Characterization of the Phosphorylation Sites in the Chicken and Bovine Myristoylated Alanine-rich C Kinase Substrate Protein, a Prominent Cellular Substrate for Protein Kinase C

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Little is known about the important cellular substrates for protein kinase C and their potential roles in mediating protein kinase C-dependent processes. We evaluated the protein kinase C phosphorylation sites in a major cellular substrate for the kinase, a protein of apparent M, 80,000 in bovine and 60,000 in chicken tissues; we have recently determined the primary sequences of these proteins and tentatively named them the myristoylated alanine-rich C kinase substrates. The proteins were purified to apparent homogeneity from bovine and chicken brains, phosphorylated with protein kinase C, digested with trypsin, and the phosphopeptides purified and sequenced. Four distinct phosphopeptides were identified from both the bovine and chicken proteins. Two of the phosphorylated serines were contained in the repeated motif FSFKK, one in the sequence LSGF, and one in the sequence SPK. All four sites were phosphorylated in the cell-free system appeared to be phosphorylated in intact cells; an additional site may have been present in the proteins from intact cells. The identity of the phosphorylation site domains from two proteins of overall 65% amino acid sequence identity suggests a potential role for this domain in the physiological function of the myristoylated alanine-rich C kinase substrate proteins.

The diacylglycerol-activated calcium-dependent protein kinase, protein kinase C, has been implicated in the control of many cellular processes such as mitogenesis, exocytosis, differentiation, and neurotransmission (for review, see Refs. 1–5). Although the kinase itself is becoming better understood, little is known about its physiologically important cellular substrates and their roles in mediating the diverse biological responses to protein kinase C activation.

We have recently begun to characterize one prominent cellular substrate for protein kinase C, formerly known as the 80- to 87-kDa protein in mammalian cells and tissues and the 60-kDa protein in chicken cells (for review, see Refs. 4–6); because the molecular masses of these proteins predicted from cDNA sequence analysis are much smaller than those determined by SDS-gel electrophoresis and for other reasons, we have recently proposed the name myristoylated alanine-rich C kinase substrate (MARCKS) (7).

The protein kinase C-mediated phosphorylation of this protein is interesting for several reasons. The phosphorylation of the MARCKS protein is dramatically stimulated within seconds after exposure of the cells to active phorbol esters, diacylglycerols, and many other agonists (4–11), and in all cases this phosphorylation is thought to be mediated by protein kinase C (10–12). Thus, although no enzymatic or other function has been ascribed to the protein, its phosphorylation state has served as a useful index or marker for protein kinase C activation in intact cells. In addition, the protein is rapidly dephosphorylated in response to receptor blockade, suggesting a potential role in the short term responses to protein kinase C activation (13). The protein is myristoylated, at least in murine macrophages (14), and there is some evidence that this modification is important in protein kinase C-dependent phosphorylation of the protein (14). Finally, we have recently shown that both the chicken and the bovine MARCKS proteins bind calmodulin in a calcium-dependent manner and that this binding is inhibited by protein kinase C-dependent phosphorylation of the protein.

For these reasons, we felt that it would be of interest to characterize the protein kinase C phosphorylation sites on this prominent and widely distributed substrate protein. In the present study, we analyzed the protein kinase C phosphorylation sites from both the chicken and the bovine MARCKS proteins. These two proteins are antigenically distinct and differ to the greatest extent in apparent molecular weights and isoelectric points of the proteins so far described (6); we felt that a comparison of their phosphorylation sites might illuminate common structural features of potential importance to the role of this protein in protein kinase C-mediated cellular responses.

