Volume 297, number 3, 285–291 FEBS 10683 February 1992

Multisite phosphorylation of the 80 kDa (MARCKS) protein kinase C substrate in C3H/10T1/2 fibroblasts

Quantitative analysis of individual sites by solid-phase microsequencing

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Received 28 November 1991

A synthetic peptide, KKKKRFSFKKSFKLSGFSFKK, containing the phosphorylation sites of the acidic 80–87 kDa protein kinase C substrate was used to identify phosphopeptides in enzyme digests of this protein from mouse fibroblast C3H/10T1/2 cells. Stimulation of phosphorylation occurred, in vivo, with TPA at Ser^7, Ser^{11} and Ser^{18}, and, with two less potent phorbol esters, at Ser^7 and Ser^{18}. Okadaic acid effected a net phosphorylation of Ser^7 and/or Ser^{11}. Solid-phase sequencing showed that, in vitro, the order of initial rate of phosphorylation was Ser^{11} > Ser^7 > Ser^{18}, while Ser^{18} was preferentially phosphorylated when either Ser^7 or Ser^{11} was occupied. No significant phosphorylation of Ser^{15} was detected.

Kinase C; MARCKS protein; Phosphorylation

1. INTRODUCTION

There have been many reports of increased phosphorylation of an acidic protein(s) of apparent molecular weight 80–87 kDa in response to treatment of intact cells with a variety of agonists which stimulate phosphatidylinositol turnover. This protein was also shown to be phosphorylated in response to phorbol esters and membrane permeable diacylglycerols [1,2] and is a major specific substrate for protein kinase C. Several groups [3–5] have compared the sites of phosphorylation of the 80 kDa protein by peptide mapping in a number of tissues in response to stimulation of intact cells with a variety of agents, and the phosphorylation of this protein provides a useful marker for the activation state of PKC in vivo [6,7]. Phosphorylation occurs on serine at similar sites (up to 2.8 moles of ^{32}P/mole of protein) in vitro and in vivo [5,8,9]. The 87 kDa protein has been cloned and sequenced from the brain and non-neuronal tissue of many species [10–13]. The gene codes for proteins of approximately 330 amino acids that are 60–70% identical in sequence although the phosphorylated region is completely conserved. The name MARCKS (Myristoylated Alanine-Rich C Kinase Substrate) [10] has been proposed in order to highlight particular features of the protein. The 80–87 kDa protein is hereafter referred to as '80 k protein'.

The mouse embryo fibroblast cell line, C3H/10T1/2, employed in the present study, has been widely used to evaluate the role of kinase C in tumour promotion. The two-stage carcinogenesis model that has been established in experiments on mouse skin has also been demonstrated in these cells [14,15]. To determine which specific sites are phosphorylated after stimulation by the different phorbol esters and okadaic acid, a synthetic peptide corresponding to the residues 152–172 of the 80 k protein containing all known phosphorylation sites was prepared and phosphorylated in vitro by PKC. This was then proteolytically digested in parallel with ^{32}P radiolabelled 80 k protein from C3H/10T1/2 fibroblasts stimulated with the various agents. In this paper we describe the use of solid-phase sequencing which enables the extent of phosphorylation to be determined at multiple sites after coupling to Sequelon-AA membranes.

Abbreviations: PKC, protein kinase C; DOPPA, 12-deoxyphorbol-13-phenylacetate-20-acetate; SAP A, sapintoxin A; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; EDC, 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride; SDS, sodium dodecylsulphate; TFA, trifluoroacetic acid.

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2. MATERIALS AND METHODS

2.1. *Materials*

C3H/10T1/2 cells were obtained from The European Collection of Animal Cell Cultures, PHLS, UK. Foetal calf serum was from Gibco BRL (cat. no. 011-06290). TPA, 12-*O*-tetradecanoylphorbol-13-acetate and histone III-S, were from Sigma Chemical Company UK. ^{32}P carrier-free phosphate was obtained from Amersham, UK. [γ-

^{32}P]ATP (6,000 Ci/mmol) was from NEN, DuPont. Phosphatidylserine was obtained from Lipid Products, Nutfield Nurseries, Nutfield, Surrey. Microcrystalline cellulose plates were Polygram Cel 400 (20 × 20 × 0.1 cm) from CamLab. Coomassie brilliant blue R250 and ampholytes were from Serva (supplied by Cambridge BioScience). X-ray film was XAR-5 from Kodak. Sequelon-AA membrane kit was obtained from Millipore (UK). Okadaic acid was isolated from cultures of *Prorocentrum lima* as in [16]. Other items were from standard supplier sources or as listed in the text.

