p42 MAPK phosphorylates 80 kDa MARCKS at Ser-113

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Abstract It is demonstrated here that p42 MAPKinase (p42 MAPK) phosphorylates the Myristoylated Alanine-Rich C-Kinase Substrate (MARCKS) at Ser-113. In permeabilised Swiss 3T3 cells activation of protein kinase C (PKC) leads to p42 MAPK activation, but only the protein kinase C sites in MARCKS become phosphorylated and not Ser-113. The mitogen platelet-derived growth factor (PDGF) elicits the same response. These results demonstrate that while Ser-113 is a substrate for p42 MAPK in vitro and can be phosphorylated in vivo as shown by Taniguchi et al. [(1994) J. Biol. Chem. 269, 18299–18302], its phosphorylation is not subject to acute regulation by p42 MAPK in Swiss 3T3 cells.

Key words: p42MAPKinase; MARCKS; Protein kinase C; Phosphorylation

1. Introduction

The Myristoylated Alanine-Rich C-Kinase Substrate (MARCKS) is a well characterised protein kinase C (PKC) substrate that is a marker for PKC activation in vivo (reviews [2,3]). Phosphorylation of MARCKS by PKC has been shown to interrupt its interaction with calmodulin and actin [4] and thereby displaces MARCKS from the cytoskeleton. Consistent with these PKC-dependent phosphorylations controlling such functions in vivo, it has been established that MARCKS is displaced from the cytoskeleton on PKC activation [5].

Recent studies have indicated that other kinases are involved in regulating MARCKS phosphorylation. In vitro, the PKC-related kinase 1 (PRK1) can phosphorylate MARCKS on serines 152, 156 and 163, the same residues targeted by PKC itself [6]. This indicates that MARCKS phosphorylation at these sites is not an exclusive monitor of PKC activation. Additionally, it has been shown that MARCKS isolated from bovine brain is phosphorylated on sites not targeted by PKC or PRK [1]. These sites are serine or threonine residues followed by a proline, suggesting the involvement of proline-directed kinases.

One of the downstream targets of PKC is the p42 MAPKinase (p42 MAPK) which phosphorylates Ser/Thr residues surrounded by two proline residues. Since many growth factors and the PKC activator 12-tetradecanoyl phorbol-13-acetate (TPA) induce PKC as well as MAPK activation and result in phosphorylation of MARCKS, it has been important to establish whether p42 MAPK itself in any way accounts for the control of MARCKS phosphorylation. It is demonstrated here that Ser-113 in MARCKS is a substrate for p42 MAPK, but that under conditions of p42 MAPK activation there is no acute increase in Ser-113 phosphorylation.

2. Materials and methods

2.1. Materials

Trypsin was purchased from Worthington. [γ-^{32}P]ATP and Hyperfilm were from Amersham and streptolysin-O from Wellcome. Cellulose thin-layer chromatography plates were supplied by Kodak and foetal calf serum was from Gibco Bethesda Research Laboratories. Mutagenesis was performed using the Chameleon double-stranded, site-directed mutagenesis kit from Promega. All other biochemical reagents were from Sigma. A polyclonal antiserum that detects p42 MAPK was raised in rabbits against the peptide ITVEEALAHPYLEQYYDPTFDEPV.

2.2. MARCKS phosphorylation in permeabilised Swiss 3T3

Swiss 3T3 cells were cultured as described previously [7]. A 15 cm dish of quiescent Swiss 3T3 cells was washed three times with prewarmed phosphate-buffered saline (PBS) and incubated with a freshly prepared mixture of 1 ml streptolysin-O (0.036 IU/ml) and 4 ml prewarmed permeabilisation buffer (6.25 mM $MgCl_2$, 10 μM ATP, 12.5 mM PIPES pH 7.4, 150 mM KCl, 1.25 mM EGTA, 0.75 mM $CaCl_2$, 37.5 mM NaCl, 20 μM leupeptin, 1.5 μM aprotinin, 1 μM microcystin) for 3 min. Subsequently [γ-^{32}P]ATP (10 μCi) was added and the cells were stimulated with TPA (400 nM) or PDGF (50 ng/ml) for a further 10 min. The cells were scraped into an Eppendorf tube and homogenized with 10 strokes through a syringe fitted with a 25 gauge needle. Extracts were heated for 10 min at 95°C, incubated on ice for 10 min and centrifuged for a further 10 min at 12000 rpm in a benchtop microfuge. The heat-stable MARCKS protein was precipitated with trichloroacetic acid (10% final concentration) for 4 h at room temperature. After centrifugation the pellet was resuspended in 1×Laemmli sample buffer [8] and loaded onto a 10% SDS-PAGE gel. The Coomassie-stainable band of 80 kDa was excised and subjected to 2-dimensional peptide mapping.