MATERIALS AND METHODS

Cells—Chicken embryo fibroblasts prepared as described (15) were grown in minimal Eagle’s medium supplemented with 4% (v/v) fetal calf serum; MARCKS, myristoylated alanine-rich C kinase substrate; PMA, phorbol 12-myristate 13-acetate; HPLC, high performance liquid chromatography; PS, phosphatidyserine; EGTA, ethylenebis(oxethylenenitri10)tetraacetic acid; HFBA, heptadecanoic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

1 The abbreviations used are: SDS, sodium dodecyl sulfate; MARCKS, myristoylated alanine-rich C kinase substrate; PMA, phorbol 12-myristate 13-acetate; HPLC, high performance liquid chromatography; PS, phosphatidyserine; EGTA, [ethylenebis(oxethylenenitri10)tetraacetic acid; HFBA, heptadecanoic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

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calf serum and 10% (v/v) tryp tener phosphate. Fetal bovine skin fibroblasts were generously provided by Dr. R. R. Minor, Cornell University, Ithaca, NY and were grown in Dulbecco’s modified Eagle’s medium containing 20% (v/v) fetal calf serum. All tissue culture media were supplemented with 2 mM glutamine, 100 units/ml penicillin, and 100 µg/ml streptomycin. Labeling of cells with 32P, serum deprivation, stimulation with phorbol 12-myristate 13-acetate (PMA), two-dimensional electrophoresis, and autoradiography were carried out as described previously (16) except that 32P was added at a concentration of 0.5 mCi/ml.

**Purification of the Chicken and Bovine MARCKS Proteins**—Fetal bovine skin fibroblasts (approximately 600 g) were homogenized in 5-6 volumes of ice-cold 10 mM Tris-HCl (pH 7.5), 0.25 M sucrose, 10 mM dithiothreitol, 2 mM EGTA, and 0.2 mM phenylmethylsulfonyl fluoride with four 30-s bursts of a Brinkmann homogenizer (Brinkmann Instruments) before centrifugation at 12,000 g at 4°C for 35 min. The supernatant was boiled for 10 min, cooled to 4°C, and centrifuged at 12,000 x g for 30 min. This supernatant was lyophilized, 100% (v/w) trichloroacetic acid added to a final concentration of 1%, the mixture stirred for 1 h at 4°C, and then centrifuged at 12,000 x g for 20 min. 100% trichloroacetic acid was added to the resulting supernatant to a final concentration of 15%, and the mixture was stirred for 2-3 h at 4°C. Following centrifugation at 12,000 x g for 35 min, the pellet was washed twice with 0.1 M NaOH, and resuspended at a protein concentration of approximately 20 mg/ml in 50 mM Tris-HCl (NaOH was added to adjust the pH to 7.5), frozen with liquid nitrogen, and stored at -70°C.

For the initial chromatographic step, a 2.5-ml sample was thawed, filtered into an Amicon stirred cell (Amicon Corp., Danvers, MA) and applied to a 200-ml Ultrogel AcA34 column with a flow rate of 2.75 ml/min in 40 mM Tris-HCl (pH 7.35), 0.4 M NaCl; fractions (2.75 ml) were collected at 1-min intervals. For the chicken MARCKS protein, peak fractions were pooled and concentrated to 1 ml on a YM-30 membrane in a 10-ml Amicon stirred cell, and applied to a 100 X 3.2-mm RP-8 Newguard column with a flow rate of 0.3 ml/min at 4°C in 200 mM ammonium bicarbonate (pH 7.8). The chicken phosphopeptides were initially separated after a 20-min delay with a gradient from 0-36% (v/v) acetonitrile in 0.1% trifluoroacetic acid over 24 min followed by a gradient from 7.2-21.9% (v/v) acetonitrile in 0.1% (v/v) trifluoroacetic acid over 147 min. Fractions (0.2 ml) were collected at 1-min intervals and handled as described above.

Some chicken and bovine peptides required a further step for purification; for this we used the same system except that 1% heptfluorobutyric acid (HFBA) replaced 1% trifluoroacetic acid in buffer B. The fractions that required further purification were pooled, lyophilized, resuspended in 250 µl of 0.1% HFBA, and injected. The peptides were separated after a 10-min delay with a gradient of 0-42% (v/v) acetonitrile in 0.1% HFBA over 140 min. Fractions (0.2 ml) were collected at 1-min intervals and handled as described above.

