Protein Kinase C-mediated Phosphorylation of the Myristoylated Alanine-rich C-kinase Substrate Protects It from Specific Proteolytic Cleavage

(Received for publication, September 7, 1995, and in revised form, October 26, 1995)

Gwendolyn Spizzi and Perry J. Blackshear

From the Howard Hughes Medical Institute, and Section of Diabetes and Metabolism, Division of Endocrinology, Metabolism and Nutrition, Departments of Medicine and Biochemistry and the Sarah W. Stedman Center for Nutritional Studies, Duke University Medical Center, Durham, North Carolina 27710

The myristoylated alanine-rich C kinase substrate (MARCKS) is a major cellular substrate of protein kinase C. Its concentration in cells is important for the normal development of the central nervous system, and perhaps other physiological processes. We found that MARCKS concentrations in cells were regulated in part by a specific proteolytic cleavage; this resulted in two fragments, each representing about half of the intact protein, that co-existed with MARCKS in cells and tissues. These fragments were present in significant concentrations in quiescent fibroblasts; they disappeared, and the amount of intact MARCKS increased, within 15 s of activation of protein kinase C by serum. In vitro experiments demonstrated that phosphorylated MARCKS was a poor substrate for a protease activity present in cell extracts, whereas dephosphorylated MARCKS was a good substrate. Both the protease activity and the specific MARCKS cleavage products were essentially absent in brain, but present in many other cells and tissues. The protease activity, which had the characteristics of a cysteine protease, cleaved MARCKS between Asn\(^1\) and Glu\(^48\) of the bovine sequence, three amino acids to the amino-terminal side of the MARCKS phosphorylation site domain. These studies demonstrate that MARCKS is subjected to specific cleavage by a cellular protease, in a manner dependent on the phosphorylation state of the substrate. This represents a novel means of regulating cellular MARCKS concentrations; these data also raise the interesting possibility that MARCKS is involved in regulating the activity of this novel cellular protease.

The myristoylated alanine-rich C kinase substrate, or MARCKS\(^1\) protein, is a prominent cellular substrate for protein kinase C (PKC) (1, 2). MARCKS and its relative, the MARCKS-related protein (also known as F52 or MadMARCKS) comprise a family of heat-stable, acidic proteins that are characterized by several common properties. These include three highly conserved regions within the primary protein sequence: The amino-terminal sequence, which is responsible for directing myristoylation; a short sequence of identity to the mannose 6-phosphate/insulin-like growth factor II receptor which surrounds the splice site of the single known intron; and an internal highly basic domain of 25 amino acids containing the PKC phosphorylation sites. This basic phosphorylation site domain (PSD) is also the binding site for calmodulin; this occurs in a calcium-dependent fashion and is inhibited by PKC phosphorylation (3). The PSD also has been implicated in binding to cytoskeletal actin, again in a phosphorylation- and calmodulin-dependent manner (4). The deduced amino acid sequences of MARCKS from various animal species predict proteins of 28–33 kDa; however, MARCKS migrates anomalously on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), at molecular mass ~ 60,000–87,000 depending on the species.

Although the precise cellular function of MARCKS remains obscure, recent data from gene targeting experiments in the mouse indicate that it is necessary for normal development of the central nervous system, as well as extrauterine life (5). Abnormalities seen in the MARCKS-deficient pups include exencephaly, agenesis of the corpus callosum and other forebrain commissures, failure of hemisphere fusion, and lamination abnormalities of the cerebral cortex and retina. The molecular basis of these abnormalities is not known; however, heterozygous mice expressing 50% of wild-type MARCKS levels are phenotypically normal, suggesting that a certain threshold MARCKS concentration is necessary for these developmental processes to occur normally.