2.3. Protein purification

Purification of the GST-MARCKS fusion protein was as described earlier [9]. Recombinant p42 MAPK was purified according to Stokoe et al. [10] and recombinant protein kinase C was purified from Sf9 cells according to Stabel et al. [11].

2.4. MARCKS phosphorylation by p42 MAPK in vitro

Active MEK (MAPK or ERK kinase) protein was immunoprecipitated from one 15 cm dish of confluent Swiss 3T3 cells that were stimulated with 250 nM TPA for 10 min. The cells were washed twice in ice-cold PBS and scraped into buffer A (50 mM Tris-HCl pH 7.5, 100 mM NaCl, 5 mM EDTA, 100 μM Na_3VO_4, 50 mM NaF, 1% Triton X-100, 50 μg/ml PMSF, 10 mM benzamidine, 125 μg/ml aprotinin, 250 μg/ml leupeptin). MEK antiserum (12.5 μl) and 70 μl of a *S. aureus* cell suspension 1:1 slurry in PBS were added and the extracts were incubated for 5 h at 4°C. After immunoprecipitation of active MEK protein, the antibody complex was washed three times in buffer A (with 150 mM NaCl). The protein was eluted from the antibody complex in 150 μl PBS containing the peptide antigen (2 mg/ml). Active MEK (20 μl) in PBS (or alternatively 0.3 μg of active MEK1 S117E S221E [12]) were incubated with 0.3 μg recombinant p42 MAPK, 10 μg of either GST-MARCKS or GST in the presence of 1 μCi [γ-^{32}P]ATP in buffer B (25 mM HEPES pH 7.5, 10 mM pNPP, 0.1 mM EDTA, 10 mM $MgCl_2$) for 30 min at 30°C. The

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samples were loaded onto a 10% SDS-PAGE gel, dried down and autoradiographed.

2.5. Site-directed mutagenesis

Mutagenesis of Ser-113 to Ala was performed with the Chameleon kit from Stratagene according to the manufacturer's instructions. To introduce a two base pair exchange into MARCKS pGEX 2T we used the following oligonucleotide – GGCCGAGCCCGCCGAGCCGGC-CTCCCCGGCCGCCGAGGC-. The second oligonucleotide – GAG-CCCGCCGAGCCCAGCGCGCCGGCCGCCGAGGCCGAG – was used for selection of mutated double stranded plasmid and was designed to destroy a *Kpn*I restriction site in the plasmid sequence of pGEX 2T.

2.6. Other procedures

Protein determination of GST-MARCKS was carried out according to the method of Bradford [13]. Other proteins were measured with the BCA protein assay reagent (Pierce). SDS-PAGE was performed according to [8]. Two-dimensional peptide mapping was performed as described in [6].

3. Results and discussion

3.1. p42 MAPK phosphorylates MARCKS in vitro

The 80 kDa MARCKS protein is a well described substrate for PKC and the related PRK family [2,6]. As recently reported by Taniguchi et al. [1], MARCKS protein isolated from bovine brain was found to be phosphorylated not only on the three serine residues targeted by PKC and PRK (Ser-152, Ser-156, Ser-163) but also on six so far unknown sites, one of which corresponds to the p42/p44 MAPK phosphorylation motif P-X-S/T-P.

In order to assess if MARCKS is a substrate for p42 MAPK, activated recombinant MAPK protein was incubated with GST-MARCKS in the presence of [γ-^{32}P]ATP. Two phosphorylated bands (Fig. 1, lane 1) migrating as 110 and 70 kDa proteins were obtained. These represent the full-length fusion protein of MARCKS and an N-terminal fragment that is generated by proteolysis during expression in bacteria. The GST moiety of the fusion protein is not phosphorylated (Fig. 1, lane 5–7).

