Vol. 190, No. 1, 1993 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
January 15, 1993 Pages 236-241

PROTEIN KINASE C PHOSPHORYLATES Ser^{152}, Ser^{156} and Ser^{163} BUT NOT Ser^{160} OF MARCKS IN RAT BRAIN

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Received November 19, 1992

SUMMARY: MARCKS is one of the major physiological substrates of PKC and was reported to be phosphorylated by PKC at 4 serine residues that are within the CaM-binding region (Graff *et al.*, J. Biol. Chem. *264*, 11912, 1989). Using MARCKS from rat brain and a synthetic peptide of 25 amino acids containing all 4 of the serine residues, we investigate the differences in phosphorylation by PKC isozymes I, II and III. Tryptic peptide analysis of PKC phosphorylated MARCKS or peptide, we found ^{32}P was in peptides of (K)S^{152}FK, (R)FS^{156}FK and $LS^{160}GFS^{163}$FK. Further digestion of LSGFSFK with α-chymotrypsin revealed that ^{32}P incorporation occurred only at Ser^{163} but not at Ser^{160}. The initial rates and stoichiomatry of phosphorylation of Ser^{152} and Ser^{156} were twice as those of Ser^{163} using either one of the three PKC isozymes. These results indicate that *in vitro*, PKC phosphorylates MARCKS only at three sites, but not at Ser^{160} as that reported previously, and there was no preferential phosphorylation of MARCKS by either PKC isozyme I, II or III.

One of the major signal transduction pathways in the brain involves activation of protein kinase C (PKC). Stimulation of this family of kinases of which several isozymes now have been identified (1) has been implicated in the responses to various hormones, neurotransmitters and growth factors. The identity and molecular characterization of its physiological substrates is much less known. An acidic protein, with an apparent molecular mass of 80-87 kDa on SDS/PAGE, known as MARCKS (2), was shown to be a physiological substrate in a number of systems (2-5). The protein has been isolated from various sources (6-8) and its myristoylation (9), binding of CaM (10) and phosphorylation-dependent subcellular localization (11) were characterized. Despite a growing number of studies show that this protein is a major PKC substrate in many cells, a functional role has not been assigned. In a recent study of the phosphorylation of chicken and bovine MARCKS by PKC, both *in vitro* and in intact cells, phosphate was reported to be incorporated into 4 serine residues in a region having clustered basic residues (10). Phosphorylation of MARCKS at these sites has been shown to antagonize the binding of CaM (10, 12).

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Abbreviations: PKC, protein kinase C; MARCKS, *m*yristoylated *a*lanine *r*ich *c*-*k*inase *s*ubstrate; CaM, calmodulin; HPLC, high pressure liquid chromatography; TFA, trifluoroacetic acid.

0006-291X/93 $4.00

In our attempt to study the differences among PKC isozymes type I, II and III in the phosphorylation of MARCKS *in vitro*, we found that, even after extensive phosphorylation, phosphate incorporation was lower than that expected for 4 phosphorylation sites. Therefore, we synthesized a peptide of 25 amano acids corresponding to the region of the chicken and bovine MARCKS containing the 4 residues that are reported to be phosphorylated by PKC (Ser^{152}, Ser^{156}, $Ser^{160}$and Ser^{163}) and examined the kinetics of phosphorylation at each individual site catalyzed by the three isozymes of PKC. We found that these isozymes phosphorylate MARCKS at only 3 serine residues.

EXPERIMENTAL PROCEDURES

The MARCKS peptide with an amino acid sequence of ^{145}KKKKKRFSFKKSFKLSGFS-FKKNKK^{169} (according to rat sequence numbering) was synthesized in an Applied Biosystems synthesizer and purified by reverse phase HPLC on a Vydac C18 column (4.6 x 250 mm) using a linear gradient of acetonitrile (0-50 %) in 0.1 % TFA. PKC isozymes and PKM from rat brain were prepared as previously described (13). MARCKS protein was purified from rat brain homogenate by heat treatment (90 °C, 10 min), acid-precipitation (2.5 % $HClO_4$) and DEAE-cellulose column chromatography.

