Chemical cross-linking of pleckstrin in human platelets: evidence for oligomerization of the protein and its dissociation by protein kinase C

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The major substrate of protein kinase C (PKC) in platelets is the 40 kDa protein, pleckstrin. Addition of the homobifunctional reagent, bis(sulphosuccinimidyl)suberate (BS^3), to platelet lysate, cytosol fraction or to electropermeabilized platelets resulted in cross-linking of pleckstrin to give higher-molecular-mass complexes of 68 kDa, 90 kDa and 100–120 kDa respectively, which were visualized by immunoblotting with an anti-pleckstrin antibody. Higher levels of cross-linking were observed in permeabilized platelets than in platelet lysates. The yields of the cross-linked complexes were much reduced after dilution of platelet lysate or lysis of electropermeabilized platelets and, in the case of the 90 kDa and 100–120 kDa species, after activation of PKC by phorbol 12-myristate 13-acetate. Similar experiments with purified pleckstrin indicated that the 90 kDa and 100–120 kDa species consist, at least in part, of pleckstrin dimers and higher oligomers. After incubation of purified pleckstrin (0.45 mg/ml) for 1 h with 2 mM BS^3, about 25 % of the protein was present in cross-linked species. The results indicate that pleckstrin undergoes a reversible self-association that can be prevented by phosphorylation of the protein, and also interacts with an unidentified platelet protein of about 28 kDa.

INTRODUCTION

Pleckstrin, a 40 kDa protein found only in cells and cell lines of haematological origin [1,2], is the major substrate of protein kinase C (PKC) in the blood platelet [3]. Although the phosphorylation of pleckstrin has long been known to be closely associated with the secretion of platelet granule contents in response to physiological agonists [3–5], the functions of this protein in platelets are not well understood. The cloning of pleckstrin revealed that this protein contains similar N- and C-terminal domains of about 100 residues that lie outside the putative phosphorylation sites [1]. These domains, now termed pleckstrin homology or PH domains, have since been identified in a wide range of proteins with roles in signal transduction or cytoskeletal function [6–8]. PH domains can bind phosphoinositol derivatives, such as PtdIns(4,5)P_2 [9] and perhaps 3-phosphorylated phosphoinositides [10], and can contribute to the binding of some proteins to the $\beta\gamma$-subunits of heterotrimeric G-proteins [11–13]. Recent evidence indicates that the N-terminal PH domain of pleckstrin can inhibit phospholipase C when the protein is expressed in COS-1 or HEK-293 cells, probably by binding to PtdIns(4,5)P_2 rather than to G-protein $\beta\gamma$-subunits [14]. The phosphorylated form of pleckstrin appeared to be the active species [15]. However, it remains uncertain whether phosphorylation of pleckstrin leads to inhibition of phospholipase C in platelets and, in any case, this is unlikely to be the sole function of the protein. Some PH domains, such as those of Bruton's tyrosine kinase (Btk) [16] and of the c-Akt (Rac) serine-threonine kinases [17], have also been reported to bind PKC, and in general PH domains seem likely to interact with other proteins, as well as with phosphoinositides. We have therefore used a homobifunctional reagent, bis(sulphosuccinimidyl)suberate (BS^3) [18], to detect protein–protein interactions involving pleckstrin in platelets. The results indicate that pleckstrin can form oligomers as well as a 68 kDa complex with an unidentified protein. Pleckstrin phosphorylation appeared to inhibit self-association of the protein. Some of these results have been published in a preliminary form [19].

