Phosphorylation of human pleckstrin on Ser-113 and Ser-117 by protein kinase C

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During platelet activation, receptor-coupled phospholipid hydrolysis stimulates protein kinase C (PKC) and results in the phosphorylation of several proteins, the most prominent being pleckstrin. Pleckstrin is composed of two repeated domains, now called pleckstrin homology (PH) domains, separated by a spacer region that contains several consensus PKC phosphorylation sites. To determine the role of PKC-dependent phosphorylation in pleckstrin function, we mapped the phosphorylation sites *in vivo* of wild-type and site-directed mutants of pleckstrin expressed in COS cells. Phosphorylation was found to occur almost exclusively on Ser-113 and Ser-117 within the sequence 108-KFARKS*TRRS*IRL-120. Phosphorylation of these sites was confirmed by phosphorylation of the corresponding wild-type and mutant synthetic peptides *in vitro*.

INTRODUCTION

Protein kinase C (PKC) has been implicated in mediating a variety of signalling events that occur after cell-surface receptor-coupled activation of phospholipid hydrolysis [1]. These events lead to divergent cellular responses including proliferation, differentiation, altered gene expression, exocytosis and long-term potentiation [2]. With the exception of receptor down-regulation, the steps that occur between PKC activation and particular cellular responses have not been clearly characterized [3,4]. To characterize these events it will be important to identify the physiological substrates of PKC and to determine the functional consequences of substrate phosphorylation.

In platelets the major substrate for PKC is pleckstrin (also called p47), a protein of unknown function that was first identified by its extensive phosphorylation in response to platelet activators [5,6]. The deduced pleckstrin amino acid sequence contains a 100 amino acid duplicated region at the N- and C-termini [7]. Several recent reports have identified so-called pleckstrin homology (PH) domains in many important signalling and cytoskeletal proteins [8–11]. The precise function of PH domains is ambiguous. Several PH domains can bind G$\beta\gamma$ subunits *in vitro* [12], and may inhibit G$\beta\gamma$-dependent signalling processes *in vivo* when overexpressed in COS-7 cells [13]. Binding studies *in vitro* also suggest that PH domains specifically bind phosphatidylinositol 4,5-bisphosphate (PIP_2) [14].

The function of pleckstrin itself in physiological responses is also unclear. Recently pleckstrin was reported to inhibit agonist-induced PIP_2 hydrolysis mediated by G$\beta\gamma$-dependent and independent signalling pathways in transfected COS cells [15], consistent with a role in PIP_2 binding. Pleckstrin expression is restricted to platelets and leucocytes, possibly indicating that its function is specific to these cell types [7,16]. Pleckstrin phosphorylation is markedly increased during platelet activation, so pleckstrin might have a role in signalling events that lead to platelet shape change, adhesion or secretion, all of which necessitate reorganization of filamentous actin. Although unphosphorylated pleckstrin was previously reported to inhibit actin polymerization *in vitro* [17], more recent experiments with actin affinity columns [18], and recombinant pleckstrin (K. L. Craig and C. B. Harley, unpublished work), have failed to demonstrate a direct interaction between pleckstrin and actin.

We report here that recombinant human pleckstrin expressed in COS-1 cells is phosphorylated by PKC. We used this system to identify the major phosphorylation sites of pleckstrin as Ser-113 and Ser-117 by comparing tryptic phosphopeptide maps from several phosphorylation site mutants of pleckstrin with wild-type pleckstrin. These phosphorylation sites were confirmed by tryptic phosphopeptide mapping of synthetic peptides corresponding to residues 108–120 of the pleckstrin sequence. The availability of pleckstrin phosphorylation site mutants should help determine the role of PKC phosphorylation in pleckstrin function.

MATERIALS AND METHODS

Plasmid construction and mutagenesis

A 1093 bp *Nco*I fragment containing the entire human pleckstrin coding region plus 44 bp of 3′ untranslated sequence, derived from a previous pleckstrin prokaryotic expression construct (pKK233-p47) [7], was treated with Klenow and the appropriate dNTPs to make the ends flush, then ligated into the *Sma*I site of the eukaryotic expression vector pEVRF2 [19], creating pEVp47(+). This cloning procedure fuses the first nine codons of the HSV thymidine kinase gene to the first translated ATG of the pleckstrin cDNA, with translation initiation potentially occurring at either ATG. The sequence of the nine additional residues is MASWGSGYP and would yield an extended polypeptide of predicted molecular mass 41023 Da.