For these and other reasons, regulating MARCKS protein concentrations in cells is important. In general, MARCKS protein concentrations closely parallel its mRNA levels; these can in turn be regulated at the level of gene transcription. For example, tumor necrosis factor \(\alpha\) and lippolylyasaccharide can cause dramatic increases in the levels of MARCKS mRNA and protein in neutrophils, macrophages, and related cells (6–8). Decreases in MARCKS mRNA and protein have been seen in fibroblasts transformed by a variety of oncogenes or by chemical carcinogenesis (9–13). At the post-transcriptional level, MARCKS mRNA and protein are decreased in Swiss 3T3 cells in response to several hours of exposure to phorbol esters, bombesin, platelet-derived growth factor, and agents that elevate cAMP (14, 15). In a different study, Lindner et al. (16) reported rapid decreases in MARCKS protein levels after short-term (less than 15 min) exposure to phorbol 12-myristate 13-acetate (PMA).

One means of controlling MARCKS protein levels is by specific proteolysis, perhaps regulated by some of the stimuli enumerated above. In the present study, we show that MARCKS...
can be cleaved into two major fragments that achieve significant concentrations in some cells and tissues. This cleavage is prevented by PKC-dependent phosphorylation of MARCKS. The putative protease involved is present in many cells and tissues, appears to be a cysteine protease, and degrades MARCKS at a site between asparagine 147 and glutamate 148 in the bovine sequence, three amino acids amino-terminal to the PSD. These results indicate that MARCKS levels in cells can be controlled by a specific protease, whose proteolytic efficacy is dependent on the phosphorylation state of its substrate. They also raise the interesting possibility that MARCKS can physically associate with this uncharacterized protease, perhaps even regulating its activity toward other substrates.

MATERIALS AND METHODS

Cell Maintenance and Radiolabeling—Human foreskin fibroblasts (HFF, Cloneteks Corp., San Diego, CA) were grown in Dulbecco’s modified Eagle’s medium (DMEM, Life Technologies, Gaithersburg, MD) supplemented with 10% (v/v) heat-inactivated fetal calf serum, 2 mM glutamine, 100 units/ml penicillin, and 100 µg/ml streptomycin (Life Technologies). Mouse LM/TK- cells (17) stably transfected with bovine MARCKS cDNA under the control of the human β-actin promoter (18) were maintained in the same medium supplemented with 400 µg/ml Geneticin (Life Technologies). To separate live cells, confluent cells were rinsed once with phosphate-buffered saline and the medium was replaced with serum-free DMEM supplemented as above except that it contained 0.1% (w/v) bovine serum albumin (BSA, lyophilized and crystallized; Sigma). To label with L-[35S]cysteine, DMEM lacking cysteine and methionine was supplemented with 0.1% BSA, glutamine, penicillin, and streptomycin as above and 10 mM methionine. L-[35S]Cysteine (DuPont NEN Research Products, Boston, MA) was added at 0.05–0.2 mCi/ml. To label with L-[35S]methionine, DMEM lacking cysteine and methionine was supplemented with 0.1% BSA, glutamine, penicillin, and streptomycin as above and 20 mM cysteine. L-[35S]Methionine (DuPont NEN) was added at 0.1 mCi/ml. To label with L-[14C]alanine, DMEM was supplemented with 0.1% BSA, glutamine, penicillin, and streptomycin as above with L-[35S]cysteine (DuPont NEN) at 5 µCi/ml. Cells were serum starved and radiolabeled for 16 h. Following the overnight incubation, dimethyl sulfoxide (Me2SO, Sigma), phorbol-12-myristate-13-acetate (PMA, Sigma), or serum was added directly to the medium. A 16 mM stock of PMA was made in Me2SO followed by a 1:100 dilution in 0.1% BSA-containing DMEM; this was then added immediately to the medium at a 1:100 dilution resulting in a final concentration of 1.6 µM. As a control, Me2SO was diluted similarly and added to the cells such that the final amount of Me2SO was 0.01% in both control and PMA-treated cells. Fetal calf serum was added directly to medium at a 1:10 dilution. Cells were incubated at 37°C in a water-jacketed incubator supplemented with 5% CO2. Following radiolabeling and hormonal stimulation, the cells were placed on ice, the medium was removed, and the cells were washed three times with ice-cold phosphate-buffered saline. Cells were lysed in 50 mM Tris-HCl, pH 7.4, containing 10% glycerol (v/v), 1% Nonidet P-40 (v/v), 1 mM EDTA, and 150 mM salt. Depending on the experiment, either 150 mM NaCl or 100 mM NaCl plus 50 mM Naf was used. A protease mixture containing 200 µg aprotinin, 1 µg benzamidine-HCl, 2 µM leupeptin, 1 µM pepstatin, and 574 µM phenylmethylsulfonyl fluoride was added to the lysis buffer. When the specificity of the protease inhibitors was evaluated, each protease inhibitor was added separately to the lysis buffer. One ml of lysis buffer was used on 100-mm dishes and 0.5 ml of lysis buffer was used on 60-mm dishes. Cells were lysed with a rubber policeman and collected into a 1.5-ml centrifuge tube. Depending on the experiment, the cell extracts were subjected to clarification with or without prior boiling. For non-boiled samples, lysates were tumbled at 4°C for 20 min followed by centrifugation at 13,500 × g for 10 min at 4°C. For boiled samples, lysates were boiled for 10 min, placed on ice for 10 min and then dialyzed by centrifugation at 13,500 × g for 10 min at 4°C. Clarified lysates were then subjected to immunoprecipitation.