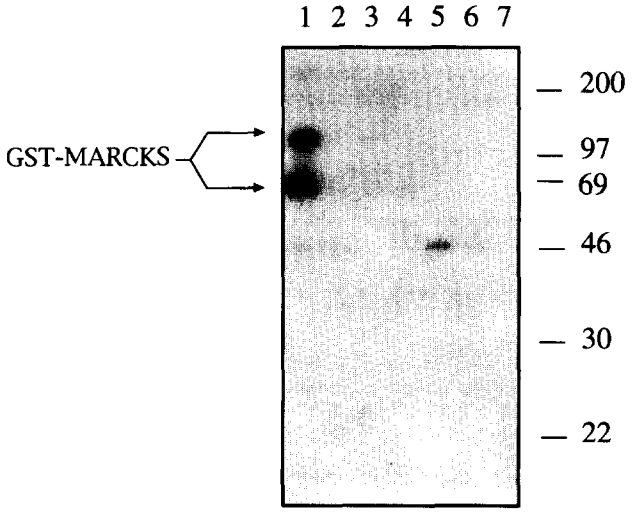

Fig. 1. Phosphorylation of GST-MARCKS by p42 MAPK. Recombinant p42 MAPK protein was activated by MEK which was immunoprecipitated from TPA-stimulated Swiss 3T3 cells. Active p42 MAPK phosphorylates GST-MARCKS (lane 1) but not the GST moiety itself (lane 5). Non-activated p42 MAPK or active MEK do not phosphorylate GST-MARCKS (lanes 2,3) or GST (lanes 6,7). A control phosphorylation with active MEK and p42 but no substrate protein is shown in lane 4.

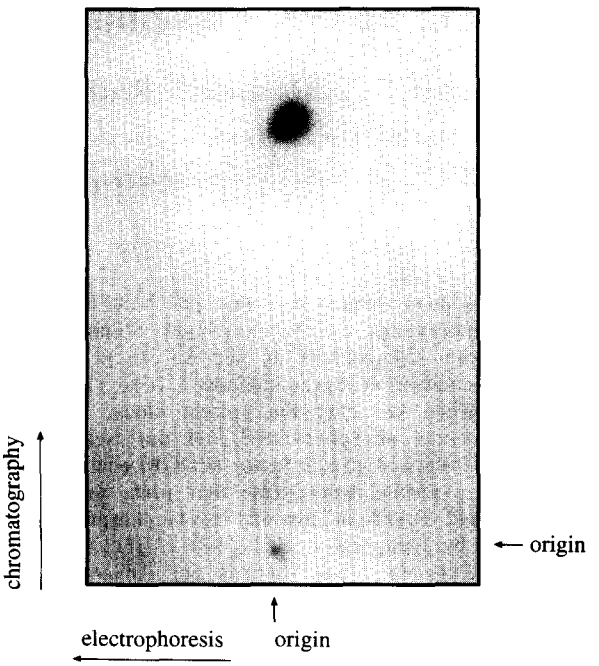

Fig. 2. Two-dimensional analysis of MARCKS phosphopeptides. GST-MARCKS was phosphorylated by p42 MAPK, the proteins were separated by SDS-PAGE and the 110 kDa full length GST-MARCKS was excised and digested with trypsin. The resulting peptides were separated on a cellulose thin-layer chromatography plate by electrophoresis for 20 min at 50 mA (1st dimension) and then ascending chromatography (2nd dimension) for 4 h until the buffer front had reached the top of the plate. The plate was then dried and autoradiographed.

To determine whether MAPK phosphorylates one of the proline-directed residues identified in vivo, full-length GST-MARCKS was isolated from an SDS-PAGE gel, digested with trypsin and subjected to 2-dimensional electrophoresis. Fig. 2 shows that p42 MAPK phosphorylates a single tryptic peptide that migrates quite differently from the four peptides targeted by PKC all of which move away from the origin in the first dimension (compare Fig. 5C).