Mutagenesis was carried out by subcloning the *Bam*HI–*Xba*I fragment of pEVp47 containing the entire pleckstrin cDNA into the multiple cloning site of the pSelect-1 phagemid (Promega). After transformation into JM 109 *Escherichia coli* cells, single-stranded template DNA was prepared by infection with helper phage R408. Six oligonucleotides were designed to change the codons for the following amino acids to alanine: Ser-40 (AGT to

Abbreviations used: PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; PH, pleckstrin homology; βARK, β-adrenergic receptor kinase; PIP_2, phosphatidylinositol 4,5-bisphosphate; GST, glutathionine *S*-transferase; FBS, fetal bovine serum.

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GCT), Ser-43 (AGC to GCC), Thr-73 (ACG to GCG), Ser-113 (TCT to GCT), Thr-114 (ACC to GCC) and Ser-117 (TCC to GCC). The double-stranded DNA made from the mutagenesis reaction was transformed into *E. coli* strain BMH 71-18 mutS, which is repair deficient. Plasmid DNA was screened for the correct mutation by dideoxynucleotide sequencing. A 445 bp *Bam*HI–*Xba*I fragment corresponding to the N-terminal half of pleckstrin was digested out of the correctly mutated phagemids and subcloned back into the wild-type eukaryotic expression vector pEVp47. The subcloned regions were sequenced in their entirety to ensure that no other mutations had been inadvertently created.

The prokaryotic expression vector pGEXp47 was created by ligating the *Bam*HI–*Bgl*II fragment from pEVp47(+) into the *Bam*HI site of the prokaryotic expression vector pGEX-2T (Pharmacia).

Antibodies

Recombinant glutathione *S*-transferase (GST)–pleckstrin fusion protein expressed from pGEXp47 in *E. coli* was purified by affinity chromatography on *S*-hexylglutathione-coupled 4% beaded agarose (Sigma), essentially as described [20]. The protein was emulsified in Freund's complete adjuvant and subcutaneously injected into New Zealand White rabbits (300 μg per rabbit). After three boosts the serum was collected and affinity-purified by the following procedure. Purified fusion protein was coupled to activated cyanogen bromide–Sepharose (Pharmacia), in accordance with the manufacturer's instructions, to make an affinity column. After the resin had been pre-equilibrated with 10 mM sodium phosphate, pH 6.8, 10 ml of serum was applied directly to the column and washed through with several bed volumes of the phosphate buffer. Pleckstrin-specific antibodies were eluted with 100 mM glycine, pH 1.8, and neutralized in 1/20 volume of 1 M phosphate buffer, pH 8.0 [21]. Each fraction was tested for its ability to immunoprecipitate pleckstrin from transiently transfected COS cells.

Cell culture and transfections

HL-60 and HEL cell lines were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) and 2 mM L-glutamine. COS cells were grown in Dulbecco's medium containing 10% FBS. COS-1 cells were transiently transfected by a DEAE-dextran technique. Briefly, 5 ml of serum-free medium containing 250 μg/ml DEAE-dextran (Pharmacia), 50 mM Tris/HCl, pH 7.5, and 25 μg of plasmid DNA was added to a 75% confluent dish of COS cells and incubated at 37 °C for 3 h. The cells were then shocked for 1 min with 10% DMSO, washed in PBS and incubated in complete medium for 2 d to allow for expression of recombinant protein.

Western blotting

Proteins were separated on SDS/polyacrylamide gels (12% gel) and transferred onto nitrocellulose; the filter was then incubated with a 1/200 dilution of the affinity-purified anti-pleckstrin antibody in Tween-20 buffer (0.1% Tween-20, 140 mM NaCl, 10 mM Tris/HCl, pH 7.5). After 2 h, the filter was washed in Tween-20 buffer for 30 min followed by four washes of 10 min each. The blots were then developed either with an enhanced chemiluminescence (ECL) detection method (Amersham) or by a colorimetric method. For chemiluminescence, a 1/20000 dilution of a goat anti-rabbit horseradish peroxidase secondary antibody (Amersham) was incubated with the blot for 1 h, followed by several washes in Tween-20 buffer. The washed blot was developed with the ECL kit reagents and exposed to Kodak XAR film for 1 min. For colorimetric development, a 1/7500 dilution of a goat anti-rabbit alkaline phosphatase secondary antibody (Zymed) was incubated with the blot for 1 h, followed by several Tween-20 washes. The reactive bands were then detected by incubation in 5-bromo-4-chloro-3-indolyl phosphate *p*-toluidine salt and *p*-Nitro Blue Tetrazolium chloride.