**In Vitro Phosphorylation of MARCKS Protein by Protein Kinase C**—The phosphorylation reactions were carried out at 20°C for 25 min unless otherwise specified and contained purified MARCKS protein (approximately 6 µM) and purified protein kinase C (approximately 30 nM), with a specific activity of 3000 cpm/pmol, ICN Biomedicals, Inc., Irvine, CA, and either 600 µg phosphatidylserine (PS), 80 µg diolein, and 1.5 mM CaCl2 or 5 mM EGTA as noted in the text. The total volume of the reactions ranged from 100 to 1500 µl. The reactions were stopped by the addition of either SDS sample buffer (final concentrations: 1% (w/v) SDS, 0.01 M EDTA, 0.25 M sucrose, 0.085 M dithiothreitol) for SDS-polyacrylamide gel electrophoresis as described previously (18) or glacial acetic acid to a final concentration of 30% for AG 1-X8 chromatography. Bands containing 32P-labeled tryptic phosphopeptides were identified as described previously (16). After electrophoresis, the gels were stained with 0.1% Coomassie Blue, destained, and then silver stained according to the instructions of the manufacturer (Bio-Rad). The arrow labeled 60K indicates the position of the chicken MARCKS protein. For the two-dimensional gel, the positions of the molecular weight standards and the approximate isoelectric focusing gradient are indicated.
ml) were collected at 1-min intervals and handled as described above. After purification, fractions corresponding to the peak of radioactivity were subjected to amino acid sequence analysis on an Applied Biosystems 470A gas-phase sequenator in combination with an Applied Biosystems 120A phenylthiohydantoin analyzer (Applied Biosystems, Foster City, CA). The wash from each cycle was collected and subjected to Cerenkov scintillation counting. Peptide Mapping of MARCKS Protein Phosphorylated in Intact Cells and in a Cell-free System—The phosphorylation of the proteins in intact cells is described above; the cell-free protein phosphorylation was as above except that the reaction products were precipitated with trichloroacetic acid (final concentration, 20%) and separated by two-dimensional gel electrophoresis exactly as in the intact cell experiment. After two-dimensional electrophoresis, the gels were dried and autoradiographed without fixing or staining. The MARCKS proteins were excised from the gels and digested with trypsin as described previously (20). The recovery of radioactivity from the gel slices averaged about 80% in these experiments. The digested samples were resuspended in 250 μl of 0.1% trifluoroacetic acid by vigorous mixing and water bath sonication, centrifuged at 12,000 × g for 15 min at room temperature, and subjected to reverse phase chromatography on the C4 column described above. The peptides were separated with a gradient from 0–36% (v/v) acetonitrile in 0.1% (v/v) trifluoroacetic acid over 120 min at a flow rate of 0.2 ml/min. Fractions (0.2 ml) were collected at 1-min intervals and subjected to Cerenkov scintillation counting.

RESULTS

Purification of the Chicken and Bovine MARCKS Proteins—Both the chicken and bovine MARCKS proteins were purified from brain extracts to apparent homogeneity using a heat treatment step in conjunction with size exclusion chromatography and a concentration step. After a single gel filtration step, the chicken MARCKS protein appeared to be pure as assessed by Coomassie Blue and silver staining of one- and two-dimensional gels (Fig. 1). For most bovine preparations, gel filtration chromatography did not achieve complete purification; however, Fig. 2 demonstrates that fractions eluting from the second column (Ultrogel AcA34) appeared to contain pure protein as assessed by silver staining. Protein Kinase C Phosphorylation of the MARCKS Protein—The protein kinase C-dependent phosphorylation of the purified chicken MARCKS protein was increased by the addition of PS and CaCl₂ (Fig. 3); similar results were obtained with the bovine protein (data not shown). The cofactor-independent phosphorylation of both the chicken and bovine MARCKS proteins was rapid at 20 °C, appearing within 30 s, and was maximal in both cases by 20 min (Figs. 3 and 4). The MARCKS protein appeared to be fully phosphorylated under these reaction conditions since the addition of 50-fold more protein kinase C produced little increase in MARCKS phosphorylation (Fig. 3A); the autophosphorylation of the kinase itself could be seen under these conditions (Fig. 3A). Phosphorylation of the MARCKS protein in the absence of PS and CaCl₂ (cofactor-independent) also occurred but more slowly and to a lesser extent and was probably due to the presence of the proteolytic catalytic fragment of protein kinase C known to be present in this preparation of protein kinase C and active under these conditions (21).