2.2. *Partial purification of PKC*

A partially purified preparation of PKC was obtained from sheep brain [17] and was assayed using the method of Parker et al. [18].

2.3. *Synthesis of peptide*

The peptide KKKKRFSFKKSFKLSGFSFKK, corresponding to residues 152–172 of the bovine brain MARCKS protein, according to the cDNA sequence determined by Stumpo et al. [10] and containing the phosphorylation site recognised by PKC, was synthesized using standard Fmoc chemistry on an Applied Biosystems 430A Peptide synthesizer using reagents supplied by the manufacturer.

2.4. *In vitro phosphorylation of synthetic peptide*

Batches of synthetic peptide (5 nmol) were incubated at 250 nM, 1 μM and 10 μM final concentration in 20.5 and 0.5 ml, respectively, in 20 mM Tris–HCl, pH 7.5, 10 mM Mg^{2+}, 1.5 mM Ca^{2+}, 10 μM ATP (containing 25, 50 and 50 μCi [γ-^{32}P]ATP, respectively), 50 μg/ml phosphatidylserine; 20% v/v PKC (1.4 U/ml); for the stated time (see Results and Figure legends) at 30°C.

Estimation of the level of phosphorylation of the phosphopeptide was achieved by spotting 50 μl of the above onto P81 paper (4 × 2 cm) after incubation. These were then washed, 3 × 10 min with 75 mM orthophosphoric acid, placed in 4 ml scintillant (Beckman Ready Safe) and radioactivity was measured by scintillation counting. The specific activity (cpm/pmol ATP) for each incubation mix was estimated by spotting 50 μl onto P81 paper (4 × 2 cm) and measuring radioactivity by scintillation counting.

2.5. *Purification of in vitro phosphorylated peptide*

The phosphorylated peptide was made 1% v/v TFA and fractionated by HPLC using a water/acetonitrile gradient (0–50% in 0.1% TFA) on an Aquapore Octyl reverse-phase column (30 × 1 cm) fitted to a Gilson HPLC System (Anachem, UK). Samples for 2D phosphopeptide mapping and FAB MS were re-chromatographed using the same gradient but on a Vydac C_{18} reverse-phase column (218TP54, 25 × 0.46 cm).

2.6. *Mass spectroscopy of phosphorylated peptide*

Fast atom bombardment mass spectra were acquired on a VG70/250SE mass spectrometer in positive ion mode at an accelerating voltage of 8 kV using a caesium ion gun operating at 20 kV [19]. Peptide in solution at a known concentration was applied to the probe tip, concentrated by air-drying to approximately 2 μl and mixed with 1 μl of *m*-nitrobenzyl alcohol containing 1% v/v trifluoroacetic acid. Several scans were summed using the multi-channel analysis facility over the mass range of 2,780–2,420 at a scan time of 11.5 s and a resolving power of 1,000. Calibration was achieved using 2 μl of a 0.5 M mixture of caesium iodide and sodium iodide (Aldrich Chemical Company).