EXPERIMENTAL

Materials

BS^3 was obtained from Pierce (Rockford, IL, U.S.A.). Phorbol 12-myristate 13-acetate (PMA), heparin, soybean trypsin inhibitor, chymostatin, PMSF and other fine chemicals were from Sigma Chemical Co. (St. Louis, MO, U.S.A.), and leupeptin, aprotinin and pepstatin from Boehringer-Mannheim Canada (Laval, Quebec, Canada). Centricon-10 micro-concentrators were obtained from Amicon (Oakville, Ontario, Canada) and Immobilon P from Millipore (Canada) Ltd. (Mississauga, Ontario, Canada). Pre-stained protein molecular-mass markers for SDS/PAGE (175, 83, 62, 47.5, 32.5, 25, 16.5 and 6.5 kDa) were from New England BioLabs (Beverly, MA, U.S.A.). Anti-pan PKC polyclonal antibody was from Upstate Biotechnology Inc. (Lake Placid, NY, U.S.A.). Horseradish peroxidase-linked anti-rabbit IgG and enhanced chemiluminescence (ECL) reagents were purchased from Amersham (Oakville, Ontario, Canada). Ro 31-8220 was a gift from Dr. P. D. Davis (Roche Products, Welwyn Garden City, Herts., U.K.).

Preparation of platelet lysate

Human platelets were isolated by a modification of the method of Mustard et al. [20]. They were washed first in Ca^{2+}-free Tyrode's solution buffered to pH 6.5 with 5 mM Pipes and containing BSA (3.5 mg/ml), heparin (50 units/ml) and apyrase (60 μg/ml), and then in the same medium buffered with 5 mM Hepes (pH 7.4) and containing only BSA and apyrase. The platelets were finally resuspended at a concentration of 2.5×10^9/ml in buffer A (100 mM KCl/2.5 mM EGTA/25 mM Hepes, adjusted to pH 7.4 with KOH) containing 0.2 mM

Abbreviations used: PKC, protein kinase C; PH domain, pleckstrin homology domain; BS^3, bis(sulphosuccinimidyl)suberate; PMA, phorbol 12-myristate 13-acetate; ECL, enhanced chemiluminescence; Btk, Bruton's tyrosine kinase.

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leupeptin and were then immediately frozen in methanol/solid CO_2. Platelets were stored at −70 °C until required, when they were thawed, frozen and thawed again to give a lysate (at 0 °C). In some experiments, particulate and cytosol fractions were prepared by centrifuging the lysate in a Beckman airfuge [30 min, 4 °C, 1518 kPa (22 lb/in²)]. The particulate fraction was washed three times in buffer A prior to use.

Electropermeabilized platelets

Human platelets were isolated and electropermeabilized as previously described [21]. The permeabilized platelets were cooled to 4 °C and then separated from the suspending medium by gel filtration at 4 °C on Sepharose CL-4B, using buffer A supplemented with 2.5 mM EDTA/3.9 mM $MgCl_2$ as eluant. ATP (final concentration 5 mM) was then added and the platelets were diluted to 5×10^8/ml. Glutamate-containing buffers [21], which quench BS^3, were not used.

Purification of pleckstrin by FPLC

Outdated platelet concentrates in plasma (5 units) were obtained from the Canadian Red Cross. The platelets were washed three times at 4 °C in a solution (pH 6.5) containing 13 mM sodium citrate/5 mM glucose/135 mM NaCl and then resuspended in 10 vol. of buffer A containing protease inhibitors (1 mM PMSF, 0.2 mM leupeptin, 3 μM pepstatin, 0.8 μM aprotinin, 15 μg of chymostatin/ml and 10 μg of soybean trypsin inhibitor/ml). The suspension was sonicated, cell debris was removed by low-speed centrifugation and a high-speed supernatant was obtained by ultracentrifugation at 100000 ***g*** at 4 °C for 1 h. This was diluted to approx. 1 mg of protein/ml and fractionated with 50 %–100 % $(NH_4)_2SO_4$. The precipitate was dissolved and dialysed in buffer B (10 mM KH_2PO_4/1 mM EDTA/1 mM EGTA/20 mM 2-mercaptoethanol, adjusted to pH 7.0 with KOH) and applied to a Mono S cation-exchange column. Protein was eluted with a 20 ml linear salt gradient (0–0.5 M KCl in buffer B). Fractions containing pleckstrin (identified after SDS/PAGE) were desalted in a Centricon-10 mini-concentrator and applied to a Mono Q anion-exchange column in buffer B adjusted to pH 8.3. Pleckstrin was eluted with a 20 ml linear salt gradient (0–0.35 M KCl). Fractions containing pleckstrin were desalted and concentrated in a Centricon-10, using buffer B adjusted to pH 7.4.

