G-protein-coupled or growth factor receptors that couple to phospholipase C show that this phenomenon is not limited to thrombin-initiated phosphoinositide hydrolysis (18).

These results raise the question of how phosphorylation regulates pleckstrin’s activity. It is conceivable that the phosphorylation of pleckstrin affects its cellular location, in the process affecting its proposed interaction with PI(4,5)P2. However, preliminary studies in platelets and pleckstrin-transfected COS-1 cells indicate that this is not the case.3 We have previously shown that the N-terminal PH domain of pleckstrin is critical for its ability to inhibit phosphoinositide hydrolysis. Since the N-terminal PH domain and the 3 sites of phosphorylation are close to each other in the linear sequence, one alternative explanation is that the first portion of the intervening sequence between the PH domains obstructs the access of ligands to binding sites within the N-terminal PH domain. Under this model, phosphorylation relieves this obstruction, allowing the N-terminal PH domain to interact with other molecules necessary for the inhibition of phosphoinositide hydrolysis. Alternatively, the first part of the intervening sequence may undergo a conformational change upon phosphorylation that affects necessary spatial relations between the N-terminal and C-terminal PH domains or an as-yet-unidentified accessory protein. The PH domain from the N-terminal PH domain of pleckstrin, like that from dynamin and β-spectrin, possesses a highly polarized electrostatic potential. It is possible that this asymmetry of charges contributes to an interaction between the N-terminal PH domain and the charged residues surrounding the protein kinase C phosphorylation site. However, regardless of which model ultimately proves to be correct, phosphorylation appears to affect pleckstrin activity by altering the charge distribution in a region proximal to the N-terminal PH domain. Somewhat surprisingly, the effects of phosphorylation could be mimicked by replacing the phosphorylation sites with either a negatively charged glutamate residue or a positively charged lysine residue. This suggests that it is the presence of charged residues in this region of pleckstrin that is important and not the negative charge carried by the phosphate groups.

Finally, what is the role of pleckstrin in cells that normally contain it, such as platelets and other blood cells? The rapid phosphorylation of pleckstrin is one of the hallmarks of platelet activation and is thought to occur when diacylglycerol and Ca2+ activate protein kinase C. This suggests that phosphorylated pleckstrin could play a regulatory role, helping to limit the duration of agonist-induced phospholipase C activity and perhaps preventing premature platelet activation. Whether it does so in blood cells remains to be demonstrated.

REFERENCES
1. Koch, C. A., Anderson, D., Moran, M. F., Ellis, C., and Pawson, T. (1991) Science 252, 668–674
2. Ren, R., Mayer, B. J., Cicchetti, P., and Baltimore, D. (1993) Science 259, 337–341
3. Haslam, R. J., Kolde, B. J., and Hemnings, B. A. (1993) Nature 363, 309–310
4. Mayer, B. J., Ren, R., Clark, K. L., and Baltimore, D. L. (1993) Cell 73, 629–630
5. Yoon, H. S., Hajduk, P. J., Petros, A. M., Olejniczak, E. T., Meadows, R. P., and Fesik, S. W. (1994) Nature 369, 672–675
6. Madias, M. J., Musacchio, A., Paunstingh, H., Nilges, M., Saraste, M., and Oschkinat, H. (1994) Nature 369, 675–677
7. Ferguson, K. M., Lemmon, M. A., Schlessinger, J., and Sigler, P. B. (1994) Cell 79, 199–202
8. Timm, D., Salim, K., Gout, I., Guruprasad, L., Waterfield, M., and Blundell, T. L. (1994) Nature Struct. Biol. 1, 782–788
9. Downing, A. K., Driscoll, P. C., Gout, I., Salim, K., Zelvehn, M. J., and Waterfield, M. D. (1994) Curr. Biol. 4, 884–891
10. Fushman, D., Cahill, S., Lemmon, M. A., Schlessinger, J., and Cowburn, D. (1995) Proc. Natl. Acad. Sci. U.S.A. 92, 816–820
11. Musacchio, A., Gilson, T., Rice, P., Thompson, J., and Saraste, M. (1993) Trends Biochem. Sci. 18, 343–348
12. Harlan, J. E., Hajduk, P. J., Yoon, H. S., and Fesik, S. W. (1994) Nature 371, 168–170
13. DeBburman, S. K., Ptasienski, J., Boetticher, E., Lomasney, J. W., Benovic, J. L., and Hasey, M. M. (1995) J. Biol. Chem. 270, 5742–5747
14. Pitcher, J. A., Touhara, K., Payne, E. S., and Lefkowitz, R. J. (1995) J. Biol. Chem. 270, 11707–11710
15. Touhara, K., Koch, W. J., Hasey, B. E., and Lefkowitz, R. J. (1995) J. Biol. Chem. 270, 17000–17005
16. Haslam, R. J., Lynham, J. A., and Fox, J. E. B. (1979) Biochem. J. 178, 397–406
17. Imakka, T., Lynham, J. A., and Haslam, R. J. (1983) J. Biol. Chem. 258, 13795–13798
18. Abrams, C. S., Wu, H., Zhao, W., Belmonte, E., White, D., and Brass, L. F. (1995) J. Biol. Chem. 270, 14485–14492
19. Leeb-Lundberg, L. M. F., Cotecchia, S., Lomasney, J. W., DeBernardis, J. F., Lefkowitz, R. J., and Caron, M. G. (1995) Proc. Natl. Acad. Sci. U.S.A. 92, 5651–5655
20. Kelley, D. J., Pessin, J. E., Rudko, A. E., and Johnson, G. L. (1994) Proc. Natl. Acad. Sci. U.S.A. 91, 4316–4320
21. Neer, E. J. (1995) Cell 80, 249–257
22. Ryu, S. H., Kim, U.-H., Wahl, M. I., Brown, A. B., Carpenter, G., Huang, K.-P., and Rhee, S. G. (1990) J. Biol. Chem. 265, 17941–17945
23. Touhara, K., Inglese, J., Pitcher, J. A., Shaw, G., and Lefkowitz, R. J. (1994) J. Biol. Chem. 269, 10217–10220
24. Landt, O., Grunert, H.-P., and Hahn, U. (1990) Science 249, 13795–13798
25. Tyers, M., Rachubinski, R. A., Stewart, M. I., Varrichio, A. M., Shorr, R. G., Haslam, R. J., and Harley, C. B. (1988) Nature 333, 470–473
26. Longo, C., Garrido, B., Balza, G., Lopez-Moratella, N., and Santiago, E. (1992) Int. J. Biochem. 24, 133–143
27. Schager, H., and von Jagow, G. (1987) Anal. Biochem. 166, 368–379
28. Morris, J. F., Madden, S. L., Tournay, O. E., Cook, D. M., Sukatme, V. P., and Rauscher, F. J. (1991) Oncogene 6, 2339–2348
29. Brach-Pei, J., Vassallo, R. J., Belmonte, E., Ahuja, M., Cichowski, K., and Hua, D. (1992) J. Biol. Chem. 267, 13795–13798
30. House, C., and Kemp, B. E. (1987) Science 236, 726–728
31. Watson, S. P., and Lapetina, E. G. (1985) J. Biol. Chem. 260, 13795–13798
32. Rittenhouse, S. E., and Sasson, J. P. (1985) J. Biol. Chem. 260, 8657–8660

3 A. Ma, L. F. Brass, and C. S. Abrams, unpublished data.