2.7. *Amino acid sequencing*

Peptides were covalently bound to Sequelon-AA membranes (Mil-

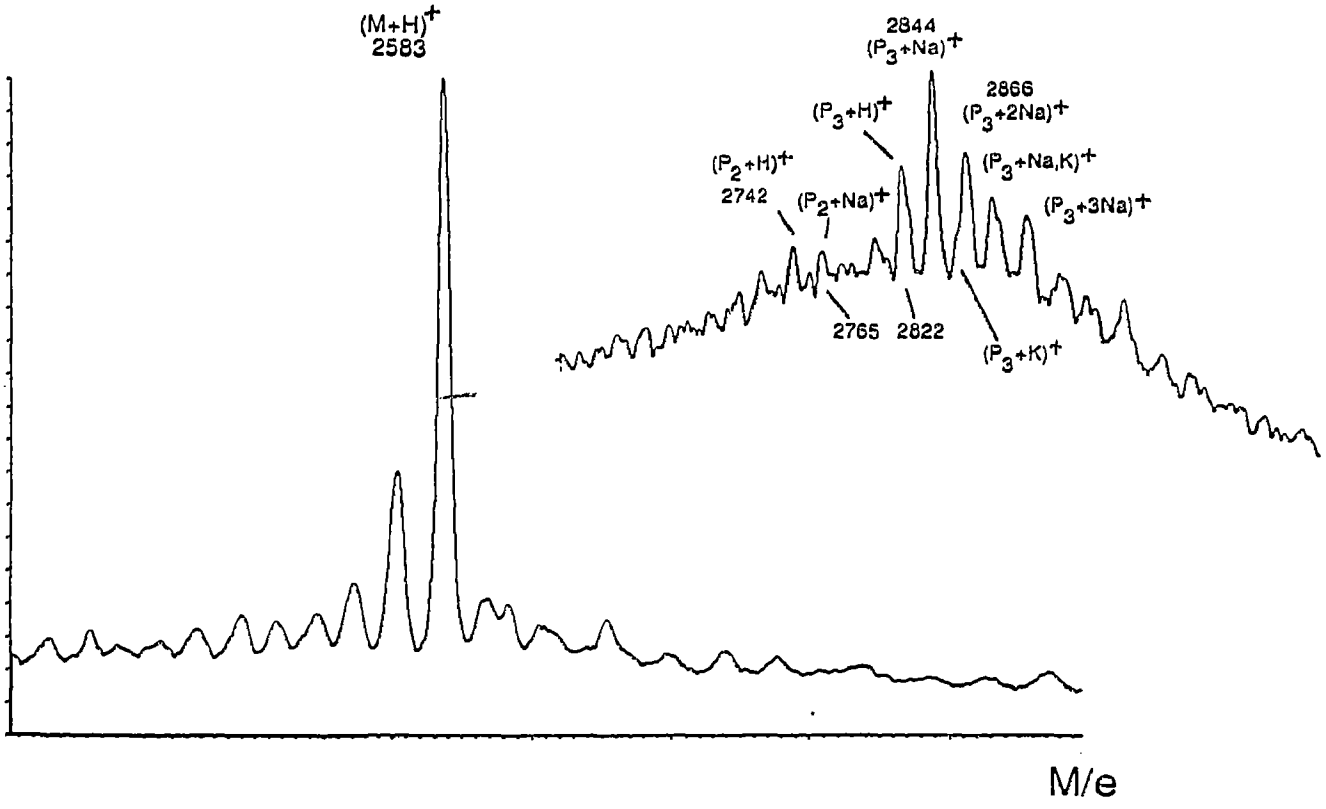

Fig. 1. Fast atom bombardment mass spectroscopy of dephosphopeptide (ca. 20 pmol) and phosphopeptide (inset; ca. 200 pmol; maximally phosphorylated, using a peptide concentration of 250 nM in a total volume of 20 ml with incubation for 2 h under the conditions described in the text). P_2 and P_3 indicate doubly and triply phosphorylated peptides, respectively, and $(P_3 + Na)^+$ indicates triply phosphorylated peptide pseudomolecular ion as the single sodium adduct. Non-phosphorylated peptide is less prone to produce metal ion adducts, consequently a major peak of $(M + H)^+$ is detected and is one reason for the greatly increased sensitivity of the unphosphorylated species.