Phosphorylation and immunoprecipitation *in vivo*

Approximately 10^7 cells per sample were washed twice in phosphate-free minimum essential medium (Gibco), then incubated at 37 °C with 200 μCi of [^{32}P]P_i (ICN) (2 h). For the staurosporine dose–response curves, phorbol 12-myristate 13-acetate (PMA) and/or staurosporine in DMSO carrier solvent was added to each sample at 1 h or 30 min respectively before harvesting. Cells were then scraped from the dishes, transferred to Eppendorf tubes and washed twice in PBS at 0 °C; then 500 μl of cold lysis buffer (1% Nonidet P-40, 150 mM NaCl, 50 mM Tris/HCl, pH 8.0) was added to each sample of cells. After a 30 min incubation on ice, the lysates were spun at 10000 ***g*** for 10 min. Affinity-purified anti-pleckstrin antibody (5 μl) was added to each lysate supernatant and gently rocked for 16 h at 4 °C. Swollen Protein A–Sepharose beads (20 μl; Pharmacia) were added to the lysate for the last hour of this procedure, then pelleted by low-speed centrifugation; the supernatant was then removed. After five washes with 400 μl of lysis buffer, loading buffer was added to the beads and the solubilized protein was separated on SDS/polyacrylamide gels (12% gel). The gels were then dried and exposed to PhosphorImager cassettes (Molecular Dynamics) or film. With Molecular Dynamics PhosphorImager software, PMA-induced phosphorylation was determined by first subtracting the non-specific background phosphorylation in each lane from the corresponding induced band; then the counts present in the uninduced band were subtracted from this value.

Phosphorylation of recombinant pleckstrin and synthetic peptides *in vitro*

Recombinant GST–pleckstrin fusion protein was phosphorylated essentially as described [22], using a mixture of purified α, β and γ PKC isoenzymes (Upstate Biotechnology, Inc.) and various amounts of the fusion protein in a final volume of 25 μl. The reaction was stopped by addition of an equal volume of loading buffer.

The peptides KFARKSTRRSIRL-NH_2 (wild-type) and KFARKSTRRAIRL-NH_2 (S117A), corresponding to residues 108–120 of the pleckstrin sequence, were synthesized by the Central Facility of the Institute for Molecular Biology and Biotechnology, McMaster University. They were phosphorylated *in vitro* as described above, and then unincorporated [γ-^{32}P]ATP was removed by ion-exchange chromatography on a DEAE-Sepharose column before trypsinization and mapping as described below.

Phosphopeptide mapping

Pleckstrin immunoprecipitated from cells labelled *in vivo* or from phosphorylation reactions *in vitro* was run on an SDS/polyacrylamide gel and transferred to nitrocellulose. After exposure to film, the radioactive band was cut out and processed as described [23], by incubation in 0.5% polyvinylpyrrolidone, molecular mass 360 kDa (Sigma), in 100 mM acetic acid for 30 min at 37 °C. The nitrocellulose was then washed in water and incubated at 37 °C in bicarbonate buffer containing 100 ng of 1-chloro-4-phenyl-3-L-toluene-*p*-sulphonamidobutan-2-one-

treated trypsin (Sigma) per μl, the concentration of which was doubled after 2 h and incubated for at least 8 h further to ensure complete digestion. The soluble tryptic peptides were freeze-dried then oxidized in performic acid. After the peptides had been dissolved in water and freeze-dried twice more, they were dissolved in 3 μl of pH 1.9 buffer composed of H_2O/acetic acid/formic acid (897:78:25, by vol.) and spotted on to cellulose plates 1 mm thick (EM Science) as described [24]. The plates were run on a Hunter Thin Layer Electrophoresis System at 1500 V for 35 min, then transferred to a chromatography tank containing phosphochromatography buffer composed of n-butanol/H_2O/pyridine/acetic acid (15:12:10:3, by vol.). The plates were developed twice in this buffer to increase resolution in the chromatographic direction, then exposed to a PhosphorImager screen or directly to film. Phosphoamino acid analysis was performed on individual spots scraped from the plates as described [24].