Both MARCKS protein and the peptide were phosphorylated using a mixed-micellar reaction of 25 µl with 30 mM Tris/HCl (pH 7.5), 6 mM $MgCl_2$, 0.4 mM $CaCl_2$, 0.04% NP40, 120 µM [^{32}P]ATP (specific activity 1000-3000 cpm/pmol), 100 µg/ml phosphatidylserine, 20 µg/ml diolein and 5-20 ng purified PKC (specific activity 1000-3000 nmol phosphate/min/mg) for the indicated times at 30 °C. Enzyme activity was determined using histone IIIS as described (13). Phosphate incorporation into peptide or MARCKS protein was monitored using P81 paper washed at least 4 times in 75 mM phosphoric acid and radioactivity determined by scintillation counting. For tryptic peptide analysis, phosphorylation reactions were terminated with acetic acid to a final concentration of 30 %. Phosphorylated peptide or protein was initially separated from [^{32}P]ATP on a small column of Dowex AG-1X8 and DEAE-cellulose by elution with 30 % acetic acid or directly on the C18 reverse phase HPLC column using a 0-50 % acetonitrile gradient in 0.1 % TFA. After repeated lyophilization to remove residual acid, the sample was dissolved in 0.1 M NH_4HCO_3 for tryptic digestion at 30 °C overnight. The resulting phosphopeptides were analyzed by reverse phase HPLC and the radioactivity was determined by Cerenkov counting. Alternatively, the tryptic phosphopeptides were analyzed by 2-dimensional (2D) peptide mapping by electrophoresis in the first dimension at 1000 V for 20 min at pH 3.5 in pyridine:acetic acid:water (1:10:189) and ascending chromatography in the second dimension in n-butanol:acetic acid:water (4:1:5). Phosphate incorporation in peptides was quantified by scanning the plates directly with an AMBIS system. Amino acid sequencing was performed in an Applied Biosystem model 470A Protein Sequencer. MARCKS protein phosphorylated by PKC was located by autoradiography following SDS/PAGE and transferred to 50 mM NH_4HCO_3 buffer (pH 7.8) for digestion with trypsin overnight at 30 °C. The phosphopeptides were analyzed by reverse phase HPLC and 2D-peptide mapping as described above for MARCKS peptide.

RESULTS AND DISCUSSION

Previously, Graff *et al.* (10) determined that all four serine residues of MARCKS in chicken, that were located close together within the CaM-binding region (2), can be phosphorylated by PKC, whereas the third serine in bovine MARCKS was probably not, in spite of the sequence identity between these two proteins surrounding the phosphorylation sites. However, these authors concluded and referenced subsequently (12,14-16) that all four serine in MARCKS are phosphorylated by PKC. In comparison with the chicken and bovine brain MARCKS sequence, these four serine residues correspond to positions 152, 156, 160 and 163 of the rat brain protein (17). Anticipating 4-site phosphorylation when studying the rat brain MARCKS protein phosphorylated by the purified PKC isozymes, we observed a stoichiometry of phosphorylation lower than that would be expected for four phosphorylation sites. In order to analyze in detail the stoichiometry of phosphorylation at each site by various

isozymes we synthesized a peptide of 25 amino acids in the CaM-binding region containing all four serine residues. This MARCKS peptide could be phosphorylated by PKC (type II) to a maximal stoichiometry of 2.5 mol phosphate/ mol peptide. The same results were obtained with isozymes type I and III and with PKM.

Three major tryptic phosphopeptides were detected by reverse phase HPLC on a C18 column (Fig. 1). Amino acid sequence analysis of the 3 peak fractions (t_R= 36, 53 and 59 min), indicated that fraction of t_R=36' actually contained 2 peptides, SFK and KSFK, apparently generated by alternative cleavage of basic residues surrounding Ser^{156}. Peak fraction of t_R=53' also contained 2 peptides, RFSFK and FSFK, both containing Ser^{152}, and fraction of t_R=59' consisted of a single heptapeptide, LSGFSFK, with Ser^{160} and Ser^{163} in its sequence. The same tryptic digest when analyzed by 2D-peptide mapping, however, yielded 3 major and 2 minor spots (Fig. 2A). Analysis of each peak from the reverse phase HPLC by subsequent 2D-peptide mapping identified the individual phosphopeptides on the peptide maps where spots 1, 1', 2, 2' and 3 correspond to SFK, KSFK, RFSFK, FSFK and LSGFSFK respectively (Fig. 2 B-D).