For lysates from HFF cells that had been boiled prior to clarification, further preclarification was unnecessary. For boiled lysates from LM/TK- cells and non-boiled lysates from HFF cells, lysates were preclarified by incubation with a 1:100 dilution of nonimmune control antibody and tumbling at 4°C for 3 h. In the case of samples immunoprecipitated with monoclonal antibodies, a secondary rabbit anti-mouse IgG antibody (2 mg/ml; Pierce) was added at a 1:10 dilution followed by tumbling at 4°C for 1 h. Protein A-Sepharose, 0.1 g, (Pharmacia Biotech Inc., Piscataway, NJ), washed and resuspended in 1 ml of lysis buffer, was then added at a 1:10 dilution and samples were tumbled for 1 h at 4°C. Protein A-Sepharose was sedimented by centrifugation at 6000 × g for 3 min and the supernatants subjected to further immunoprecipitation.

Samples that were preclarified by boiling or by treatment with non-competitive antibodies were subjected to immunoprecipitation with a 1:100 dilution of the appropriate antibodies and tumbled at 4°C overnight. When competing peptides were used, an equal volume of antibody and 5 mM solutions of the peptides were mixed and incubated for 1 h at 4°C prior to adding to preclarified lysates. In the case of immunoprecipitations with monoclonal antibodies, lysates were treated with a rabbit antibody as the secondary antibody as described above. All samples were treated with a 1:10 dilution of protein A-Sepharose as described above. Protein A-Sepharose was precipitated by centrifugation at 6000 × g for 3 min and the supernatants were discarded. The pellets were washed three times with 0.5–1 ml of lysis buffer followed by centrifugation at 6000 × g for 3 min. The final protein A pellet was resuspended in 100 µl of SDS sample buffer containing 10% sucrose (w/v), 100 mM dithiothreitol, 1.2% SDS (w/v), 12 mM EDTA, and 0.0012% (w/v) Pyronin Y and boiled for 3 min. The immunoprecipitates were analyzed by electrophoresis on 10% SDS-polyacrylamide gels. Gels were fixed in 40% methanol and 10% acetic acid for 20 min, rinsed extensively with water for 20 min, and treated with Autoluf (National Diagnostics, Atlanta, GA) for 20 min. Gels were dried and exposed to Kodak X-Omat XAR film at −80°C.

