PRK1 phosphorylates MARCKS at the PKC sites: serine 152, serine 156 and serine 163

Ruth H. Palmer[a], Dorothee C. Schönwaßer[a], Dinah Rahman[b], Darryl J.C. Pappin[b], Thomas Herget[c], Peter J. Parker[a,*]

[a]*Protein Phosphorylation Laboratory, Imperial Cancer Research Fund, PO Box 123, Lincoln's Inn Fields, London, WC2A 3PX, UK*
[b]*Protein Sequencing Laboratory, Imperial Cancer Research Fund, PO Box 123, Lincoln's Inn Fields, London, WC2A 3PX, UK*
[c]*Institute of Physiological Chemistry, University of Mainz, Mainz, Germany*

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Abstract The 80kDa Myristolated Alanine-Rich C-Kinase Substrate (MARCKS) is a major in vivo substrate of protein kinase C (PKC). Here we report that MARCKS is a major substrate for the lipid-activated PKC-related kinase (PRK1) in cell extracts. Furthermore, PRK1 is shown to phosphorylate MARCKS on the same sites as PKC in vitro. Thus, control of MARCKS phosphorylation on these previously identified 'PKC' sites may be regulated under certain circumstances by PRK as well as PKC mediated signalling pathways. The implications for MARCKS as a marker of PKC activation and as a point of signal convergence are discussed.

Key words: Protein kinase C; PKC; PRK; MARCKS; Phosphorylation

1. Introduction

The cellular substrates for the recently identified PRK (PKC-related kinase) family [1–3] are not yet well characterised. The PRK family members are closely related to the PKC superfamily isotypes in the catalytic domain whilst retaining a distinct amino terminal regulatory domain. Unlike the PKCs which characteristically retain a cysteine-rich C1 domain responsible for effector binding, the PRKs do not encode a C1 domain but possess two distinct conserved regulatory domains termed HR1 and HR2 [3,4]. Consistent with the lack of a C1 domain it has become clear that while the PRKs resemble PKCs in being activated by proteolysis [5,6], they are not activated by phorbol esters. Various fatty acids and phospholipids have now been shown to activate PRKs in vitro [6–9] although the signalling pathways leading to the activation of PRK1 in vivo remain unclear.

The Myristoylated Alanine-Rich C-Kinase Substrate (MARCKS), has been studied extensively and characterised as a PKC-specific substrate (for reviews see [10,11]). The MARCKS protein comprises three domains: (i) an amino-terminal domain which is modified by myristoylation and is responsible for the membrane binding properties of MARCKS, (ii) an MH2 domain, conserved within MARCKS family members, with unknown function and (iii) a basic effector domain. This effector domain contains the calmodulin and actin binding sites of the MARCKS protein in addition to the PKC phosphorylation sites. PKC phosphorylation of MARCKS on serine residues within the effector domain results in the disruption of both the calmodulin and actin binding properties of MARCKS as well as loss of membrane binding. In addition to phosphorylation by PKC isotypes, MARCKS has been reported recently to be phosphorylated in vivo by proline-directed protein kinases [12], the effect of these phosphorylations on MARCKS function has not yet been elucidated.

In order to determine the potential role of PRKs in signal transduction pathways we have investigated cellular PRK substrates. In this paper we show that purified PRK1 is able to phosphorylate MARCKS. Moreover the resulting phosphopeptides are identical to those produced when MARCKS is subjected to phosphorylation by PKC. The results