As the LSGFSFK fragment contains 2 serine residues it would be expected to incorporate twice as much phosphate as the other two fragments after extensive phosphorylation; however this result was not obtained. Instead, the ratio of phosphate incorporation for (R)FSFK : (K)SFK : LSGFSFK was 1.7 : 1.8 : 1. To investigate whether Ser^{160} and/or Ser^{163} was phosphorylated we further digested the peptide of t_R=59' from the reverse phase HPLC column with α-chymotrypsin. Analysis of this α-chymotrypsin digest by 2D-peptide mapping revealed only one radioactive fragment, which migrated as would be expected from SFK (Fig. 2E) and comigrated with $S^{156}FK$ of t_R=36. Separation of the α-chymotrypsin digest on reverse phase HPLC revealed 2 prominent 215 nm absorption peaks in excess to the background absorption observed in a control run of α-chymotrypsin alone (Fig 3). The peak of t_R=33 min,

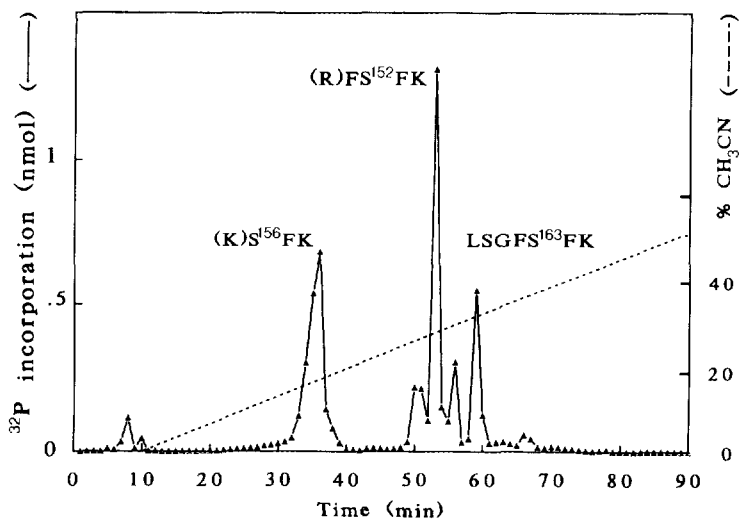

Fig. 1. Reverse phase HPLC of tryptic fragments of MARCKS peptide extensively phosphorylated by PKC type II. By amino acid sequencing the fraction of t_R=36 min contained both KSFK and SFK, the fraction of t_R=53 min contained both RFSFK and FSFK and the fraction of t_R=59 min contained LSGFSFK. Residues produced from alternative tryptic digestions are indicated by parentheses.