\[ M_r \times 10^3 = 200 - 160 - 93 - 67 - 45 - 31 - 21.5 - 14.4 \]

\[ 80K \]

**Fig. 2.** Silver stain of peak fractions of the bovine MARCKS protein eluting from the Ultrogel AcA34 column. Pooled peak fractions from the TSK 3000 fractionation were concentrated and applied to a 200-ml Ultrogel AcA34 column. Equal volumes of the fractions from the Ultrogel AcA34 column were separated by SDS-polyacrylamide gel electrophoresis and the gel silver stained as described in the legend to Fig. 1. The positions of the bovine MARCKS protein (80K) and the molecular weight standards are indicated. The bovine MARCKS protein is known to migrate with an unusually low mobility on this type of gel (10).

**Fig. 3.** Time course of protein kinase C (PKC) phosphorylation of the chicken MARCKS protein in the presence or absence of PS, diolein, and calcium. Purified chicken MARCKS protein (60K) was phosphorylated in vitro at 20 °C for the time indicated in the presence of either PS, diolein, and CaCl₂ (A) or 5 mM EGTA (B) as described under “Materials and Methods.” The reactions were terminated by the addition of SDS sample buffer, and the reaction products were separated on a 15% SDS-polyacrylamide gel. The autoradiographs are shown here. In A, the asterisk after one 90-min sample denotes a sample in which 50-fold more protein kinase C was included during the phosphorylation reaction; auto-phosphorylated protein kinase C is indicated. For C, the bands were identified by autoradiography, excised, rehydrated in 2 ml of water by shaking for 15 min, and subjected to scintillation counting in 15 ml of Biofluor (Du Pont). The radioactivity was then plotted as a function of time.
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Fig. 4. Time course of protein kinase C phosphorylation of bovine MARCKS protein. Purified bovine MARCKS protein (80K) was phosphorylated in the presence of PS, diolein, and calcium at 20 °C for the specified times. The reactions were terminated with SDS sample buffer and subjected to SDS-polyacrylamide gel electrophoresis. The autoradiograph is shown in A; the position of the 80-kDa MARCKS protein is indicated by the arrow. For B, the bands were identified by autoradiography, excised, and subjected to scintillation counting in water. The radioactivity was then plotted as a function of time.

The phosphorylation of the MARCKS protein in the presence of PS and CaCl₂ was maximal, whereas the contribution of cofactor-independent phosphorylation was minimized in reactions conducted for 25 min at 20 °C (Fig. 3). Therefore, these conditions were used to generate phosphorylated proteins for tryptic cleavage. Furthermore, Figs. 3 and 4 demonstrate that under these conditions of autoradiographic exposure, the MARCKS protein appeared to be the only phosphorylated protein in the reaction mixture.