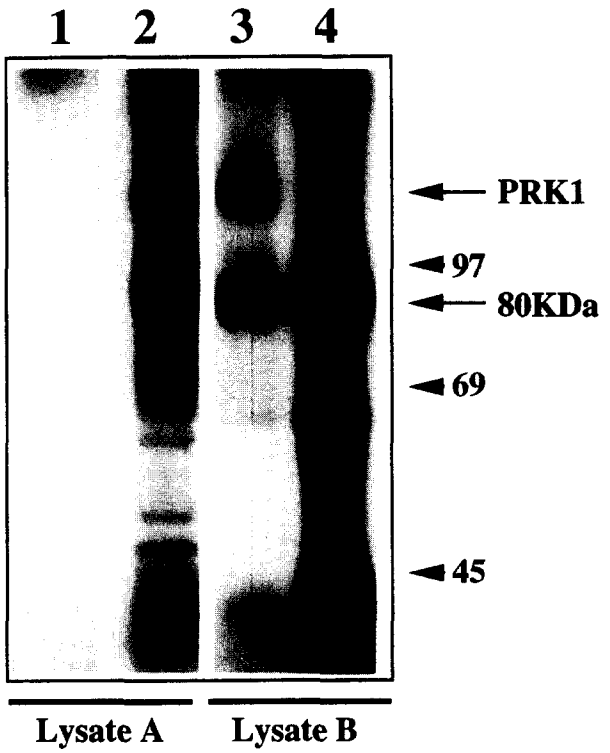

Fig. 1. PRK1 phosphorylates an 80 kDa cellular protein. Swiss3T3 cell lysates were treated as follows: Lysate A: Incubated at 65°C for 10 min; Lysate B: Incubation at 65°C for 10 min followed by 95°C for 5 min, incubation at 4°C for 5 min and finally centrifugation at 15,000g for 5 min. Lysates were then incubated for 10 min at 30°C with the following: Lane 1: no enzyme control; Lane 2: PRK1; Lane 3: PRK1 and Lane 4: PKC-β1. Reactions were stopped by the addition of 10 μl 4× SDS sample buffer. Results were then analysed by running the samples on 8% SDS-PAGE and autoradiography at −80°C overnight. The phosphorylated 80kDa cellular protein and autophosphorylated PRK1 are indicated by arrows.

*Corresponding author. Fax: (44) (71) 269 3092.

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provide evidence that the MARCKS protein may act as a point of convergence for different signalling pathways.

2. Materials and methods

2.1. Materials

Trypsin was from Worthington. Protamine sulphate, glutathione-agarose and glutathione were from Sigma. [γ-^{32}P]ATP was from Amersham, cellulose thin layer chromatography plates were from Kodak. Foetal calf serum was obtained from Gibco Bethesda Research Laboratories.

2.2. Tissue culture

Swiss3T3 fibroblasts were seeded and made quiescent as described previously [13].

2.3. Protein purification

In order to obtain recombinant MARCKS protein, GST-MARCKS fusion protein was purified from *Escherichia coli* cells (XL-1 Blue, Stratagene) by glutathione affinity chromatography as described previously [14]. Recombinant PRK1 was purified from COS 7 cells as described [6]. Recombinant PKC-β1 was expressed and isolated from baculovirus-infected insect cells [15].

2.4. Two-dimensional peptide mapping

Phosphorylated GST-MARCKS protein was digested overnight at room temperature with 20 μg/ml trypsin (Sigma). The resulting tryptic phosphopeptides were dried under vacuum and resuspended in 20 μl of electrophoresis buffer (pH 3.5; pyridine/acetic acid/water (1:10:189). The samples containing 0.1–1.0 × 10^6 cpm were spotted on TLC plates and subjected to electrophoresis at 50 mA for 20 min. After drying, the TLC plate was chromatographed in the second dimension in chromatography buffer (n-butanol/pyridine/acetic acid/H_2O (75:50:15:60)). The plate was then dried and phosphorylated peptides detected by autoradiography at −80°C.