RESULTS AND DISCUSSION

Expression of pleckstrin in COS cells

To achieve expression of pleckstrin in a eukaryotic system, a recombinant cDNA encoding the entire 350 amino acids of pleckstrin (predicted molecular mass 40087 Da) was subcloned into the eukaryotic expression vector pEVRF2 [19], to create pEVp47(+). Western analysis indicated that COS cells, which do not normally express pleckstrin, expressed full-length recombinant pleckstrin when transiently transfected with pEVp47(+) (Figure 1, lane 2). The level of expression of pleckstrin in this system was compared with endogenous pleckstrin levels found in the human erythroleukaemia HEL and human promyelocytic HL-60 cell lines, and in platelets (Figure 1, lanes 3, 4 and 5 respectively). As seen in Figure 1, the transiently transfected COS cells express approximately the same amount of pleckstrin per μg of total protein as do HL-60 cells. The high level of pleckstrin in HEL cells is not unexpected because this line expresses several megakaryocyte markers [25].

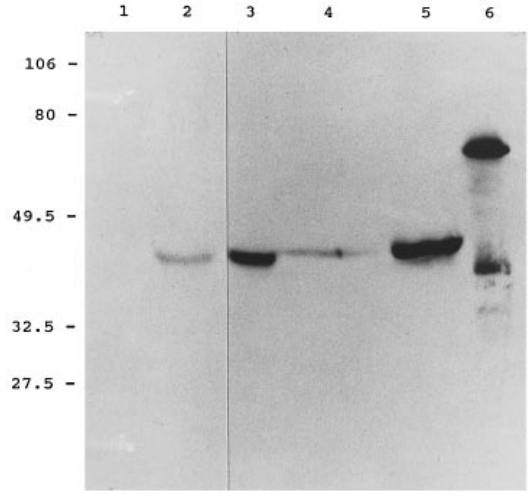

Figure 1 Western analysis of pleckstrin expression

Total protein from the indicated sources was run on a 12% polyacrylamide gel, then electroblotted onto nitrocellulose and probed with the affinity-purified anti-pleckstrin antibody and detected by an enhanced chemiluminescence method. Lane 1, untransfected COS cells (25 μg); lane 2, COS cells transfected with pEVp47(+) (25 μg); lane 3, HEL cells (25 μg); lane 4, HL-60 cells (25 μg); lane 5, 10 μg of total platelet protein; lane 6, 25 ng of purified recombinant GST–pleckstrin fusion protein. The 39 kDa protein in lane 6 is a degradation product of GST–pleckstrin. Molecular masses (kDa) of markers are shown at the left.

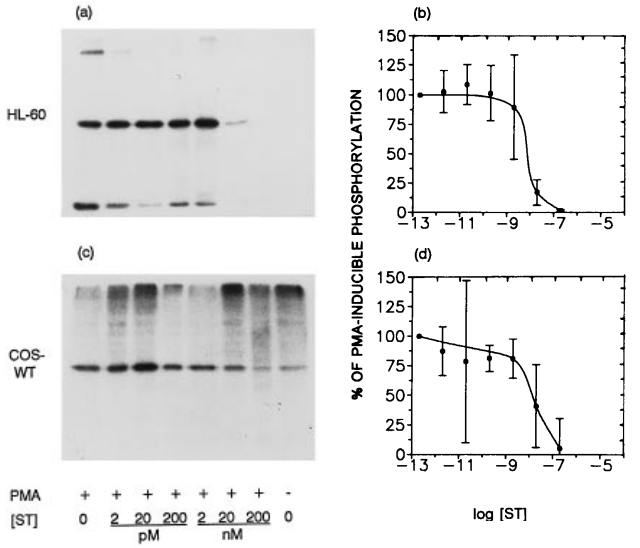

Figure 2 Inhibition of PMA-induced pleckstrin phosphorylation by staurosporine

Pleckstrin from ^{32}P-labelled HL-60 (**a**) or COS pEVp47(+) transfected cells (**c**) was immunoprecipitated after treating the cells with or without PMA (100 nM, 30 min) and staurosporine (ST, indicated concentrations, 1 h), then run on SDS/polyacrylamide gels. These experiments were repeated three times and quantified with a Molecular Dynamics PhosphorImager as described in the Materials and Methods section. The percentage of PMA-inducible phosphorylation remaining at each concentration (M) of ST is shown in (**b**) and (**d**).

The absolute amount of pleckstrin in the various cell lines was estimated by comparison with 25 ng of GST–pleckstrin (Figure 1, lane 6). The mass amount of pleckstrin expressed in COS cells was estimated at 0.02% of total COS cell protein; this is equivalent to the amount previously determined in undifferentiated HL-60 cells (4.4 ng of pleckstrin per 25 μg of total protein [26]). Because typically 5% of the COS cells were successfully transfected (as determined by immunofluorescence microscopy; results not shown), we estimate that in each individually transfected COS cell 0.4% of the total protein is pleckstrin. This is comparable to the abundance of pleckstrin in platelets [27].