3.2. p42 MAPK phosphorylates MARCKS at Ser-113

Partial sequencing of the tryptic peptide indicated that it is the peptide ^{102}-EAAEAEPAEPSSPAAEAEGASASSTSSPK-^{130} which is phosphorylated by p42 MAPK (data not shown). This peptide contains two of the six residues identified to be phosphorylated in vivo [1], including one (Ser-113) that matches the MAPK phosphorylation motif. Site-directed mutagenesis of Ser-113 generated a protein that was phosphorylated poorly by MAPK compared to the wild-type protein (Fig. 3A). This residual phosphorylation was contributed by low stoichiometry phosphorylation on sites other than Ser-113 and was not detectable after separation of the phosphorylated peptides by electrophoresis and thin-layer chromatography (Fig. 3B).

3.3. p42 MAPK does not phosphorylate MARCKS in permeabilised Swiss 3T3 cells

In order to assess if MAPK regulates the phosphorylation

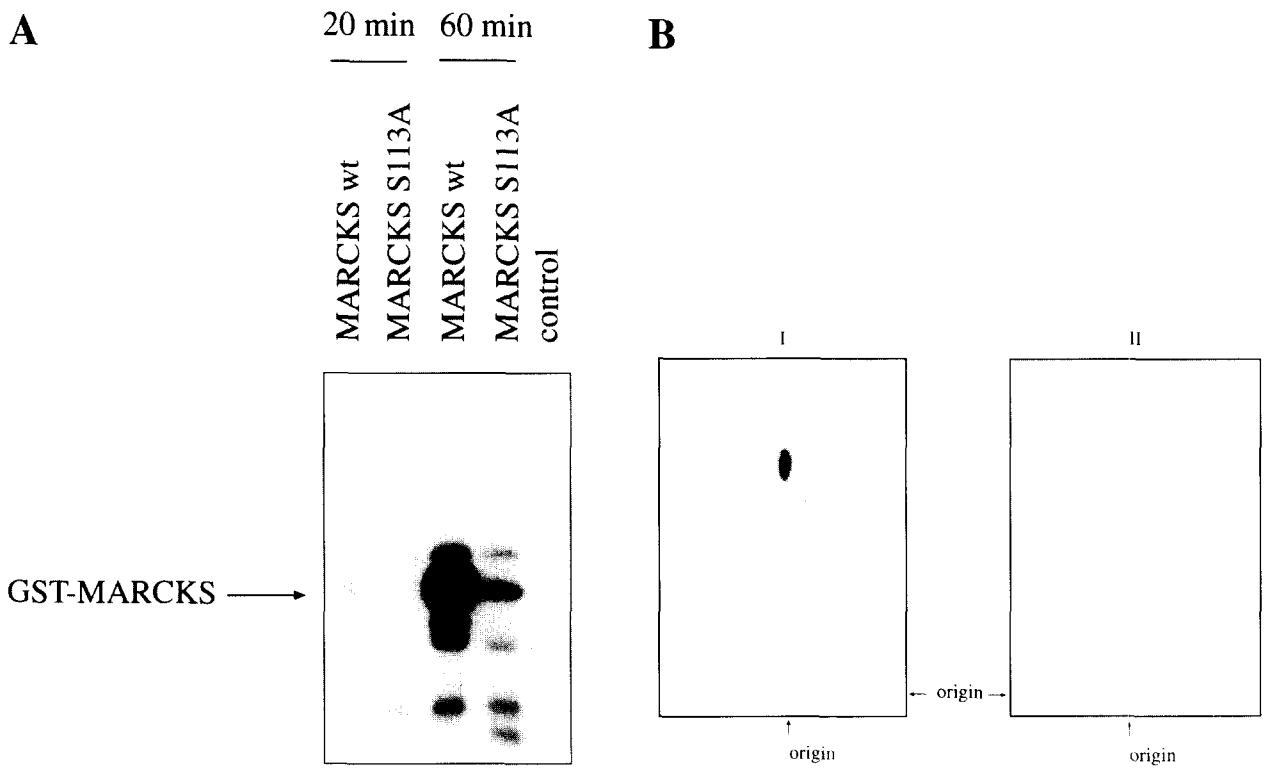

Fig. 3. (A) p42 MAPK can phosphorylate MARCKS wt efficiently but not MARCKS S113A. Recombinant p42 MAPK activated by MEK S117E S221E phosphorylates GST-MARCKS wt (lanes 1,3) but to a far lesser extent the mutated GST-MARCKS S113A (lanes 2,4). Lane 5 shows a control reaction without MARCKS substrate protein. (B) Equal amounts of GST-MARCKS wt (I) and GST-MARCKS S113A (II) protein shown in lanes 3 and 4 (panel A) were excised from the SDS-PAGE gel, digested with trypsin and subjected to 2-dimensional peptide mapping and autoradiographed, as described in Fig. 2.