Cross-linking and immunoblotting of pleckstrin complexes

BS^3 (5 μl in buffer A) was added to 100 μl of platelet fraction or to 100 μl of a suspension of electropermeabilized platelets. The cross-linking reaction was carried out for various periods at 0 °C and was terminated either by quenching with 1 vol. of 100 mM Tris/HCl (pH 8.0), followed by 1 vol. of 10 % trichloroacetic acid 30 min later or, when indicated, by the immediate addition of trichloroacetic acid. Acid-precipitated protein was dissolved in electrophoresis sample buffer [22]. FPLC-purified pleckstrin was cross-linked at 0 °C with 2 mM BS^3 for 1 h, after which the reaction was quenched with electrophoresis sample buffer. Protein (10–20 μg/lane) was analysed by SDS/PAGE [22] using 10 % acrylamide. Proteins were then blotted on to Immobilon P and probed with a polyclonal rabbit-antibody to purified pleckstrin [23]. Immunoreactive proteins were visualized by ECL. Alternatively, gels containing FPLC-purified pleckstrin were stained with silver [24] or Coomassie Brilliant Blue R [5]. In the latter case, the gels were dried between porous cellophane sheets and the protein was quantified by one-dimensional scanning using an LKB UltroScan XL laser densitometer. The molecular masses of cross-linked protein complexes were calculated by linear regression, using pre-stained protein standards. Protein was determined by the Lowry method.

RESULTS

Studies with platelet lysate

Immunoblotting of platelet protein with an antibody to purified pleckstrin [23] revealed a major 40 kDa pleckstrin band (PLK)

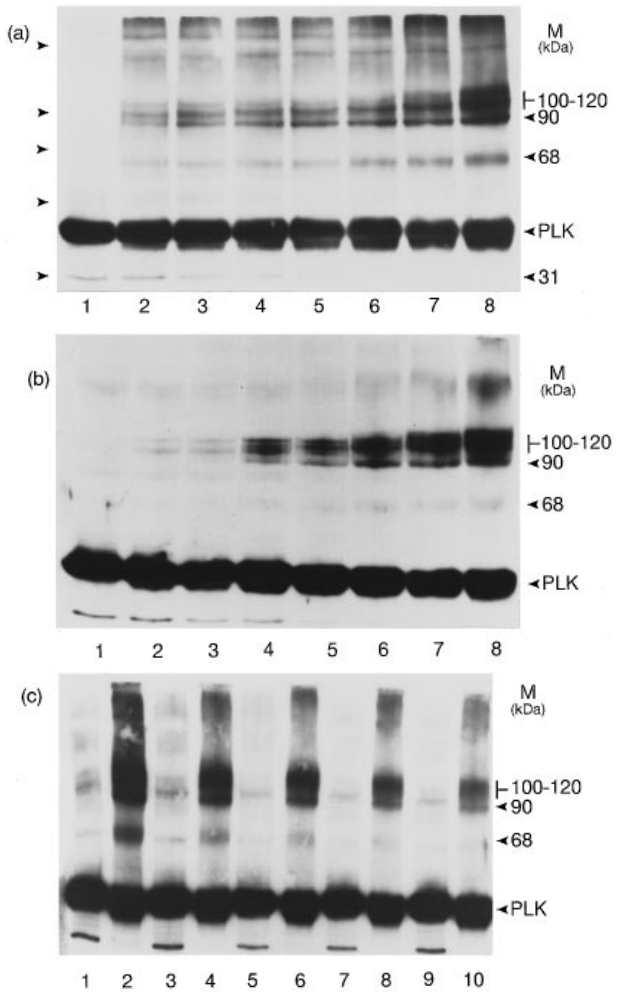

Figure 1 Factors affecting the cross-linking of pleckstrin in platelet lysate by BS^3