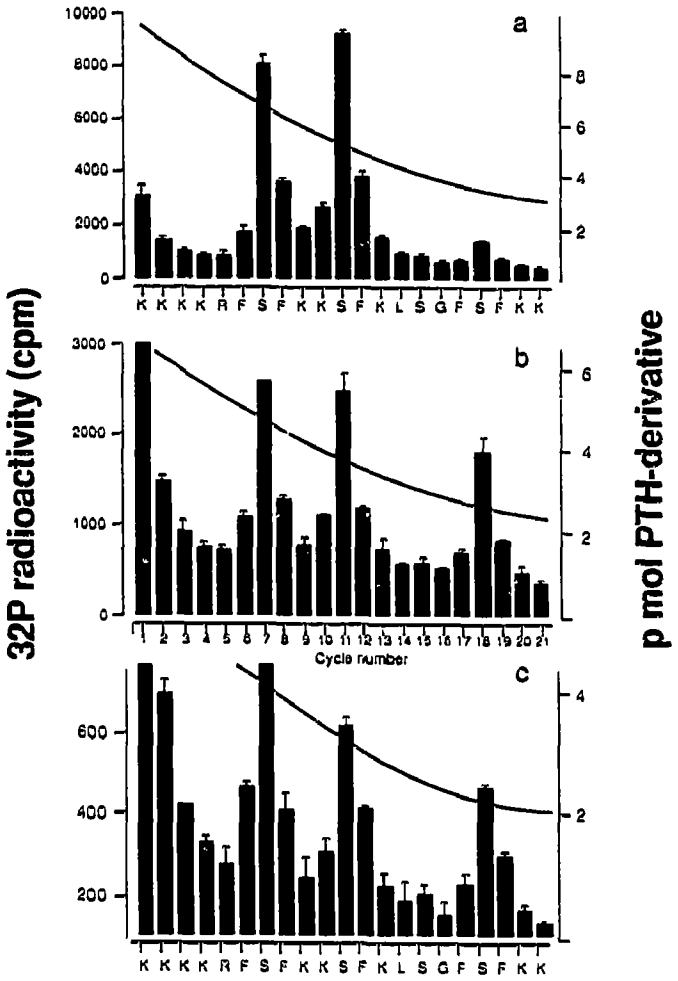

Fig. 2. Phosphopeptides with different levels of phosphorylation were sequenced after covalent coupling to Sequelon-AA on Milligen 6600 Prosequencer, following incubation for 1 h at an initial peptide concentration of: (a) 250 nM in a final volume of 20 ml, 744,851 cpm loaded; (b) 1 μm in a final volume of 5 ml, 299,323 cpm loaded; and (c) 10 μM in a final volume of 0.5 ml, 85,016 cpm loaded. The cpm and yield at each residue through the sequence are displayed.

ligen) following the manufacturer's instructions. Sequelon-AA membrane is a modified PVDF (polyvinylidene difluoride) membrane containing aryl amino groups which may be coupled to both the C-terminal and side-chain carboxyl groups of peptides or proteins via reaction with the water soluble carbodiimide, EDC.

Peptides were subjected to solid-phase microsequencing on a Mil-

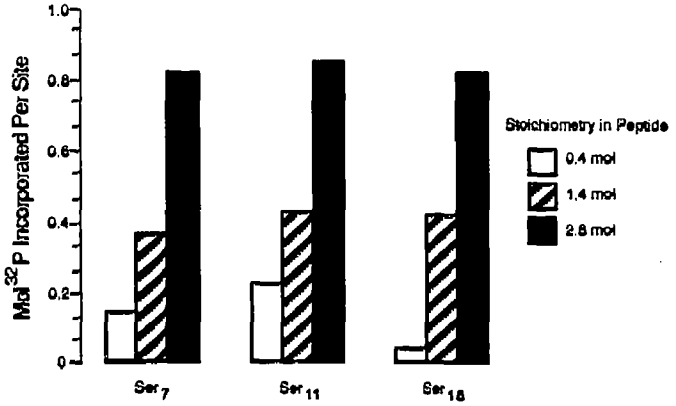

Fig. 3. The relative phosphorylation at each serine residue in the synthetic peptides is shown after incorporation of the following total radioactivity into the peptide: 0.4, 1.4 and 2.8 mol/mol ^{32}P-phosphopeptide.