PKC-dependent phosphorylation of heterologously expressed pleckstrin

Endogenous pleckstrin in HL-60 cells undergoes a greater than 5-fold increase in phosphorylation in response to PMA treatment, as shown by immunoprecipitation of pleckstrin from [^{32}P]-labelled cells (Figure 2). Staurosporine inhibited the PMA-inducible phosphorylation of pleckstrin with an IC_{50} of about 6 nM, as calculated from the dose–response curve in Figure 2. Although staurosporine is not a highly specific inhibitor, this IC_{50} value is identical to that reported for the inhibition of PKC by staurosporine *in vitro* [28], and comparable to results for the inhibition of PMA-induced pleckstrin phosphorylation in the platelet (IC_{50} 28 nM; [29]). Recombinant pleckstrin expressed in COS cells displays a similar increase in phosphorylation in response to PMA treatment (Figure 2). Staurosporine pre-treatment also antagonized PMA-induced pleckstrin phosphoryl-

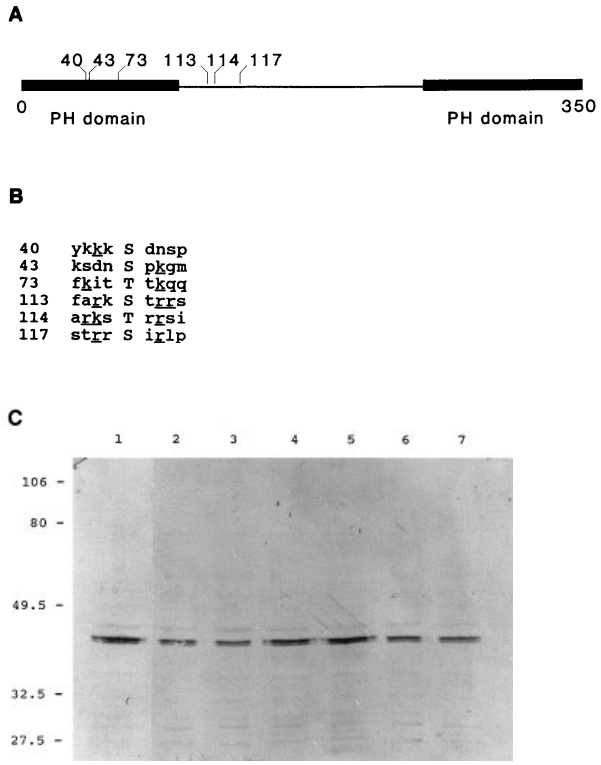

Figure 3 Candidate phosphorylation site mutants of pleckstrin

(**A**) Diagram of pleckstrin sequence indicating the position of the two pleckstrin homology domains (PH) (solid bars), and the six residues tested as candidate PKC phosphorylation sites. (**B**) Amino acid sequence surrounding each of the six potential PKC phosphorylation sites, with the potential phosphoacceptor residue in the centre and capitalized. All of these sequences contain at least one arginine or lysine group in the +2 or −2 position, which are the two positions most commonly occupied by a positively charged amino acid for substrates known to be phosphorylated by PKC [37]. (**C**) Western analysis of site-directed mutants expressed in COS cells. COS cell lysate (25 μg) derived from transfection with each of the six mutant constructs was probed with anti-pleckstrin antibodies and developed by using a colorimetric reaction after probing with an alkaline phosphatase secondary antibody. Lane 1, wild-type; lane 2, mutant S40A; lane 3, mutant S43A; lane 4, mutant T73A; lane 5, mutant S113A; lane 6, mutant T114A; lane 7, mutant S117A. Molecular masses (kDa) of markers are shown at the left.

ation in COS cells, with an IC_{50} of about 10 nM. These results confirm that the phosphorylation of pleckstrin in COS cells occurs in a similar manner to that of endogenous pleckstrin in HL-60 cells or in platelets [29].

Phosphorylation of site-specific mutants of pleckstrin

Pleckstrin is phosphorylated by PKC-dependent pathways during physiological activation of platelets, as well as after direct activation of PKC by PMA treatment [30]. Therefore we chose to mutate six sites in pleckstrin that were potential PKC phosphorylation sites (Figures 3A and 3B), as determined by multiple sequence-alignment comparisons with known PKC substrates [31]. The three highest-scoring sites, Thr-114, Ser-113 and Ser-117, are clustered together between the two PH domains. Western analysis of lysates from transient expression of these six mutants in COS cells shows that their expression levels were comparable to the wild-type protein (Figure 3C).