Platelet lysate was incubated with BS^3 and protein was analysed by SDS/PAGE. Each experiment was repeated 3–4 times with comparable results; representative immunoblots are shown. The positions of standard proteins are indicated on the left (**a** only) and the calculated molecular masses (M) of immunoreactive complexes on the right. PLK, pleckstrin monomer. (**a**) Time course of cross-linking of pleckstrin. Samples of platelet lysate (250 μg of protein) were incubated at 0 °C without (lane 1) or with 2 mM BS^3 (lanes 2–8). The reactions were stopped by direct addition of trichloroacetic acid after the following times: lane 1, 10 min; lane 2, 1 min; lane 3, 3 min; lane 4, 5 min; lane 5, 10 min; lane 6, 15 min; lane 7, 30 min; lane 8, 60 min. (**b**) Effect of BS^3 concentration on the cross-linking of pleckstrin. Platelet lysate (280 μg of protein) was incubated at 0 °C for 10 min with buffer A (lane 1) or various BS^3 concentrations (lanes 2–8), as follows: lane 2, 0.05 mM; lane 3, 0.1 mM; lane 4, 0.25 mM; lane 5, 0.5 mM; lane 6, 1.0 mM; lane 7, 2 mM; lane 8, 4 mM. (**c**) Dependence of cross-linking on the dilution of platelet lysate in buffer A. Different volumes of platelet lysate were mixed with buffer A to give 100 μl. The samples were incubated at 0 °C for 10 min with buffer A (lanes 1, 3, 5, 7, 9) or 2 mM BS^3 (lanes 2, 4, 6, 8, 10). The protein content of the incubations was: lanes 1 and 2, 200 μg; lanes 3 and 4, 100 μg; lanes 5 and 6, 50 μg; lanes 7 and 8, 25 μg; lanes 9 and 10, 12.5 μg. The same amount of protein (12.5 μg) was loaded in each lane of the gel.

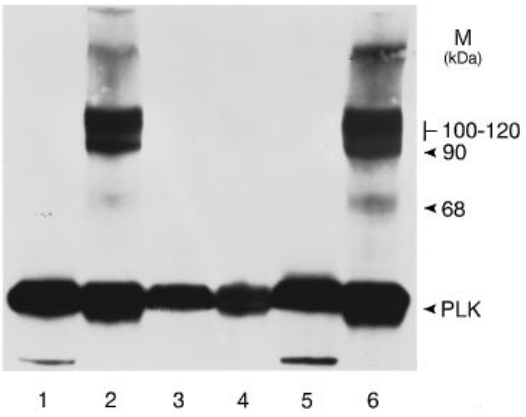

Figure 2 Cross-linking of pleckstrin in platelet particulate and cytosolic fractions by BS^3

Platelet lysate (lanes 1 and 2), particulate fraction (lanes 3 and 4) and cytosolic fraction (lanes 5 and 6) were incubated with buffer A (lanes 1, 3, 5) or 2 mM BS^3 (lanes 2, 4, 6) for 12 min at 0 °C. All incubations contained 140 μg of protein. Precipitated protein was analysed by SDS/PAGE. The Figure shows an immunoblot with the calculated molecular masses (M) of immunoreactive complexes on the right. PLK, pleckstrin monomer. This Figure is representative of three identical experiments.

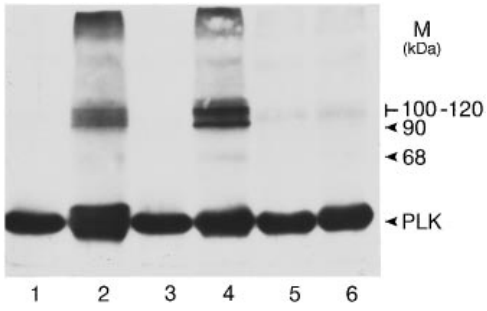

Figure 3 Comparison of the cross-linking of pleckstrin in platelet lysate with that in electropermeabilized or intact platelets