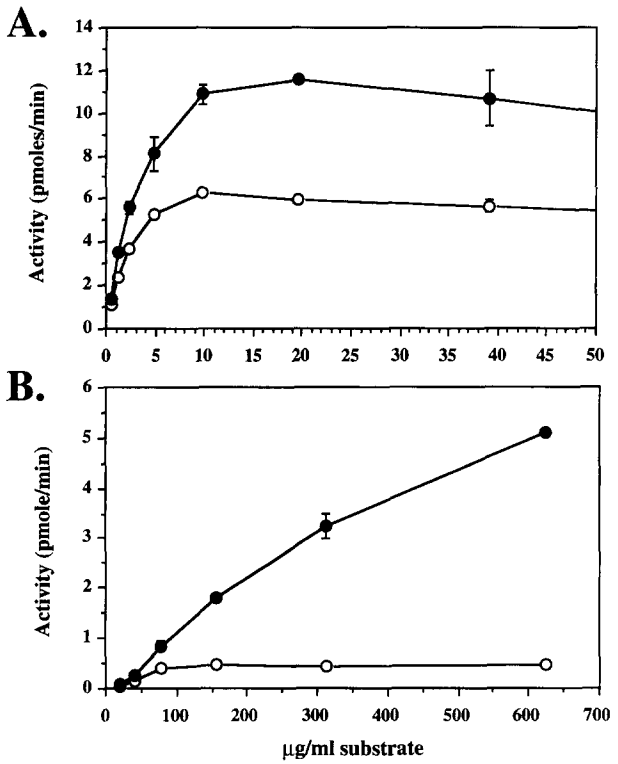

Fig. 2. PRK1 phosphorylates MARCKS in vitro. The kinetics of MARCKS phosphorylation by intact PRK1 and trypsinised PRK1 are compared. **(Panel A)** PRK1 activity towards MARCKS peptide for intact PRK1 (○–○) and PRK1 trypsinised with 8 μg/ml trypsin (●–●) are shown. **(Panel B)** PRK1 activity towards GST-MARCKS fusion protein for intact PRK1 (○–○) and PRK1 trypsinised with 8 μg/ml trypsin (●–●) are shown. Assays were carried out in duplicate at 30°C for 10 min and stopped by spotting onto Whatman P81 paper and placing in 10% acetic acid. The filters were washed 3 × 10 min in 30% acetic acid, and incorporation determined by Cerenkov radiation.

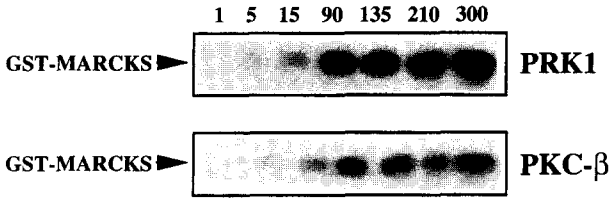

Fig. 3. Relative activities of PRK1 and PKC-β1 for MARCKS. Equal units of both PRK1 and PKC-β1 were activated, by limited proteolysis or with phosphatidylserine/TPA respectively, and a time-course of GST-MARCKS phosphorylation carried out. Reactions were stopped by the addition of 4 × SDS sample buffer and subsequent boiling for 5 min, samples were then analysed by 8% SDS-PAGE followed by autoradiography at −80°C.

2.5. Preparation of COS 7 cell lysates for phosphorylation

Cell lysates were prepared by lysis of COS 7 cells (two plates of 1 × 10^6 cells) into 500 μl lysis buffer (60 mM Tris-HCl pH 7.5, 100 mM NaCl, 5 mM EDTA, 0.3% (v/v) β-mercaptoethanol, 100 μM Na_3VO_4, 50 mM NaF, 1% (v/v) Triton X-100, 50 μl/ml phenylmethylsulfonyl fluoride (PMSF), 10 mM benzamidine, 125 μg/ml aprotinin, 250 μg/ml leupeptin). The extracts were then treated for 10 min at 65°C to inactivate endogenous protein kinases. After this, lysate A (50%) was stored at 40°C. Lysate B was further treated with a 5-min incubation at 95°C followed by centrifugation for 10 min at 15,000 × g to remove heat insoluble proteins.

2.6. Other procedures

SDS-PAGE was according to Laemmli [16]. Protein was determined by the method of Bradford [17] using bovine serum albumin as a standard.