of MARCKS at Ser-113 acutely, we used permeabilised Swiss 3T3 cells that were labelled with [γ-^{32}P]ATP. Fig. 4 shows that phorbol ester treatment of permeabilised cells can activate p42 MAPK in this system. Furthermore, p42 MAPK remains in the cytosol of the cells after activation. This means the active kinase stays in the appropriate compartment relative to its potential substrate (i.e. MARCKS).

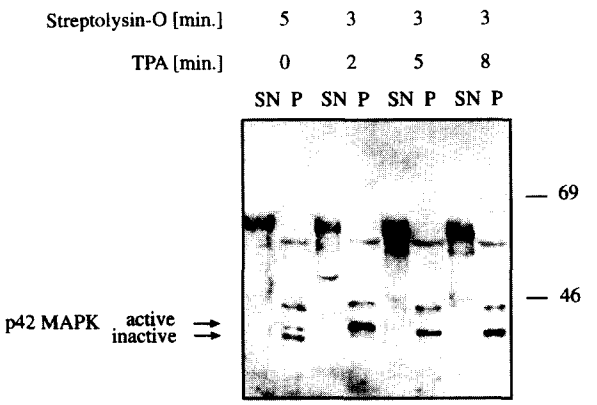

Fig. 4. TPA activates MAPK in permeabilised Swiss 3T3. Swiss 3T3 cells were permeabilised with streptolysin-O and then treated for 2, 5 or 8 min with TPA as described in Section 2. Subsequently cells were centrifuged for 10 min at 12000 rpm. Pellets (P) were resuspended in 1×sample buffer. Supernatants (SN) were trichloroacetic acid-precipitated and resuspended in 1×sample buffer and were loaded on a 10% SDS-PAGE gel. In non-stimulated cells the majority of p42 MAPK is in the inactive, faster migrating form (as indicated), whereas 2, 5 or 8 min treatment with TPA shifts almost all the enzyme to the active, slower migrating form. A similar shift can be observed for p44 MAPK.

Permeabilised Swiss 3T3 cells were stimulated by TPA or PDGF in order to activate p42 MAPK. MARCKS protein was purified, digested with trypsin and peptides subjected to electrophoresis and thin-layer chromatography. The resulting phosphopeptide maps were compared to phosphopeptide maps of MARCKS after in vitro phosphorylation by PKC (Fig. 5A–D). Analysis of the labelled peptides shows that PDGF and TPA treatment do not induce acute phosphorylation of MARCKS on Ser-113 (compare Fig. 2).

3.4. Discussion

It is established that p42MAPK can phosphorylate residue Ser-113 in MARCKS. This is consistent with the known specificity of this protein kinase. Although this serine in MARCKS is known to be phosphorylated in vivo in bovine brain, it is shown here that acute activation of p42 MAPK in permeabilised Swiss 3T3 cells does not alter Ser-113 phosphorylation or that of other 'non-PKC' sites. This distinction could be explained by tissue-specific differences in MAPK function, by the fact that Ser-113 might be fully phosphorylated in quiescent Swiss 3T3 cells or by the action of a brain-specific proline-directed kinase.

It is concluded that phosphorylation of MARCKS in murine fibroblasts is not subject to direct, acute control by p42 MAPK.