Platelet lysate (lanes 1 and 2), electropermeabilized platelets (lanes 3 and 4) and intact platelets (lanes 5 and 6) were incubated for 10 min at 0 °C with buffer A (lanes 1, 3, 5) or 2 mM BS^3 (lanes 2, 4, 6). This platelet lysate was prepared by freezing and thawing the electropermeabilized platelets. All incubations contained 55 μg of protein. Precipitated protein was analysed by SDS/PAGE. The Figure shows an immunoblot with the calculated molecular masses (M) of immunoreactive complexes on the right. PLK, pleckstrin monomer. This Figure is representative of three identical experiments.

and a minor species of 31 kDa, probably a fragment of pleckstrin (Figure 1). The latter was not seen when an anti-peptide antibody, prepared to the C-terminal 15 residues of pleckstrin, was used (results not shown). Incubation of platelet lysate with BS^3, followed by immunoblotting with anti-pleckstrin antibody, reproducibly led to the appearance of additional immunoreactive bands, indicating that cross-linking had occurred between pleckstrin and specific platelet proteins. The extent of this cross-linking depended on both the time of incubation with BS^3 (Figure 1a) and the concentration of BS^3 (Figure 1b). The major novel immunoreactive species had apparent molecular masses of 90 kDa and 100–120 kDa (nine determinations). The latter band consisted of several components; those with the higher molecular masses were only detected after extended periods of cross-linking or with high concentrations of BS^3 (Figures 1a and 1b). In addition to these major cross-linked species, an immunoreactive complex with an apparent molecular mass of 68 kDa (nine determinations) was seen, particularly under the conditions favouring more extensive cross-linking (Figure 1). Trace amounts of high-molecular-mass species (e.g. 150 kDa) were also seen in some experiments. The 31 kDa immunoreactive protein disappeared after extensive cross-linking. A broadening or slightly increased mobility of the 40 kDa pleckstrin band, attributable to intramolecular cross-linking, was usually observed. The amounts of cross-linked complexes that were formed decreased in parallel with the platelet protein concentration, even when the same total amounts of protein were analysed (Figure 1c). This result suggests that these complexes partially dissociated on dilution of the platelet lysate. As a whole, the experiments in Figure 1 show that the pleckstrin complexes described above can be readily demonstrated by cross-linking with 2 mM BS^3 for 10 min, using at least 0.5 mg of total platelet protein/ml. Very similar results were obtained when the anti-peptide antibody was used. The nature of these complexes was studied by cross-linking with 2 mM BS^3 after pre-incubation of platelet lysate with 2 mM dithiothreitol, 20 mM 2-mercaptoethanol or 6 M urea (for 1 h at 0 °C). Dithiothreitol had no effect and 2-mercaptoethanol only slightly decreased the intensity of the cross-linked immunoreactive bands. In contrast, urea abolished all cross-linking of pleckstrin. It follows that the complexes identified do not contain disulphide-linked pleckstrin and are only formed when protein secondary and tertiary structure is intact.

Cross-linking of pleckstrin in platelet subcellular fractions

Platelet lysate and the corresponding particulate and cytosol fractions were cross-linked with BS^3 using the same final protein concentrations in all samples (Figure 2). Although some pleckstrin remained associated with the particulate fraction, no cross-linked species were detected. In contrast, marked cross-linking occurred in the cytosolic fraction and all the complexes described above were found in this fraction. Identical results were obtained when platelet lysate was fractionated after cross-linking with BS^3 (results not shown).

Comparison of the cross-linking of pleckstrin in platelet lysate with that in electropermeabilized platelets

The same cross-linked pleckstrin complexes were observed in electropermeabilized platelets and in lysates prepared from them, whether the former were incubated with BS^3 at 0 °C (Figure 3) or at 25 °C (results not shown). However, the extent of cross-linking was consistently much lower in the lysates, suggesting that the higher intracellular concentration of pleckstrin in permeabilized platelets favoured complex formation. In contrast with lysate (Figure 1c), dilution of electropermeabilized platelets (up to 16-fold) did not affect the extent of cross-linking observed. As expected, BS^3 did not cross-link pleckstrin in non-permeabilized platelets (Figure 3).