We labelled the COS cells transfected with these mutants with [^{32}P]P_i and immunoprecipitated the mutant proteins. Of the six

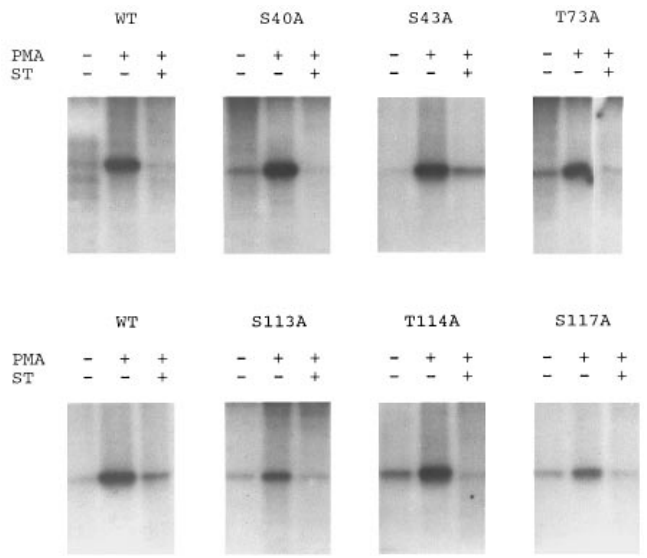

Figure 4 Phosphorylation of pleckstrin mutants *in vivo*

Wild-type (WT) or the indicated phosphorylation-site mutants of pleckstrin were immunoprecipitated from [^{32}P]P_i-labelled transfected COS cells. In each set, cells were either untreated (left-hand lane), treated with PMA (100 nM, 30 min) (middle lane) or treated with PMA in the presence of staurosporine (ST; 200 nM, 1 h) (right-hand lane).

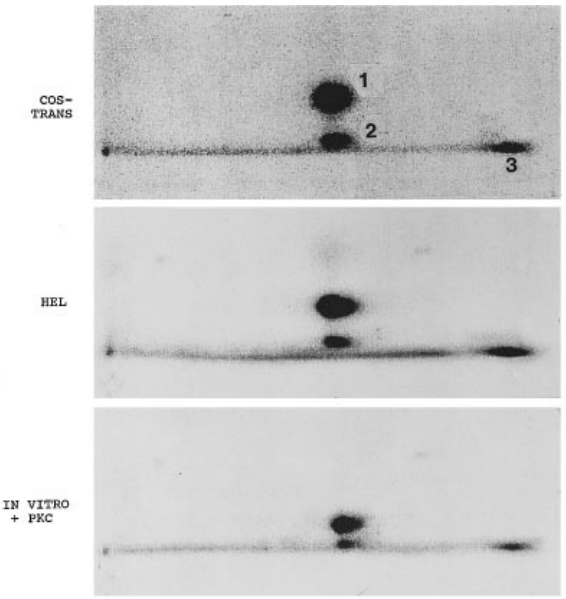

Figure 5 Tryptic phosphopeptide analysis of pleckstrin phosphorylated *in vivo* and *in vitro*

^{32}P-labelled pleckstrin was digested with trypsin and the resulting peptides were separated in two dimensions (see the Materials and Methods section). Wild-type pleckstrin from the following sources was used: upper panel, COS cells transfected with pEVp47(+); middle panel, endogenous pleckstrin from HEL cells; lower panel, GST–pleckstrin fusion protein purified from *E. coli* and phosphorylated *in vitro* with purified PKC. The three major spots were designated 1, 2 and 3.

mutants, only Ser-113A and Ser-117A had a lower level of PMA-induced phosphorylation than the wild-type protein (Figure 4). This experiment suggested that at least these two sites contribute to the total phosphorylation observed.