Purification of Tryptic Phosphopeptides—Tryptic phosphopeptides from the phosphorylated MARCKS proteins were separated by reverse phase HPLC. Chromatography of tryptic fragments of the phosphorylated chicken protein (Fig. 5) produced three major peaks of radioactivity: one centered at fraction 5, one at fraction 76 (I-II), and the last at fraction 92 (III). The peak centered at fraction 5 corresponds to the elution position of both [³²P]ATP or P₃ and the family 3 phosphopeptide (see below). The peak at fraction 92 was pure enough to provide useful amino acid sequence information, whereas the peak at fraction 76 was not. To purify the phosphopeptides that eluted from the first reverse phase column near fraction 76, appropriate fractions were pooled and reappied to the same column in 0.1% HFBA instead of 0.1% trifluoroacetic acid (Fig. 6). This change in solvent system allowed for the purification and sequencing of two phosphopeptides (I and II).

Three of the bovine phosphopeptides were purified using the two-step fractionation procedure with reverse phase chromatography in buffers originally containing trifluoroacetic acid and subsequently HFBA. Fig. 7 shows that the initial reverse phase chromatography of the cleaved bovine protein produced four major peaks of radioactivity (IV, V, VI, and VII). The peak at fraction 86 was only seen in one of three separate preparations; sequencing revealed that it was a contaminating phosphopeptide from the GAP-43 protein (22) IQASF (see “Discussion”). Fig. 8 demonstrates that after reinfusion, three of the bovine phosphopeptides eluted as a single peak of radioactivity; peak fractions from each were then sequenced. The fourth peak (VII) could be sequenced without further purification.

Determination of Phosphorylation Site Sequences—The amino acid sequences of the peptides corresponding to each of the major peaks of radioactivity, I–VII, were determined with a gas-phase sequenator. Two methods were employed to ascertain which residues were phosphorylated (23–28). In one method, waste fractions were collected from the sequenator, subjected to Cerenkov scintillation counting, and the radioactivity profile compared with the sequence information. In the other, a low step yield of serine coupled with a large amount of dehydroalanine in the cycle indicated that a serine was phosphorylated. The sequences of the phosphorylated peptides and the serine residues that were phosphorylated are shown in Table I. The sequences demonstrate that either or both the chicken and bovine MARCKS proteins contained the same families of phosphorylation site peptides: FSFK, LSGFS, or SFK. The variability of sequence within a peptide family was due to the presence or absence of either a lysine or arginine residue, suggesting that trypsin, active at these basic residues, cleaved at variable positions. In the family 1 peptides, the single serine was phosphorylated. In the family 2 peptides, 2 serines were present. In every case, the 2nd...
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A

FIG. 6. Reinjection of chicken phosphopeptides in HFBA. Fractions 75 (A) or 77 and 78 (B) from Fig. 5 were lyophilized and resuspended in 0.1% HFBA. After resuspension, the phosphopeptides were reinjected onto the same C4 column with HFBA replacing trifluoroacetic acid in the buffers. The peptides were separated at a flow rate of 0.2 ml/min with a linear gradient of acetonitrile in trifluoroacetic acid as shown by the dashed line and described under "Materials and Methods." Fractions were collected at 1-min intervals and the radioactivity determined by Cerenkov scintillation counting and plotted as a function of fraction number. The peaks of radioactivity were labeled I or II according to elution position.

serine in the family 2 peptide was phosphorylated. In the chicken family 2 peptide, there was a large step increase of radioactivity for the 1st serine, suggesting that it was also phosphorylated; Cerenkov scintillation counting of the washes from each sequencing cycle yielded counts of 340, 3370, 4932, 4628, 7461, and 3868 cpm for the sequence LSGFS, respectively. There was, however, no direct evidence for phosphorylation of this serine in the two bovine family 2 peptides. In the bovine family 3 peptide, the single serine was phosphorylated. Thus, at least 3 serines, 2 in the context of the repeated motif FSFKK and all 3 in the context of the SFK motif, were phosphorylated, whereas a 4th serine (in the LSGF motif) also appeared to be phosphorylated, at least in the chicken protein.