Effects of activation and inhibition of platelet PKC on the cross-linking of pleckstrin

Intact platelets (2.5×10^9/ml) were incubated at 37 °C for 2 min with 1 μM PMA to activate PKC and to stimulate the phosphorylation of pleckstrin fully. Lysates were then prepared

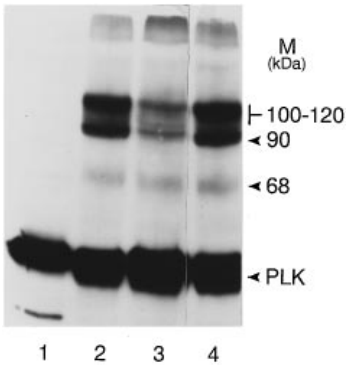

Figure 4 Effects of activation and inhibition of PKC in intact platelets on the cross-linking of pleckstrin by BS^3 in platelet lysate

Intact platelets (2.5×10^9/ml) were incubated at 37 °C with vehicle (0.2% DMSO) (lanes 1 and 2), 1 μM PMA (lane 3) or 3 μM Ro 31-8220 and 1 μM PMA (lane 4). Incubations with vehicle or PMA were for 2 min; Ro 31-8220 was added 1 min before PMA. Lysates were then prepared and samples (200 μg of protein) were incubated for 10 min at 0 °C with buffer A (lane 1) or 2 mM BS^3 (lanes 2–4). Precipitated protein was analysed by SDS/PAGE. The Figure shows an immunoblot with the calculated molecular masses (M) of immunoreactive complexes on the right. PLK, pleckstrin monomer. This Figure is representative of four identical experiments.

and protein cross-linked with 2 mM BS^3 for 10 min (Figure 4). The immunoreactivity of the 40 kDa pleckstrin band was not affected by PMA treatment. However, PMA markedly decreased the amounts of the 90 kDa and 100–120 kDa pleckstrin complexes that were detected, though it did not affect the 68 kDa complex. Addition of 3 μM Ro 31-8220, a selective inhibitor of PKC [25], 1 min before the addition of PMA completely prevented this inhibition of cross-linking (Figure 4). Identical results were obtained using electropermeabilized platelets. These observations suggest that phosphorylation of pleckstrin by PKC dissociates the major complexes detected by cross-linking.

The possibility that pleckstrin forms complexes with PKC was investigated. However, cross-linking of protein in platelet lysate or electropermeabilized platelets did not generate higher-molecular-mass species that could be detected with an anti-PKC antibody that recognizes the catalytic domains of at least the α, β, γ and δ isoenzymes.

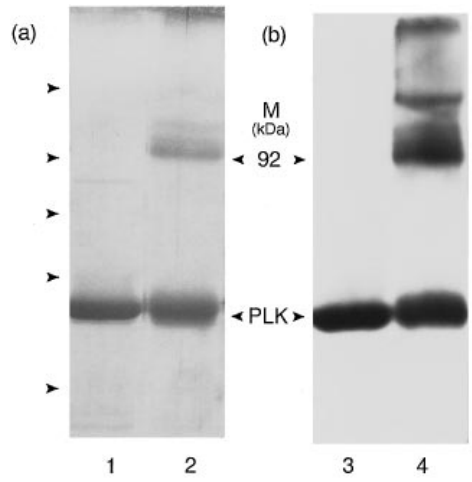

Figure 5 Cross-linking of purified pleckstrin by BS^3

Purified pleckstrin (approx. 5 μg in 40 μl of buffer B, pH 7.4) was incubated at 0 °C for 1 h without (lanes 1 and 3) or with 2 mM BS^3 (lanes 2 and 4). Protein was analysed by SDS/PAGE; (**a**) shows a silver-stained gel and (**b**) an immunoblot of identical samples. The positions of standard proteins are shown to the left of (**a**) and the calculated molecular mass (M) of the stained and immunoreactive complex between (**a**) and (**b**). PLK, pleckstrin monomer. This Figure is representative of three identical experiments.