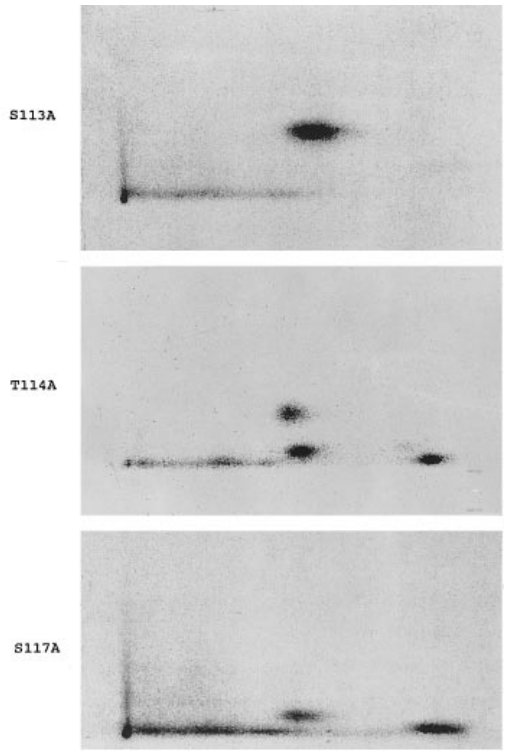

Figure 6 Tryptic phosphopeptide analysis of phosphorylation-site mutants

COS cells transfected with each mutant construct were labelled with [^{32}P]P_i and immunoprecipitated after treatment of the cells with PMA (100 nM, 30 min), and prepared for tryptic phosphopeptide mapping as described in the Materials and Methods section.

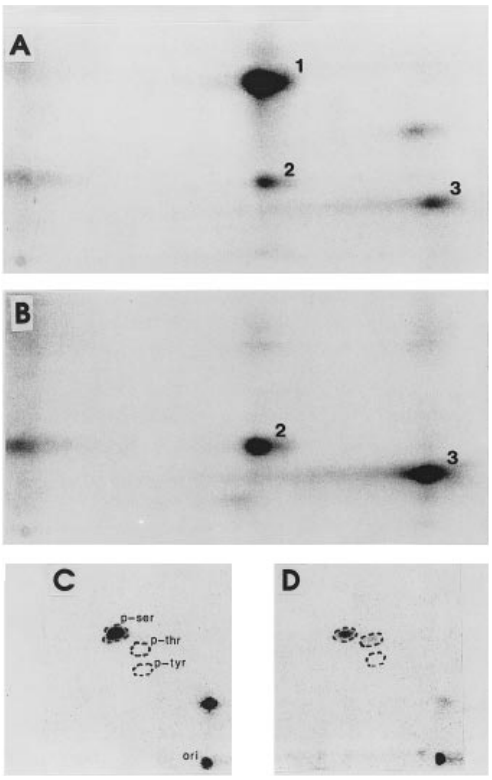

Figure 7 Tryptic phosphopeptide analysis of synthetic peptides

Peptides were phosphorylated with purified PKC *in vitro* as described in the Materials and Methods section. (**A**) Phosphopeptide map of the synthetic peptide KFARKSTRRSIRL. (**B**) Phosphopeptide map of KFARKSTRRAIRL. (**C, D**) Phosphoamino acid analysis of spots 2 and 3 respectively, scraped from the plate shown in (**B**).

Tryptic phosphopeptide mapping

To precisely determine phosphorylation sites in pleckstrin, we performed phosphopeptide mapping experiments with the wild-type and mutant proteins. Importantly, the same pattern of spots was observed for wild-type pleckstrin expressed in COS cells as for endogenous pleckstrin immunoprecipitated from HEL cells (Figure 5). There were three major spots, which we designated spots 1, 2 and 3. Phosphorylation of GST–pleckstrin *in vitro* with purified PKC produced an identical phosphopeptide map with that from pleckstrin phosphorylated *in vivo* (Figure 5). Therefore it is likely that PKC mediates all or most of pleckstrin phosphorylation *in vivo*.

Phosphorylation of specific residues was assigned by two-dimensional phosphopeptide analysis of the mutant pleckstrin proteins expressed in COS cells. For mutant S117A, the major spot (spot 1), is completely absent (Figure 6). Therefore spot 1 should correspond to a predicted tryptic peptide containing Ser-117, namely the peptide S*IR (asterisk indicating the phosphorylated residue). For mutant S113A, spot 1 remains intact, whereas both spots 2 and 3 are completely absent (Figure 6). This indicates that spots 2 and 3 derive from tryptic fragments containing phosphorylated Ser-113 in the wild-type protein. In contrast, the map of mutant T114A appeared the same as wild-type.