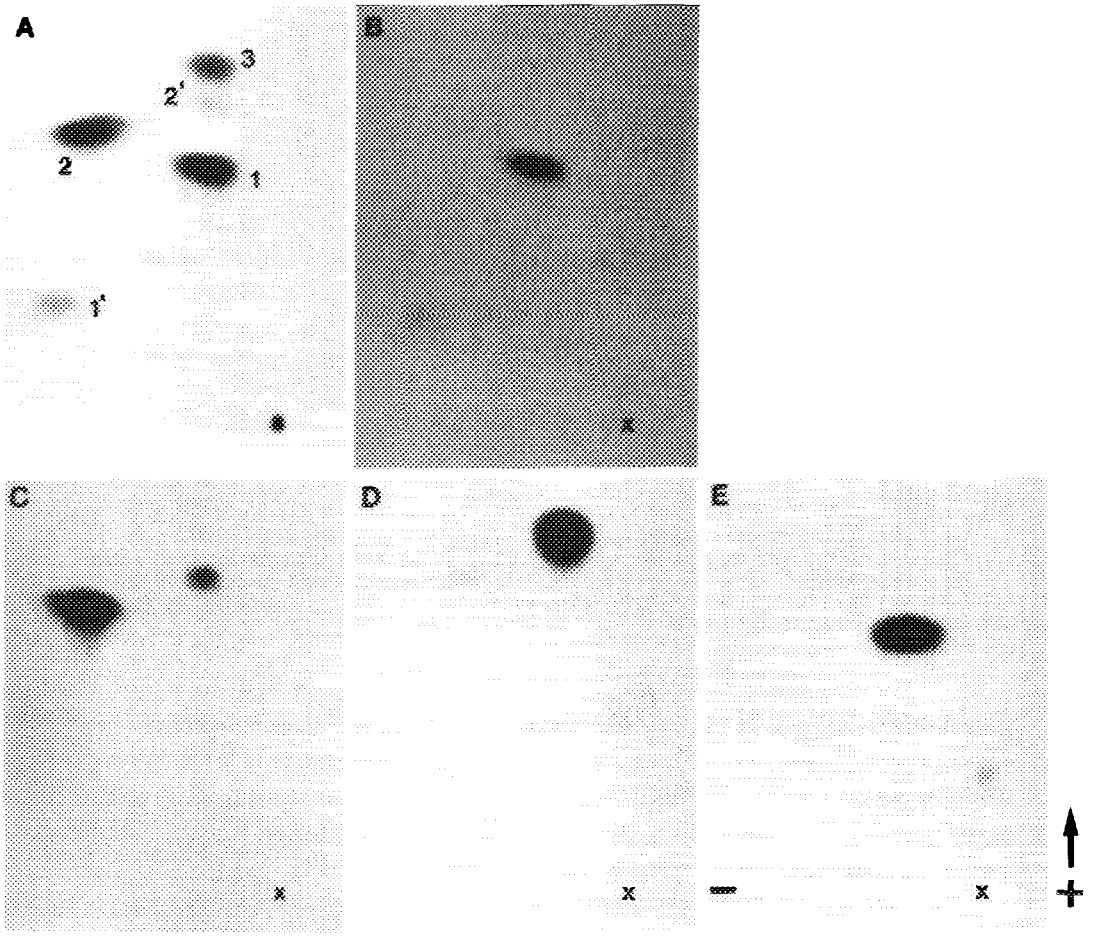

Fig. 2. Autoradiograms of tryptic peptides analyzed by 2-dimensional peptide mapping. A: whole tryptic digest after phosphorylation of MARCKS peptide with PKC (type III). Spots labeled as 1, 1', 2, 2' and 3 correspond to SFK, KSFK, RFSFK, FSFK and LSGFSFK, respectively. B-E: fractions from reverse phase HPLC fractionated tryptic digest as in Fig. 2A. B: fraction 36; C: fraction 53; D: fraction 59; E: fraction 59, further digested with α-chymotrypsin. The origin is indicated by (x); the cathode (-) and anode (+) of electrophoresis and the direction of chromatography (↑) are as indicated in E.

corresponding to the only peak of radioactivity, contained a radioactive peptide comigrating with $S^{156}FK$ on 2D-peptide map, and the other peak of tR=50 min was devoid of any radioactivity. The latter peak was identified as LSGF by amino acid sequencing. Peptide extensively phosphorylated with either PKC type I, II or III or with PKM all showed similar results. These results indicate clearly that Ser^{160}had not been phosphorylated.

Reverse phase HPLC analysis of the tryptic peptides derived from rat brain MARCKS protein extensively phosphorylated with PKC (type III) also revealed 3 peaks of radioactivity similar to that of Fig. 1. Each radioactive fraction was also analyzed by 2D-peptide mapping and compared with the (K)S^{156}FK, (R)FS^{152}FK and $LS^{160}GFS^{163}$FK standards for identification. The ratio of phosphate incorporation in the tryptic phosphopeptides of MARCKS protein was similar to those from the extensively phosphorylated MARCKS peptide: (R)FSFK : (K)SFK : LSGFSFK = 1.9 : 2 : 1. Further digestion of LSGFSFK (t_R=59') with α-chymotrypsin as