Cross-linking of purified pleckstrin by BS^3

Previous results have shown that phosphorylated pleckstrin is monomeric [26]. However, the molecular mass of the 90 kDa complex suggested that non-phosphorylated pleckstrin might form a dimer. We therefore purified pleckstrin to near homogeneity and incubated this protein with BS^3. As shown in both a silver-stained gel and an immunoblot (Figure 5), incubation of pleckstrin with BS^3 generated a major cross-linked species with a calculated molecular mass of 92 kDa (three determinations). This complex was indistinguishable on SDS/PAGE from the 90 kDa cross-linked species detected in platelet lysate. Cross-linking of purified pleckstrin also generated additional species of higher molecular mass (Figure 5), which could reflect either different extents of cross-linking of a pleckstrin dimer or, in the case of the larger complexes, the formation of trimers and tetramers. These species had molecular masses similar to those of the 100–120 kDa and minor 150 kDa complexes observed in cross-linked platelet lysate. Coomassie Blue-stained gels of purified pleckstrin (0.45 mg/ml) that had been incubated for 1 h with 2 mM BS^3 were analysed by laser densitometry. In four such experiments a mean of 25% of the pleckstrin (range 16–30%) was found in high-molecular-mass cross-linked complexes. These results suggest that the 90 kDa and larger species observed in platelet lysate and electropermeabilized platelets represent mainly pleckstrin oligomers.

DISCUSSION

BS^3 cross-links primary amine groups and is effective in the detection of non-covalently-associated protein subunits [18]. Following incubation of platelet lysate or cytosol with BS^3, pleckstrin was found in cross-linked protein complexes of 68 kDa and 90 kDa, as well as in several species in the 100–120 kDa range. The appearance of these discrete, cross-linked species indicates that pleckstrin was present in pre-existing protein complexes and was not cross-linked non-specifically to other platelet proteins. Generation of these complexes did not depend on disulphide-bond formation and required intact protein secondary and tertiary structure. Both the effects of dilution of platelet lysate on cross-linking by BS^3 and comparison of cross-linking in lysate and electropermeabilized platelets, which do not leak protein [21], suggested that pleckstrin participates in relatively low-affinity and reversible protein–protein interactions. The observation of cross-linking of pleckstrin in electropermeabilized platelets, which can respond to physiological stimuli with the secretion of granule contents [21], is consistent with roles for these complexes in platelet function.

Under defined conditions, namely incubation of 0.45 mg/ml of pleckstrin with 2 mM BS^3 for 1 h, about 25% of the protein was cross-linked to give higher-molecular-mass species. The extent to which this represents the actual degree of oligomerization of pleckstrin under these conditions is uncertain. Thus the cross-linking reaction does not proceed to completion rapidly, and the equilibrium between monomeric and associated forms of pleckstrin may be disturbed by the intramolecular or inter-

molecular cross-linking of the protein. The net result of these opposing effects is not easily determined. Additional factors must be considered in intact platelets. First, quantification suggests that the concentration of pleckstrin, which accounts for approx. 1% of platelet protein (R. J. Haslam, unpublished work), is probably 10-fold higher in intact platelets than in our experiments *in vitro* with the purified protein. Secondly, the self-association of pleckstrin could be regulated by low-molecular-mass compounds present in the platelet cytosol. At present, we can only conclude that a substantial fraction of pleckstrin is likely to exist as dimers, and possibly higher oligomers, in intact platelets.