In Vitro Transcription and Translation—Construction of wild-type and mutant bovine MARCKS cDNA constructs were as described previously (2, 19). Mutations used for this study included replacement of the amino-terminal glycine with alanine, and replacement of the four serines within the PSD with aspartic acid, asparagine, glycine, or alanine. Messenger RNA was transcribed in vitro as described previously (19). The resulting mRNA was translated in vitro using 50 µl of rabbit reticulocyte lysate (Promega, Madison, WI), 2–4 µg of mRNA, and 25 µCi of [35S]cysteine with incubation at 30°C for 1 h. In certain instances, the translation reaction was diluted with phosphate-buffered saline to 0.5 ml, and a clarified supernatant was prepared by boiling for 15 min at 30°C on ice for 10 min, and centrifugation at 107,000 × g for 40 min at 4°C.

Phosphorylation of In Vitro Translated MARCKS with Protein Kinase M—Boiled and cleared translation reaction products were subjected to phosphorylation with the catalytic fragment of PKC, protein kinase M (PKM). Translated proteins were incubated with 40 µl Hepes, pH 7.5, 12.5 mM MgCl2, 75 µM ATP, 1 µM EGTA, and 1/120 dilution of purified PKM for 30 min at 30°C on ice. Reaction products were subjected to pro tease inhibition with HSST lysates.

Analysis of Protease Activity with Exogenously Added Substrate—To analyze the protease activity in whole cell lysates, lysates were prepared as described above for immunoprecipitations, except that the cells were serum starved in the absence of isotope, and the lysates were not boiled. The absence of protease inhibitors from the lysates to proteolyze wild-type or mutant MARCKS translation reaction products, 100 µl of buffer or lysate was mixed with 15 µl of undiluted translation reaction. To analyze the effect of specific protease inhibitors, 25 µl of diluted and clarified translation reaction was mixed with 100 µl of lysate with the addition of specific inhibitors. To analyze the effect of phosphorylation on in vitro translated products, 25 µl of diluted clarified and phosphorylated translation product was mixed with 100 µl of lysis. All reactions were incubated at 30°C for 30 min followed by the addition of 30 µl of a 5 times concentrated SDS sample buffer (final concentrations described above). Samples were boiled and then analyzed by SDS-polyacrylamide gel electrophoresis. Gels were fixed, stained, and destained as described above.

Tissue Preparation—Bovine tissue was obtained fresh from the slaughterhouse, frozen immediately in liquid nitrogen, kept on dry ice during transport, and stored at −135°C. Approximately 0.5–1 g of frozen tissue was ground into a fine powder using a mortar and pestle in the presence of liquid nitrogen. The powder was thawed and homogenized using 2 ml of buffer containing 0.5 M Tris-HCl, 5 mM KCl, 5 mM β-glycerophosphate, pH 8.1, containing 250 mM sucrose, 1 mM EDTA, 1 mM EGTA, 1 mM diethiothreitol, 100 mM NaCl, 50 mM NaF, and protease inhibitors as described above except at 2-fold higher concentrations. Homogenization involved 5 up and down strokes with the Wheaton overhead stirrer set at 6. Volumes were brought up to 3 ml with buffer, and Nonidet P-40 was added to a final concentration of 1% (w/v). The samples were then incubated on ice for 1 h, followed by centrifugation at 1500 × g for 10 min. Supernatants were boiled for 10 min, incubated on ice for 10 min, and centrifuged at 13,500 × g for 1 h.

Protein
concentrations in the remaining supernatants were determined by Bio-Rad assay (Bio-Rad).