Phosphorylation Site Domain—To characterize the location of the phosphopeptides within the bovine and chicken proteins, we compared the sequences of the phosphopeptides with the amino acid sequences of the proteins from both species (7). In both the chicken and bovine MARCKS proteins, all of the sequenced phosphorylation sites were clustered in a small extremely basic domain of this acidic protein (Table I). Lysine and arginine comprised over half of the residues in this 25-amino acid phosphorylation site domain, which had a calculated pI of 12.2. This region represents the longest stretch of amino acid identity in the two proteins and contains the repeated sequences FSFKK and SFK (Table I).

Comparison of Intact Cell and in Vitro Phosphorylation—We also compared tryptic maps of the 32P-protein isolated from PMA-stimulated chicken or bovine fibroblasts with those from the brain protein phosphorylated in vitro by protein kinase C. The phosphorylated MARCKS proteins from all sources were then isolated by two-dimensional gel electrophoresis, localized by autoradiography, and digested with trypsin. Fig. 9 shows the reverse phase HPLC maps of the phosphopeptides from the various sources and demonstrates that the sites phosphorylated in vitro by protein kinase C were similar to most of the major sites phosphorylated in intact cells in response to PMA. An additional phosphopeptide that eluted between the family 1 and 3 peptides in the gradient was sometimes seen in the intact cell experiments; it is not clear whether this represents an additional phosphopeptide or a contaminant (see below).

DISCUSSION

This study describes the characteristics of several protein kinase C phosphorylation sites in the MARCKS proteins from both chicken and bovine brain and reveals several interesting facts about these sites. First, it appears that four protein kinase C phosphorylation sites in both proteins are contained within a 25-amino acid basic domain (calculated pI, 12.2) which lies in the middle of the proteins' primary sequence (7). This domain is identical in the proteins from both species as well as in the human protein and appears in the longest

3 D. M. Harlan and P. J. Blackshear, unpublished data.
stretch of sequence identity between the chicken and bovine proteins; otherwise, the amino acid sequence identity is 65%.\(^2\)

Two of the phosphorylated serines are contained within a repeated motif FSFKK, and 3 are in the context of the sequence SFK; the fourth probable site is contained within the sequence LGFS. All four sites are enclosed between blocks of basic residues, and general a-helical conformation (30). Amino acids in close proximity to the phosphorylated serine or threonine residue are important features of sites phosphorylated by protein kinase C, although in contrast to protein kinase A, a strict consensus sequence for protein phosphorylation has not been determined (31). Studies based on synthetic peptides have reported conflicting results; some groups have concluded that the critical basic residues are amino-terminal to the phosphorylated residue, whereas others have stated that the critical bases are carboxyl-terminal (32–36). Many of the known physiological phosphorylation sites (24, 25, 37–40) have basic amino acids on both sides of the phosphorylated residue; based on studies with synthetic peptide substrates, the best substrates for protein kinase C have basic residues on both sides of the phosphorylated serine or threonine residue (31, 41). The phosphorylation sites in the MARCKS proteins are in general species, it seems plausible that these domains might be crucial for the still unknown biological function of the MARCKS proteins.

One such potential function is suggested by our recent demonstration that the MARCKS proteins from both chicken and bovine brain could bind calmodulin with reasonable affinity in a calcium-dependent manner and that this binding could be prevented by prior phosphorylation of the proteins by protein kinase C.\(^3\) This finding suggests but does not prove that the phosphorylation site domain in the MARCKS protein is also the calmodulin-binding domain. This suggestion is supported by the similarity of this domain to other calmodulin-binding domains, especially in its predominance of basic residues and general a-helical conformation (30).