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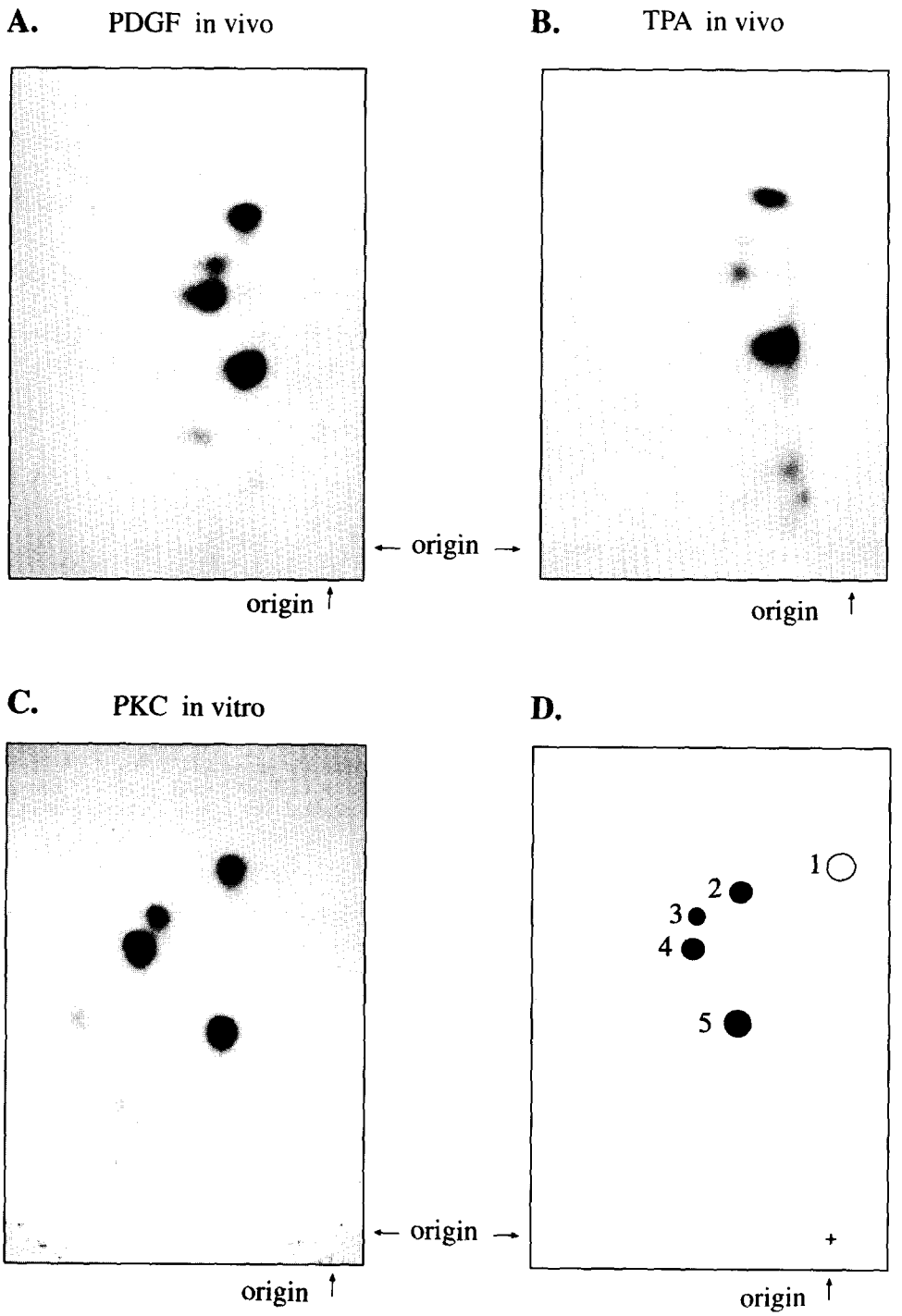

Fig. 5. Comparison of MARCKS phosphorylation in Swiss 3T3 cells after stimulation by TPA or PDGF and in vitro phosphorylation by PKC. Swiss 3T3 cells were permeabilised and stimulated by PDGF (A) or TPA (B) in the presence of [γ-^{32}P]ATP. MARCKS was isolated by SDS-PAGE, digested and subjected to 2-dimensional peptide mapping. The resulting phosphopeptide maps were compared to maps of GST-MARCKS protein phosphorylated by PKC in vitro (C). A diagram of the major tryptic MARCKS phosphopeptides characteristic for MAPK phosphorylation (peptide 1, EAAEAEPAEPS*SPAAEAEGASASSTSSPK; compare Fig. 2) or PKC phosphorylation (peptides 2–5: peptide 2, LSGFS*FK; peptide 3, RFS*FK and/or FS*FKK; peptide 4, LSGFS*FKK, peptide 5, S*FK) is shown in (D).

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