Western Blot Analysis—To analyze for the presence of MARCKS in bovine tissues, 100 (spleen and brain) or 400 (liver and kidney) μg of heat-stable protein was boiled with SDS sample buffer for 3 min and separated on 10% SDS-polyacrylamide gels. Separated proteins were electrophoretically transferred to nitrocellulose filters (Schleicher and Schuell) in 192 μl glycine, 25 μl Tris, and 20% (v/v) methanol using a Hoefer Transphor apparatus. All steps in the following immunoblotting method were performed at room temperature. Nonspecific sites on filters were blocked by incubation and shaking for 1 h with 5% (w/v) instant milk in 20 μl Tris, pH 7.6, containing 137 μl NaCl and 0.3% (v/v) Tween 20 (TBST). Filters were briefly rinsed with TBST followed by incubation with primary antibody at a 1:100 dilution in TBST for 1–2 h with shaking. Filters were rinsed three times by shaking in TBST for 10 min each time. The filters were incubated with horseradish peroxidase-conjugated secondary antibody (Bio-Rad) at a 1:5000 dilution in TBST for 30 min with shaking. Filters were again rinsed three times in TBST for 10 min each time. Immunoreactive bands were visualized by enhanced chemiluminescence (Amersham) following the manufacturer’s instructions.

Purification and Sequencing of p40—Boiled supernatants from bovine spleen were further purified by fractionation over a Mono Q anion exchange column (Pharmacia) as described previously (21). To assay for the presence of p40, aliquots of fractions were boiled with SDS sample buffer for 3 min and separated on 10% SDS-polyacrylamide gel. Proteins were electrophoretically transferred to nitrocellulose filters and analyzed by Western blot analysis as described above. Fractions positive for p40 were pooled and lyophilized. The combined fractions were resuspended in SDS sample buffer, boiled for 3 min, and separated on a 10% SDS-polyacrylamide gel. Proteins were electrophoretically transferred to polyvinylidene difluoride membranes (Bio-Rad) as described above for nitrocellulose membranes. The membranes were stained for 5 min with 0.1% Coomassie Brilliant Blue in 50% methanol, followed by destaining with 50% methanol and 10% acetic acid. A slice of filter containing p40 was subjected to Edman degradation using an Applied Biosystems (Foster City, CA) sequencer, model 470A.

RESULTS

Identification of a Unique Protein Band in Immunoprecipitates of MARCKS—These experiments were initially designed to identify novel proteins associated with MARCKS and to determine whether or not the association of these proteins was regulated by PKC-mediated phosphorylation of MARCKS. We therefore analyzed radiolabeled proteins potentially associated with MARCKS after immunoprecipitation of whole cell lysates using MARCKS-specific antibodies. For most of these studies a monoclonal antibody, 2F12, was used. This was generated against purified, recombinant human MARCKS and its epitope has been mapped to the carboxyl terminus of MARCKS.2

Human fibroblasts, serum starved and radiolabeled overnight with [35S]cysteine, were stimulated with Me2SO or PMA for 10 min followed by immunoprecipitation of cell lysates under low-stringency conditions. A radiolabeled protein band corresponding to MARCKS was present in lysates from both Me2SO- and PMA-stimulated cells, and was specifically immunoprecipitated by 2F12 and not by a control monoclonal antibody, 6F6 (Fig. 1). In addition, 2F12 specifically immunoprecipitated a [35S]-labeled protein of approximately 40 kDa (p40) that was present in lysates from Me2SO-treated cells but absent in lysates from PMA-stimulated cells. In analogous experiments using polyclonal antibodies generated against a peptide representing the first 15 amino acids of MARCKS (22), MARCKS was detected in the immunoprecipitates from both PMA-stimulated and control cells. However, in contrast to the data shown in Fig. 1 using the carboxyl-terminal specific antibody, no band migrating at 40 kDa was detected (data not shown).

p40 Is the Carboxyl-terminal Fragment of MARCKS—We performed several experiments to determine whether p40 was a MARCKS-associated protein or a carboxyl-terminal fragment of MARCKS. MARCKS lacks methionines in the mature protein since the initiator methionine is processed off during cotranslational myristoylation of the amino terminus. HFF were therefore serum-starved and radiolabeled with [35S]methionine overnight, followed by Me2SO and PMA treatment. Immunoprecipitation of these lysates with 2F12 did not result in the detection of a p40 (Fig. 2A, lanes 1–4), despite immunoprecipitation of p40 after [35S]cysteine labeling in a parallel experiment (Fig. 2A, lanes 5–8). The lack of p40 labeling by [35S]methionine supported the possibility that p40 was a carboxyl-terminal fragment of MARCKS or a MARCKS-like protein and argued against p40 being a MARCKS-associated protein.