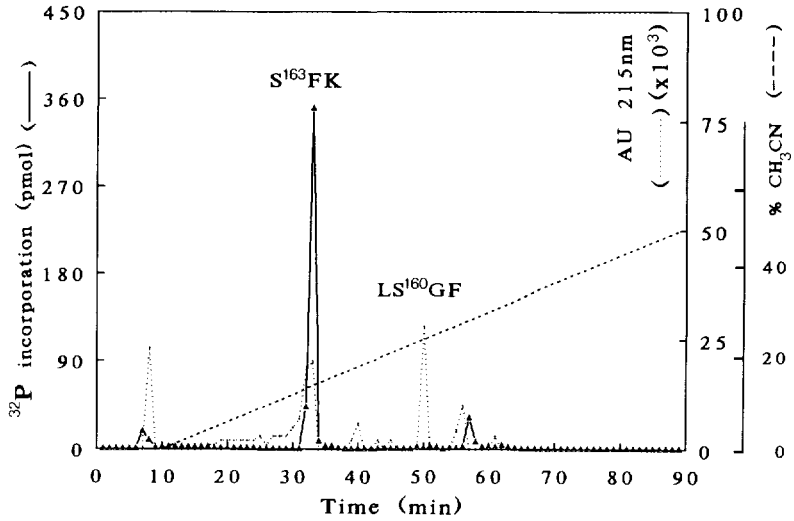

Fig. 3. Reverse phase HPLC separation of α-chymotrypsin digestion of fraction 59. The separation profile of absorption at 215 nm indicates that all but two peaks are contributed by α-chymotrypsin. These two peaks are at 33 min, which coincides with the only radioactive peak (see also Fig. 2E) and at 50 min, which corresponds to LSGF, as determined by amino acid sequencing.

described above to analyze the phosphorylation of Ser^{160} and Ser^{163}, again, indicated only S^{163}FK was radioactive but not LS^{160}GF (data not shown). Therefore, similar to MARCKS peptide phosphorylation, Ser^{160} in holo MARCKS protein was not phosphorylated by PKC either. It is not clear why the corresponding Ser^{160} of chicken MARCKS, having identical sequences within the CaM-binding region, can be phosphorylated by PKC (10).

Using 2D-peptide mapping to separate the tryptic phosphopeptides after phosphorylation of the MARCKS peptide, we were able to determine the initial rate of phosphorylation at each phosphorylation site in the peptide by each of the PKC isozymes. We quantified the phosphate incorporation during a time course of 10 s to 120 min. In Table I, the rate of phosphate incorporation during the first 3 min (in which the reaction is linear) is shown for each phosphorylation site. Apparently not every site was phosphorylated at the same rate; Ser^{152} and Ser^{156} seemed to be phosphorylated more readily than Ser^{163}. These differences in initial rates of phosphorylation were found using either one of the PKC isozymes type I, II and III (Table I) or PKM. The ratio of phosphate incorporation in histone (during the first 3 min) for PKC type I : type II : type III was 1 : 2.5 : 2.3, respectively, in our routine experiments. As can be seen in Table I, more or less the same ratio is found for each of the phosphorylation sites in MARCKS: type II and III PKC always show 2-3 times higher initial rates of phosphorylation than that of type I. This result indicates that at least *in vitro* there is no preference of any one of these PKC isozymes for MARCKS as substrate when compared with histone.

In general, it is to be expected that in a region with multiple phosphorylation sites, not all sites will be phosphorylated equally. Ser^{152} and Ser^{156} have basic residues on both sides of the serine, thereby creating a strong consensus sequence for PKC phosphorylation (17). In contrast, C-terminal to Ser^{163} there are 2 basic residues in its proximity, while Ser^{160} has only one basic residue at a -2 N-terminal position. Although Ser^{160} is a predictable phosphorylation site of PKC, lack of phosphorylation at this site may be due to the previous phosphorylation

Table I

Initial rate of phosphorylation of each individual site

Peptide fragment	PKC I		PKC II		PKC III	
(K)S[152]FK	8.5[a]	1	17[a]	2[b]	15[a]	1.8[b]
(R)FS[156]FK	17.5	1	26	1.5	24	1.4
LS[160]GFS[163]FK	5	1	15	3	11	2.2

[a] pmol phosphate/ min. [b] relative rate of phosphate incorporation if that by PKC type I was set as 1.

Peptide phosphorylated for 15s, 30s, 1 min or 3 min by equal amounts of PKC type I, II or III (20 ng each with activities of, respectively, 22, 54 and 50 pmol phosphate/ min towards histone) was digested with trypsin. Phosphate incorporation into each peptide fragment was determined by reverse phase HPLC or by 2D-peptide mapping.

at neighboring sites that introduce negative charges to block the recognition of Ser[160] by PKC. These results are in line with many other studies of PKC phosphorylation sites in other proteins, where serines having basic residues on both sides are best phosphorylated by PKC, followed by those serines having basic residues at one side only (18).

In conclusion, we did not find a clear preference of any one of these PKC isozymes for MARCKS as substrate when compared with histone. However our data indicate that PKC isozymes phosphorylate Ser[152] and Ser[156] more than Ser[163] in the CaM-binding region of MARCKS, while no phosphorylation was found on Ser[160].

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