**TABLE I**

| Family 1 peptides             |
|-------------------------------|
| Chicken I                     |
| Chicken II                    |
| Bovine IV                     |

| Family 2 peptides             |
|-------------------------------|
| Chicken III                   |
| Bovine V                      |
| Bovine VI                     |

| Family 3 peptide              |
|-------------------------------|
| Bovine VII                    |

**A. Sequenced phosphopeptides from tryptic digest**

| Family 1 peptides             |
|-------------------------------|
| Chicken I                     |
| Chicken II                    |
| Bovine IV                     |

| Family 2 peptides             |
|-------------------------------|
| Chicken III                   |
| Bovine V                      |
| Bovine VI                     |

| Family 3 peptide              |
|-------------------------------|
| Bovine VII                    |

**B. Phosphorylation site domain derived from cDNA sequence**

|                 |
|-----------------|
| KKKKRFSSFKSKFKLSGFSDKNNKK |

**FIG. 8.** Reinjection of bovine phosphopeptides in HFBA. Peak fractions from the first reverse phase column (Fig. 7) were pooled, lyophilized, and resuspended in 0.1% HFBA. These phosphopeptides were then reinjected onto the C4 column with HFBA replacing trifluoroacetic acid in the buffers. The peptides were separated at a flow rate of 0.2 ml/min with a linear gradient of acetonitrile in HFBA as shown by the dashed line and described under "Materials and Methods." One-min fractions were collected, the radioactivity determined by Cerenkov scintillation counting, and the radioactivity was plotted as a function of fraction number. The peaks of radioactivity were labeled IV, V, and VI according to Fig. 7.
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One of three preparations of phosphorylated bovine brain protein yielded one other phosphoryopeptide peak after reverse phase HPLC, eluting earlier in the gradient than the MARCKS peptides (see Fig. 7). Sequence analysis of this peptide revealed the sequence IQASF; this sequence was not contained within the bovine protein sequence predicted from cDNA cloning but instead was found near the amino terminus of the neuron-specific protein kinase C substrate protein GAP-43 (the peptide is located at amino acid position 38 in Ref. 22). This phosphopeptide also conforms to the loose consensus sequence for protein kinase C substrates, being contained in a larger domain bracketed by the presumed tryptic cleavage sites, KIQASF. The GAP-43 protein was a frequent contaminant of the brain heat-stable protein preparation, and thus this peak presumably represents a protein kinase C phosphorylation site from GAP-43. Interestingly, it is thought that there is only a single protein kinase C phosphorylation site, known to be a serine, in the GAP-43 protein (44). In addition, the protein kinase C phosphorylation of GAP-43 is believed to inhibit calmodulin binding to GAP-43, and the phosphorylation site we identified is adjacent to the proposed calmodulin-binding region of GAP-43 (45). It seems likely, therefore, that we inadvertently sequenced the physiologically relevant protein kinase C phosphorylation site on this protein, which, to our knowledge, has not been reported.

We attempted to compare peptide maps of the chicken and bovine proteins phosphorylated in a cell-free system with those phosphorylated in intact cells from the same animal species after exposure of the cells to PMA. In general, the four major peptide peaks seen after in vitro phosphorylation of the purified protein were also observed with the proteins phosphorylated in the intact cell experiments. This supports the contention that the sites phosphorylated by protein kinase C in vitro are the same as those phosphorylated in vivo, consistent with earlier suggestions. However, we also noted at least one minor phosphopeptide peak in the profiles from the cell-labeling experiments, eluting between the family 1 and 3 peptides in the acetonitrile gradient. These have not always been noted (see for example, ref. 46) and could be due to a number of artifacts including major differences in the amount of MARCKS 32P-protein loaded into the gels between the in vitro and in vivo experiments, perhaps resulting in more extensive tryptic cleavage in the latter case; the possible presence of other 32P-proteins underlying the large diffuse MARCKS spot in the gels, which would not be seen in experiments involving purified protein; and differences in the sample preparation such as the fact that the pure proteins had been subjected to boiling, whereas the cell extracts had not. However, it also remains possible that there are additional phosphorylation sites on the protein in intact cells for protein kinase C or other kinases. We are attempting to address this question in studies using site-directed mutagenesis of the cDNA clones for the MARCKS proteins followed by expression in cells and PMA-stimulated phosphorylation. These studies also should help to determine whether there is cooperativity among the sites and whether specific sites are involved in the apparent interaction of this domain with calmodulin.

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