To further confirm that p40 was a fragment of MARCKS or a MARCKS-like protein we took advantage of the heat stability of MARCKS. In the event that an associated protein was itself heat stable, it seemed unlikely that the putative association of this protein with MARCKS would withstand boiling. MARCKS-containing lysates from [35S]cysteine-labeled HFF were boiled for 10 min followed by precipitation of the denatured proteins. MARCKS remains soluble and immunoreactive following this procedure. Fig. 2B shows an autoradiograph of proteins present in immunoprecipitates of boiled and cleared lysates. Boiling removed most of the nonspecific bands detected in immunoprecipitates of non-boiled lysates. Full-length MARCKS was readily detected in immunoprecipitates from Me2SO- and PMA-treated cells. In addition, a [35S]-labeled protein migrating similarly to p40 was seen in the immunoprecipitates of lysates from Me2SO-treated but not PMA-treated cells. The presence of p40 in the immunoprecipitates of boiled lysates supports the possibility that this protein is a fragment of MARCKS or a MARCKS-like protein, rather than an associated protein.

To determine whether the presence of p40 in 2F12 immunoprecipitates was dependent upon the presence of MARCKS in the lysates, cells lacking MARCKS were tested for the presence of p40. LM/TK+ fibroblasts, a mouse cell line that lacks endogenous MARCKS, were stably transfected with either vector alone or a plasmid expressing full-length bovine MARCKS, which cross-reacts with the 2F12 antibody.3 Immunoprecipita-

Fig. 1. Immunoprecipitation of [35S]cysteine-labeled proteins from control and PMA-stimulated human fibroblasts. Human fibroblasts labeled overnight with [35S]cysteine, followed by stimulation for 10 min with 0.01% Me2SO (lanes 1 and 3) or PMA (1.6 μM in 0.01% Me2SO; lanes 2 and 4) were subjected to immunoprecipitation with control (6F6) or MARCKS carboxyl-terminal specific (2F12) antibodies. Cell extracts were preblocked with 6F6, then immunoprecipitated with 6F6 (lanes 1 and 2) or 2F12 (lanes 3 and 4). Labeled proteins in immunoprecipitates were analyzed by SDS-PAGE followed by fluorography. The arrows indicate MARCKS (top) and p40 (bottom). The positions of protein molecular weight standards are indicated.

2 P. J. Blackshear, unpublished observations.
Phosphorylation-regulated Proteolysis of MARCKS

Human fibroblasts were labeled overnight with [35S]cysteine and then stimulated with Me2SO (DMSO) or PMA as described in the legend to Fig. 1. Detergent extracts of the radiolabeled cells were boiled prior to clarification. The clarified supernatants were subjected to immunoprecipitation with a polyclonal antibody generated to the amino-terminal portion of MARCKS. Immunoprecipitations were performed in the absence (−) or presence (+) of competing peptide. Proteins present in immunoprecipitates were analyzed by SDS-PAGE followed by fluorography. The arrow indicates MARCKS and the bracket indicates the amino-terminal fragment of MARCKS.

These findings indicated that p40 was probably a carboxy-terminal fragment of MARCKS.