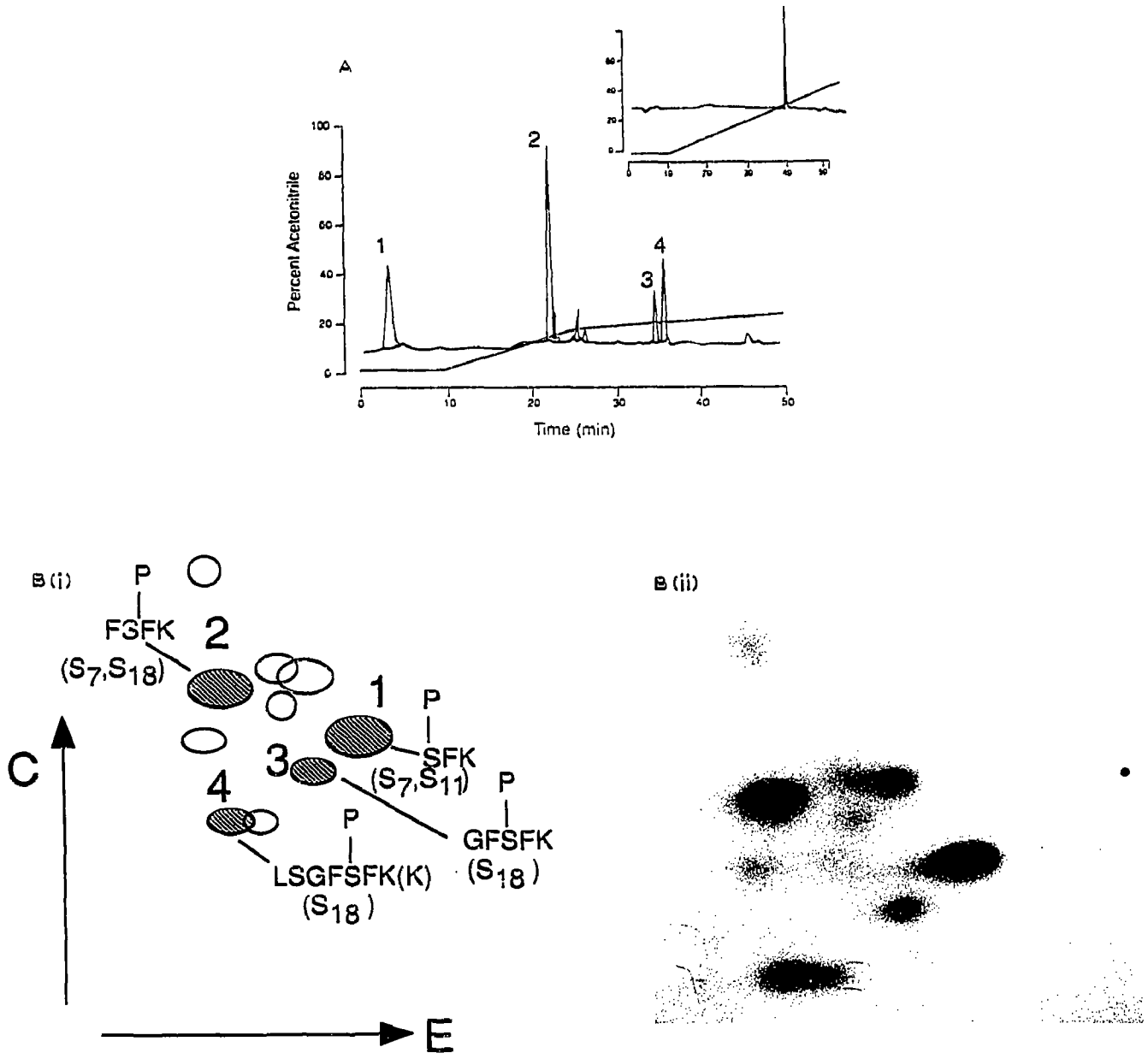

Fig. 4. Identification of sites of phosphorylation within the 80 k MARCKS protein. (A) Reverse-phase HPLC and FAB MS of enzyme digests of phosphorylated synthetic peptide. The inset shows a chromatogram of the intact phosphorylated MARCKS peptide purified by reverse-phase HPLC using a Vydac C_{18} reverse-phase column eluted with a water/acetonitrile gradient (0.1% TFA) while monitoring eluant by uv detection at 215 nm. Gradients are indicated by the continuous line —. Unusually, the dephospho- and all phospho-forms of the intact synthetic peptide co-eluted under the conditions used. (B) Diagramatic representation (i) of 2D phosphopeptide map (ii) with peptide assignments. After treatment of C3H/10T1/2 cells with phorbol ester or okadaic acid, the 80 k protein was partially purified by heat treatment and SDS PAGE, before it was digested with proteinases (trypsin and subtilisin) as described in the text. The peptides were separated on thin layer microcrystalline cellulose in the direction indicated (E, electrophoresis; C, chromatography). The presence of many minor phosphopeptides in the 2D map may also represent multiple sites of cleavage by trypsin giving rise to more than one peptide for a single phosphorylation site. This would be a consequence of the KKKKR and two KK sequences within the region containing the phosphorylation sites. Since trypsin is an endoprotease, after cleavage at one of the adjacent basic residues, subsequent trimming of a single exposed N- or C-terminal lysine or arginine, does not occur.

ligen 6600 Prosequencer. This was done by placing the disk in the reaction chamber and applying the standard Edman chemistry using the 50:50 sequence protocol which involves splitting the resulting PTH-amino acid eluant equally between the on-line Waters 600 HPLC system and a fraction collector. Samples from the fraction collector were monitored by scintillation counting. The cpm/pmol was calculated for each residue to take account of the decreasing yield through the run.