3. Results and discussion

3.1. PRK1 phosphorylates an 80 kDa protein from COS 7 cell lysates

To assess the substrate specificity of PRK1, crude COS 7 cell lysates (25 μl) were incubated with 5 μl PRK1 or PKC-β (equivalent to 2.5 Units), 5 μl 10 mM $MgCl_2$ and reactions started by the addition of [γ-^{32}P]ATP. Reactions were then incubated for 10 min at 30°C, and stopped by the addition of 10 μl 4 × SDS sample buffer. The phosphorylated proteins were seperated on 8% SDS-PAGE and detected by autoradiography at −80°C

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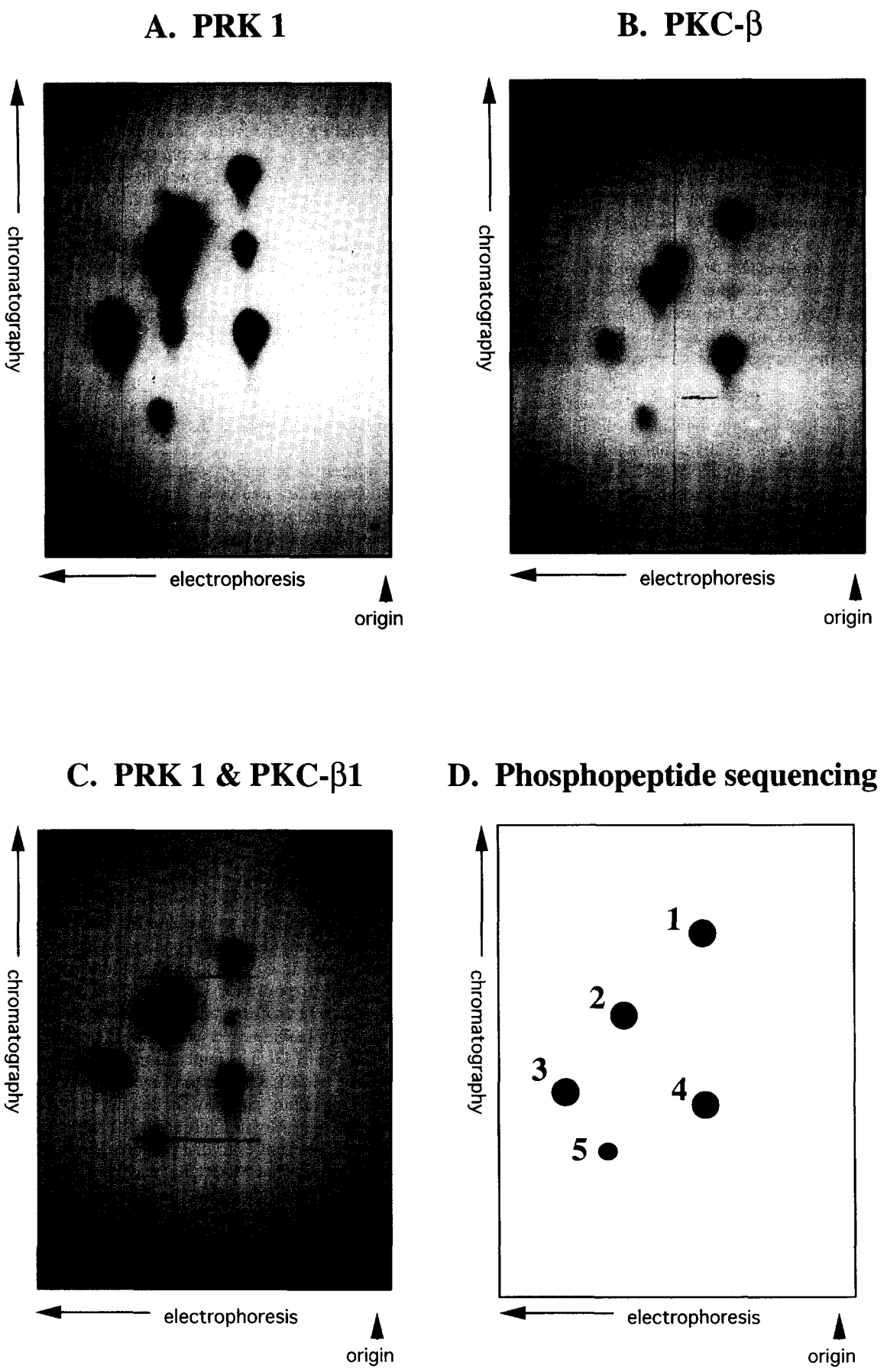
Fig. 4. Two-dimensional analysis of MARCKS phosphopeptides. Recombinant GST-MARCKS was maximally phosphorylated (4 hours) by PRK1 (Panel A) and PKC-β1 (Panel B) resolved by PAGE and the protein digested with trypsin at 37°C overnight. Tryptic phosphopeptides were resolved in the first dimension by electrophoresis at pH3.5 and by thin-layer chromatography in the second dimension. The arrow marks the origin. Equal cpms of PRK1 and PKC-β1-derived tryptic phosphopeptides were mixed and analysed as before (Panel C). TLC plates were dried and analysed by autoradiography at −80°C. Panel D indicates the results of phosphopeptide sequencing of the major tryptic phospopeptides: Peptide 1: LSGFS*FK, Peptide 2: LSGFS*FKK, Peptide 3: RFS*FKK, Peptide 4: S*FK and Peptide 5: KS*FK. * denotes modification of the serine residue by phosphorylation as identified by sequencing (from reference [19]).