Incubation of platelets with physiological agonists, such as collagen or thrombin, or with PMA causes the rapid phosphorylation of pleckstrin by PKC [3–5]. It was therefore of particular interest that pretreatment of intact or electropermeabilized platelets with PMA markedly decreased the amounts of 90 kDa and 100–120 kDa protein complexes observed after cross-linking with BS^3. Since the effect of PMA was blocked by Ro 31-8220, a selective inhibitor of PKC [25], it is likely that phosphorylation of pleckstrin by PKC leads to dissociation of the 90 kDa and 100–120 kDa protein complexes. The evidence we have obtained suggests that these complexes consist, at least in part, of pleckstrin oligomers. Thus the molecular masses of these complexes correspond roughly to those of pleckstrin dimers and trimers and incubation of purified pleckstrin with BS^3 generates significant amounts of cross-linked protein indistinguishable, on SDS/PAGE, from the complexes observed in platelet lysate. Previous work in this laboratory has shown that phosphorylated pleckstrin purified from thrombin-stimulated platelets behaves as a monomer and cannot be cross-linked by dimethylsuberimidate [26]. We found that the latter reagent generated the same pleckstrin-containing complexes in platelet lysate as BS^3, but was less effective (A. M. McDermott and R. J. Haslam, unpublished work). Thus our experiments with purified pleckstrin support the view that the effect of PMA treatment of platelets on the amounts of 90 kDa- and 100–120 kDa-pleckstrin complexes detected by immunoblotting, is attributable to dissociation of pleckstrin oligomers as a result of the phosphorylation of the protein. A preliminary report that high concentrations of non-phosphorylated pleckstrin can aggregate is consistent with our findings [27].

The roles of the N- and C-terminal PH domains of pleckstrin in the proposed oligomerization of the protein are unknown and must be studied with mutant forms of pleckstrin. However, the serine and threonine residues phosphorylated by PKC are immediately C-terminal to the N-terminal PH domain [1,15] and their phosphorylation might therefore regulate interactions of this PH domain with various ligands, including sequences within other pleckstrin molecules. Pleckstrin self-association might prevent interactions with other ligands. In this context, it is significant that the binding of PtdIns(4,5)P_2 to the N-terminal PH domain of pleckstrin appears to be facilitated by phosphorylation of the protein [14,15]. It is also possible that phosphorylation of pleckstrin could disrupt an inhibitory effect mediated by pleckstrin dimers or oligomers in resting platelets. Evidence that a PH domain and adjacent sequences can be involved in protein oligomerization has been obtained for the c-Akt serine-threonine kinase [28]. In this case, complex formation is associated with increased phosphorylation and activation of the enzyme [28,29].

PH domains not only bind phosphoinositol derivatives [9] but also contribute to the binding of G-protein $\beta\gamma$-subunits by some proteins [11–13,30]. Thus the PH domain of the β-adrenergic receptor kinase is necessary for targeting of the enzyme to free $\beta\gamma$-subunits in the plasma membrane [13], whereas that of Btk may play a role in the stimulation of tyrosine kinase activity by $\beta\gamma$-subunits [30]. Although $\beta\gamma$-subunits may bind to glutathione S-transferase fusion proteins containing the PH domains of pleckstrin [15], there is no evidence of such an interaction in intact cells [14,15]. In the present work, we did not detect any cross-linked complexes in platelet particulate fractions, though G-protein activation may be required before $\beta\gamma$-subunits are free to bind to pleckstrin. Studies with Btk [16] and c-Akt [17] have indicated that their PH domains bind various PKC isoenzymes. Platelets contain multiple PKC isoenzymes, including α, β, δ, ζ, η' and θ [31–34], and it is certainly possible that the cross-linked pleckstrin complexes in the 100–120 kDa range could include, in addition to pleckstrin oligomers, species containing PKC isoenzymes. However, we were unable to detect any cross-linked complexes containing PKC using an antibody that recognizes the α, β, γ and δ (and possibly other) isoenzymes. It is possible, however, that cross-linking of PKC to pleckstrin could have masked the epitopes recognized by this antibody. Studies with additional antibodies to PKC will be required.

We did detect a 68 kDa cross-linked protein complex containing pleckstrin that was not affected by activation of PKC. This result implies that pleckstrin monomers can bind to a platelet protein of about 28 kDa. This is unlikely to be the 31 kDa protein recognized by the anti-pleckstrin antibody because in many experiments (e.g. Figure 1a), after cross-linking with BS^3 there was a poor correlation between the decrease in the strength of the signal attributable to the 31 kDa protein and the increase in that attributable to the 68 kDa species. Further studies are in progress to identify this protein.

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