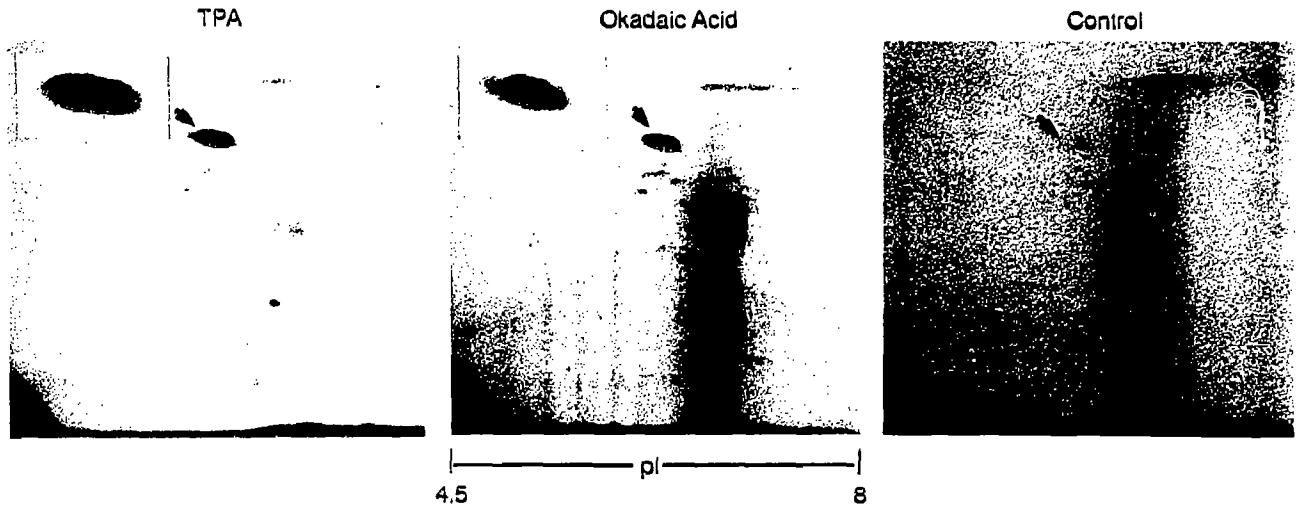

Fig. 5. To verify that the phosphorylation obtained after treatment of C3H/10T1/2 cells with phorbol esters or okadaic acid corresponded to the acidic 80 k MARCKS protein, 2D PAGE and autoradiography were performed. The 80 k protein separated as multiple isoelectric forms with pI between 4.9 and 5.1 as a result of multisite phosphorylation. The figure shows the autoradiograph from unstimulated cells (control) or after stimulation with the phorbol esters (TPA) or okadaic acid (OA).

2.8. *Enzyme digest of phosphorylated synthetic peptide*

Purified phosphopeptide was prepared in 0.1 M *N*-ethylmorpholine acetate, pH 8.6; trypsin was added to give a ratio of 2% w/w trypsin/protein and incubated overnight at 37°C. Further cleavage with subtilisin was achieved with the addition of enzyme to give a ratio of 4% w/w subtilisin/protein and continued incubation at 37°C for a further 6 h. The reaction was terminated by adding TFA to 1% v/v and freezing on dry-ice.

2.9. *Reverse-phase HPLC of enzyme digested phosphopeptides*

Phosphopeptides were loaded onto a Vydac C_{18} reverse-phase column (218TP54, 25 × 0.46 cm). Initially, elution was isocratic at 0.1% v/v aqueous TFA (buffer A) for 10 min at a flow rate of 1 ml/min. The column was developed with a linear gradient of 0–50% v/v buffer B (0.082% v/v TFA/acetonitrile) over 50 min, followed by 50–100% v/v buffer B for 20 min at the same flow rate. The eluant was monitored at 215 nm and fractions containing peptides were collected manually. Prior to amino acid microsequencing samples were concentrated in a Gyrovap (UniScience).

2.10. *Amino acid analysis*

Samples were hydrolysed at 110°C, 24 h with 6 M HCl, 2 mM phenol in the vapour phase under nitrogen. Amino acids were detected with an Applied Biosystems 420A Derivatiser-Analyzer fitted with an on-line 130A Analyzer for detection of PTC (phenylthiocarbamyl) amino acids using reagents and solvents supplied by the manufacturer.