A. PRK 1
chromatography
electrophoresis
origin
B. PKC-β
chromatography
electrophoresis
origin
C. PRK 1 & PKC-β1
chromatography
electrophoresis
origin
D. Phosphopeptide sequencing
chromatography
1
2
3
4
5
electrophoresis
origin

overnight (Fig. 1). PRK1 was found to phosphorylate several cellular proteins including the prominent phosphorylation of an 80 kDa protein. The following observations pointed to the identification of this protein as MARCKS: (i) the PRK1 substrate was of equal apparent molecular mass (80 kDa) as previously described PKC substrate MARCKS, (ii) the C substrate was a heat stable protein – a characteristic of MARCKS (Fig. 1; lane 3), and (iii) the 80 kDa PRK1 substrate co-migrated in SDS-PAGE together with the prominent band of 80 kDa phosphorylated by PKC-β (Fig. 1; lane 4).

3.2. PRK1 can phosphorylate MARCKS in vitro

In order to determine whether MARCKS is a substrate for PRK1, purified MARCKS (see section 2) and a MARCKS peptide corresponding to ^{145}-KKKKKRFSFKKSFKLSGF-SFKKSKK-^{169} (according to the murine sequence; [18]) were employed.

Phosphorylation of MARCKS was carried out using intact PRK1 or PRK1 which was fully activated by limited proteolysis [6]. PRK1 was tested for its ability to phosphorylate MARCKS peptide (Fig. 2; Panel A) and GST-MARCKS (Fig. 2; Panel B). Full length PRK1 was able to phosphorylate GST-MARCKS although inefficiently when compared to PRK1 which had been activated by proteolysis. Importantly, PRK1 was not able to phosphorylate the GST moiety alone (data not shown). Proteolysis of PRK1 resulted in a >10-fold increase in phosphorylation of GST-MARCKS with a dramatic increase in Vmax. In contrast the proteolytic activation of PRK1 resulted in only a 2-fold increase in activity towards the MARCKS effector domain peptide (Fig. 2A). This peptide, which contains the three in vivo PKC serine phosphorylation sites, was efficiently phosphorylated by the full length PRK1 enzyme. This probably reflects the ability of the MARCKS peptide to diffuse more easily into the active site of the kinase, therefore overcoming to some extent the inhibitory regulation imposed on the kinase domain of PRK1 by its amino-terminal regulatory domain. This clearly constrasts with intact MARCKS which is a poor substrate for the PRK1 holoenzyme. The PRK1 (intact or following partial proteolysis) K_m for the MARCKS peptide is ~1 μM which compares with values of between 0.4 and 10 μM for different PKC isotypes determined in this laboratory [19].