To confirm Ser-113 and Ser-117 unambiguously as phosphorylation sites and rule out the possibility that mutation of either of these sites interferes with phosphorylation of other residues elsewhere in the protein, we generated tryptic phosphopeptide maps from two synthetic peptides that were phosphorylated by PKC *in vitro* (Figure 7). The peptide KFARKSTRRSIRL (residues 108–120 of wild-type pleckstrin) yielded an identical tryptic map with that from full-length wild-type pleckstrin (compare Figure 5 with Figure 7A). Because there are only three phosphorylatable residues in this peptide, phosphorylation of pleckstrin must be restricted to all or a subset of these three sites (Ser-113, Thr-114 and Ser-117). To determine whether phosphorylation of Ser-117 was indeed responsible for spot 1 (as suggested by the absence of spot 1 in the full-length S117A mutant), we mapped another synthetic peptide that had Ser-117 replaced by alanine, KFARKSTRRAIRL. The tryptic map for this peptide lacked spot 1 but retained spots 2 and 3, as previously seen for the full-length S117A mutant (compare Figure 6 with Figure 7B). Because this latter peptide has only two phosphorylatable residues (Ser-113 and Thr-114), spots 2 and 3 must be accounted for by phosphorylation of one or both of these residues. Because these two sites are physically inseparable by tryptic cleavage, phosphoamino acid analysis was performed on the individual spots 2 and 3 scraped from the TLC plate to determine whether Ser-113, Thr-114 or both are phosphorylated. Spot 2 contained 100% phosphoserine, whereas spot 3 contained a small amount of phosphothreonine (6% of total radioactivity incorporated into this peptide), in addition to its predominant phosphoserine content (Figures 7C and 7D). Therefore spots 2 and 3 represent related tryptic phosphopeptides containing phosphorylated Ser-113, in agreement with the disappearance of these two spots in the full-length S113A mutant. Because the full-

length T114A mutant retained both spots 2 and 3, phosphorylation of Thr-114 occurs only to a minor extent *in vitro* under favourable conditions.

Quantification of the two major phosphorylation sites revealed that Ser-117 (spot 1) was slightly favoured over Ser-113 (spots 2 and 3), with the percentage of total radioactivity incorporated being 56 % for Ser-117 and 40 % for Ser-113 (± 2 %, $n = 3$). For the mutant T114A, preference for the two sites was reversed, with Ser-113 at 65 % of total phosphorylation and Ser-117 at 32 %. The only difference between phosphorylation *in vitro* of the synthetic peptide substrates and that of full-length pleckstrin was that PKC favoured Ser-117 over Ser-113 (85:15) for the wild-type peptide in comparison with the full-length protein. [An N-terminally truncated version of the wild-type peptide, ARKSTRRSIRL, favoured the 117 position even more, with virtually all (99 %) of the phosphate being incorporated onto Ser-117; results not shown]. The stoichiometry of phosphorylation of the synthetic peptides approached 1 phosphate per peptide under conditions of low substrate and high enzyme concentration (results not shown). This suggests that only one of the two sites was phosphorylated on any given molecule. From previous two-dimensional isoelectric-focusing gel analysis of pleckstrin metabolically labelled with ^{35}S or ^{32}P, we and others [32] have observed a quantitative acidic shift in the pI of pleckstrin isolated from cells treated with PKC activators, implying that nearly all pleckstrin molecules become at least singly phosphorylated after physiological stimulation [33].

In summary, we have expressed pleckstrin, the major PKC substrate of platelets, in COS cells and have shown that it can be phosphorylated in a PKC-dependent fashion in accord with its behaviour in thrombin-stimulated platelets. We have shown that purified PKC phosphorylates the same residues of pleckstrin *in vitro* as observed *in vivo*, confirming that pleckstrin phosphorylation is an excellent indicator of PKC activity *in vivo*. This is important because numerous platelet studies have used the prominent phosphorylation of pleckstrin as a correlative measure of PKC activity [34–36]. We have also unambiguously determined that phosphorylation occurs on Ser-113 and Ser-117 by the complementary methods of site-directed mutagenesis and tryptic phosphopeptide mapping of a defined peptide containing only these phosphorylatable residues. The synthetic peptides employed in this work are excellent PKC substrates (K_m 3 μM; results not shown) and may be well suited for determining PKC activity in cell lysates or permeabilized cells. Lastly, phosphorylation does not occur in either the N- or C-terminal PH domains of pleckstrin, indicating that these domains are not modulated directly by phosphorylation. The attractive hypothesis that phosphorylation of the PKC sites in the spacer region of pleckstrin may regulate PH domain access to target molecules remains to be proven.

We thank Drs Mike Tyers, Ryuji Kobayash, Karl Freeman and Mike Moran for helpful commentary on the manuscript. This work was supported by grants from the Medical Research Council of Canada to C. B. H. and Dr. Tyers.

Received 30 October 1995; accepted 13 November 1995

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