2.11. *Phorbol ester-stimulated phosphorylation of the 80 k protein by kinase C in vivo*

C3H/10T1/2 cells were grown to confluence on 5 cm dishes in Dulbecco's Modified Eagle's medium (DMEM) containing 10% foetal calf serum, under 5% CO_2 at 37°C in a humidified atmosphere. Cells were maintained in 0.5% serum overnight and then incubated for 4 h in 2 ml of phosphate-free DMEM and 1 mCi/ml of carrier-free ^{32}P orthophosphate. The cells were exposed to the phorbol esters (50 nM) for 10 min, or okadaic acid (100 nmol) for 5 h (since there is a low basal level of PKC activity in C3H/10T1/2 cells). Phosphoproteins were analysed on 10% w/v SDS PAGE gels [20] or 2D PAGE [21] after lysis of the cells in buffer containing protease inhibitors according to the method in [6]. The buffer composition was as follows: 50 mM Tris–HCl, pH 7.8, 50 mM benzamidine HCl, 50 mM NaF, 2.5 mM sodium pyrophosphate, 5 mM β-glycerophosphate, 2 mM EDTA, 20 mg/ml leupeptin, 2 mg/ml pepstatin A, 1.5% Triton X-100, 0.1% SDS.

2.12. *2D Phosphopeptide mapping*

To minimise contamination of the 80 k phosphoprotein with others of similar M_r, it was selectively purified by virtue of its heat stability. The cell lysate was heated on a boiling water bath for 10 min. Denatured proteins were pelleted by high speed centrifugation in an MSE microfuge. The heat stable proteins remaining in the supernatant were boiled with an equal volume of SDS-PAGE sample buffer for 5 min, before being electrophoresed on 7.5 or 10% w/v SDS-polyacrylamide gels. The phosphorylated 80 k protein, localized by autoradiography of the dried gel, was excised. The phosphopeptide was digested at 37°C using 100 μg/ml trypsin in 0.1 M *N*-ethylmorpholine acetate, pH 8.6. This represented approximately 1:10 enzyme to substrate by weight. The partially digested protein was extracted from the gel fragments by centrifugation and was then further digested with subtilisin (200 μg/ml) for 6 h at 37°C. The *N*-ethylmorpholine buffer was removed by freeze-drying the phosphopeptide mixture overnight, before re-dissolving in 20 μl of electrophoresis buffer (pyridine/acetic acid/water; 5:50:945, pH 3.5). The phosphopeptides were monitored for radioactivity by Cerenkov counting, and equal counts were loaded onto thin layer microcrystalline cellulose sheets (20 cm × 20 cm × 0.1 mm). Peptides were electrophoresed at 300 V for 3 h followed by ascending chromatography in pyridine/acetic acid/water/butanol (10:3:12:15). The peptide maps were then dried and autoradiographed at −70°C using Kodak XAR-5 film.

The peptides collected by reverse-phase HPLC of enzyme-digested synthetic phosphopeptide were concentrated to dryness (Gyrovap) and diluted with electrophoresis buffer (20 μl). These were subjected to 2D peptide mapping in parallel with the 80 k protein digest.

3. RESULTS AND DISCUSSION

A maximally phosphorylated synthetic peptide was purified by reverse-phase HPLC and analysed by FAB MS (Fig. 1). This spectrum indicated that the main product was a triphosphorylated species with some diphospho-form present: no tetraphosphorylated form

was detected. The observed proportions of di- and triphosphopeptide by FAB MS, while not quantitative, suggest that the actual level of phosphorylation is near 3. This figure is in good agreement with the value of 2.8 mol/mol according to other authors [5,8,9]. The presence of multiple peaks in the spectra for each phosphoform is attributed to Na^+ and K^+ adducts. Minor amounts of metal ion adducts of Mg^{2+} and Ca^{2+} (not annotated) may also be present. After coupling to Sequelon-AA membrane, sequencing was carried out on a solid-phase Milligen 6600 Prosequencer. Coupling efficiency was low for this particular peptide, about 10% according to both the incorporation of ^{32}P and the initial yields of the peptide during sequencing, possibly due to the presence of only one carboxyl group. However, the subsequent recovery of radioactivity from each site was reproducible, as measured by cpm/pmol.