3.3. PRK1 and PKC-β1 phosphorylate MARCKS on the same sites

The ability of PRK1 to phosphorylate MARCKS was compared with that of PKC-β1. Equal units of PRK1 and PKC-β1 activity were titrated using the substrate protamine sulphate which is phosphorylated in an effector independent manner by both of these kinases (data not shown). Equivalent units of both PRK1 and PKC-β1 were activated by limited proteolysis or with phosphatidylserine/TPA respectively, and a time course of GST-MARCKS phosphorylation activity carried out (Fig. 3). Both PRK1 and PKC-β phosphorylated GST- MARCKS to a similar extent and the reaction was essentially complete by 90 min. This data demonstrates that both PRK1 and PKC-β1 are equipotent in their ability to phosphorylate the full length MARCKS protein.

In order to further study the phosphorylation of MARCKS by PRK1, GST-MARCKS was incubated with both activated PRK1 and PKC-β1 to achieve full phosphorylation of MARCKS. Phosphorylated GST-MARCKS was then purified by 8% SDS-PAGE and the corresponding gel band digested overnight with 2 μg of trypsin. The resulting samples were then subjected to 2-dimensional electrophoresis as described in the materials and methods (Fig. 4; Panels A and B). Examination of the tryptic phosphopeptides of GST-MARCKS for both PRK1 and PKC-β1 revealed a similar pattern in each case. In order to confirm that these phosphopeptides were identical, equal cpm of (i) PRK1 and (ii) PKC-β1-phosphorylated MARCKS tryptic peptides were mixed and once again analysed by 2-dimensional electrophoresis (Fig. 4; Panel C). The phosphopeptides derived from PRK1 and PKC-β1 phosphorylated MARCKS did indeed co-migrate and were therefore judged to be identical. The major tryptic phosphopeptides have been analysed previously [19] and identified as: Peptide 1: LSGFS*FK, with one phosphate; Peptide 2: LSGFS*FKK, with one phosphate – this peptide is identical to Peptide 1 apart from the inclusion of an extra lysine residue; Peptide 3: RFS*FKK, with one phosphate; Peptide 4: S*FK, with one phosphate and Peptide 5: KS*FK, with one phosphate (Fig. 4D) [19].

Confirmation of the phosphorylation sites was obtained by direct sequence analysis. The MARCKS peptide was phosphorylated to completion (3.0 mol phosphate/mol peptide) by proteolytically-activated PRK1 and purified on a C18 cartridge. Sequential Edman degradation yielded [^{32}P]phosphate release at cycles 8, 12 and 19 (data not shown). This identifies the phosphorylated serine residues as 152, 156, and 163. The serines at 160 and 167 were not phosphorylated as observed for PKC (see [19]).

3.4. Conclusions

The data shown here clearly demonstrate the ability of PRK1 to phosphorylate the MARCKS protein in vitro. Indeed in Swiss 3T3 cell extracts this protein is a major substrate for PRK1. The sites phosphorylated by PRK1 are identical to those previously characterised as PKC phosphorylation sites within the MARCKS effector domain. Thus the phosphorylation of MARCKS at these sites may not provide unequivocal evidence for the action of PKC.

The identical site specificity demonstrates that PRK, like PKC, has the capacity to regulate the ability of MARCKS to bind calcium/calmodulin [20] and crosslink actin [21], both of which are disrupted by phosphorylation at these sites. That PRK1 is able to phosphorylate MARCKS is consistent with the domain structure of this family of kinases, which comprises a carboxy-terminal kinase domain that is most highly related to the PKC family [3]. The implication of these results is that the control of MARCKS effector function may be regulated by different signalling pathway inputs, i.e. via both a lipid-activated PRK pathway in addition to the well characterised and established phorbol ester/diacylglycerol-activated PKC-mediated pathways. That MARCKS may be a physiological target for PRK is consistent with the finding that in crude lysate preparations it appears to be the major substrate. A dissection of the role of PRK family kinases in the regulation of MARCKS in vivo awaits identification of a specific activator for these kinases.

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