Analysis on the Milligen sequencer showed that the peptide was phosphorylated on Ser^7, Ser^{11} and Ser^{18} (using numbering of residues for the synthetic peptide which correspond to Ser^{158}, Ser^{162} and Ser^{169} in bovine MARCKS protein [10]. After normalizing for the decrease in yield through the sequencing run, the order of phosphorylation was $Ser^{11} > Ser^7 > Ser^{18}$ (Fig. 2). The progress of incorporation of phosphate with increasing extent of phosphorylation is shown in Fig. 3. This analysis indicated that Ser^{11} became phosphorylated most rapidly, followed by Ser^7. When either of these sites was occupied, the second mole of phosphate was preferentially incorporated into Ser^{18}. The sequence analysis was carried out at least twice for each level of phosphorylation.

The results obtained by sequencing confirmed the stoichiometry suggested by FAB MS. It is evident that Ser^{15}, the third in the sequence, was not phosphorylated. This is in contrast to previous reports [5,22]. These authors analysed tryptic subdigest peptides to identify the phosphorylated serines. The peptides (F)SFK(K) (Fig. 4) could have been assigned to the region containing Ser^{15} without information from direct sequence analysis of the intact fragment. Lack of phosphorylation at Ser^{15} is not particularly surprising since this residue is not in a good consensus sequence for PKC [23].

Previous analysis employed phosphopeptide maps and reverse-phase HPLC after stimulation with various phorbol esters [6,24], at a time when the sequence of the phosphorylation domain was not known. It is now realized that identical phosphorylated subdigested peptides (this paper and [5]) can arise from different phosphorylation sites (Fig. 4). Although the particular proteinases and conditions were again used for consistency with previous analysis of phosphopeptides from this protein [6,24] the results of this study clearly demonstrated that the solid-phase methodology is better at analysing levels of phosphorylation at each site. Thus, in the light of the solid-phase sequencing results, the ability of phorbol esters to stimulate the phosphorylation of specific sites of the 80 k protein can now be assessed. TPA is a potent tumour-promoting derivative [25], whereas neither SAP A or DOPPA possess promoting activity [26,27]. Both TPA and SAP A, but not DOPPA, were reported to stimulate the phosphorylation of the 80 k protein although the number and the pattern of 80 k protein spots on 2D PAGE (Fig. 5) after stimulation of C3H/10T1/2 fibroblasts by TPA and SAP A was not identical [26], suggesting that the relative phosphate incorporation into distinct sites may be different. Reverse-phase HPLC peptides from the trypsin/subtilisin-digested maximally phosphorylated synthetic peptide (Fig. 4A) were analysed by FAB MS and amino acid analyses. In parallel with 2D phosphopeptide mapping (Fig. 4B), this enabled the assignment of individual sites to phosphopeptides found in the various 2D maps, with the knowledge that Ser^{15} is not phosphorylated. The observation of multiple phosphopeptides in the control sample indicates that the 80 k protein is basally phosphorylated in vivo at multiple sites.

Treatment of the fibroblast cells with okadaic acid results in the net phosphorylation of Ser^7 and possibly Ser^{11} (peptides 1 and 2 in Fig. 4B). Peptide 2, phosphorylated FSFK, could have also arisen from Ser^{18} but since the radioactivity associated with peptides 3 and 4 (also containing Ser^{18}) did not increase (data not shown) it is concluded that the increase in phosphorylation associated with peptide 2 derives from Ser^7. This indicates that phosphatases 1 and 2A are responsible for removal of phosphate from sites Ser^7 and/or Ser^{11}. Analysis by 2D PAGE of the fibroblast cells after treatment with okadaic acid (Fig. 5) supports the finding that ^{32}P radioactivity is incorporated into fewer sites than after TPA stimulation. Instead of three radiolabelled forms of the 80 k protein there is a predominance of less acidic isofocusing form(s).

The present study shows the advantages of solid-phase sequencing in analyzing multiply phosphorylated peptides, resulting in good recovery of ^{32}P and accurate quantitation of incorporation into individual sites. This technique is applicable to the interpretation of multisite phosphorylation patterns is a wide range of substrate proteins.

Acknowledgements: We thank Alan Harris, NIMR, for amino acid sequencing (Applied Biosystems model 477A Sequencer) and Peter Fletcher, NIMR, for synthesis of the peptide. This work was supported by MRC and UNAM to H.A. Manjarrez-Hernandez.

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