We also wished to determine if a corresponding amino-terminal fragment of MARCKS could be detected whose formation was prevented by activation of PKC. Only three cysteines are present in human MARCKS and these all reside very close to the carboxy terminus; therefore, an amino-terminal fragment would not be detected following [35S]cysteine labeling of cells and immunoprecipitation with the amino-terminal specific antibody. However, when the cells were radiolabeled with [14C]alanine, full-length MARCKS as well as a smaller protein were precipitated with the amino-terminal antibody (Fig. 3). This smaller protein or protein fragment was present only in unstimulated cells and not in PMA-stimulated cells. This smaller fragment migrated as a diffuse band of Mr ~44,000. When similar immunoprecipitations were done in the presence of competing peptide, both intact MARCKS and the entire diffuse band were competed by the peptide (Fig. 3). In addition, this same diffuse band was detected when HFF were labeled with [3H]myristate and the lysates subjected to immunoprecipitation with the amino-terminal MARCKS antibody (data not shown). These data indicate that the diffuse band at Mr ~44,000 is an amino-terminal fragment of MARCKS, whose formation is also prevented by PMA-stimulation of the cells (Fig. 3). The sum of the Mr of both the amino-terminal and carboxy-terminal fragments add up to the apparent Mr of full-length MARCKS. Interestingly, both fragments migrated anomalously on SDS-polyacrylamide gels, as does full-length MARCKS.

Physiological Agonists of PKC also Cause the Disappearance of p40—To establish that the disappearance of p40 in the immunoprecipitates from PMA-stimulated cells was not an artifact of nonphysiological activation of PKC by this phorbol ester, HFF were stimulated with 10% fetal calf serum as a source of physiological growth factors (Fig. 4). Both full-length MARCKS and p40 were detected in similar amounts in immunoprecipitates of lysates from from either untreated cells or cells treated with Me2SO, but only full-length MARCKS was detected in cells stimulated with serum between 15 s and 2 min. p40 began to reappear 5–10 min following serum addition (Fig. 4). The identity of the band appearing at 5 min as p40 was confirmed by competition experiments with the epitope-specific

![Evidence that p40 is a fragment of MARCKS](image_url)

**Fig. 2.** Evidence that p40 is a fragment of MARCKS. A, human fibroblasts were labeled overnight with either [35S]methionine (lanes 1–4) or [35S]cysteine (lanes 5–8), stimulated with Me2SO (lanes 1, 2, 5, and 6) or PMA (lanes 3, 4, 7, and 8), and then subjected to immunoprecipitation with control (6F6; lanes 1, 3, 5, and 7) or anti-MARCKS (2F12; lanes 2, 4, 6, and 8) antibodies as described in the legend to Fig. 1. The arrows point to intact MARCKS (top) and p40 (bottom). B, human fibroblasts were labeled overnight with [35S]cysteine and then were stimulated with Me2SO (D) or PMA (P) as described in the legend for Fig. 1. Extracts of the radiolabeled cells were boiled prior to clarification, and the clarified supernatants were subjected to immunoprecipitation with 2F12 (anti-MARCKS) monoclonal antibodies. The arrows indicate intact MARCKS (top) and p40 (bottom). C, control LM/TK cells expressing vector alone (vector) and LM/TK cells heterologously expressing bovine MARCKS (p80) were labeled overnight with [35S]cysteine. Boiled and clarified cell extracts from unstimulated cells were subjected to immunoprecipitation with 2F12 monoclonal antibodies. Also shown is an immunoprecipitation using 2F12 of boiled and clarified lysate from unstimulated HFF. The arrows indicate intact MARCKS (top) and p40 (bottom).
peptide (data not shown). The disappearance of p40 thus occurs in response to physiological activators of PKC as well as with PMA.

p40 Is Generated by Specific Cleavage of Endogenous MARCKS in Cell Lysates—We next assayed for the presence of a cellular protease that could cleave endogenous MARCKS and generate p40. Lysates were prepared from control and PMA-treated [35S]cysteine-labeled cells in the presence or absence of protease inhibitors; the lysates were then incubated at 30 °C for varying times. The reactions were stopped by boiling the samples, and equal amounts of trichloroacetic acid precipitable radioactive activity were subjected to immunoprecipitation with 2F12. Almost all full-length MARCKS had disappeared after 20 min of incubation of lysates prepared from control cells and incubated in the absence of protease inhibitors (Fig. 5A, lanes 1–3). There was a concomitant increase in the amount of radiolabeled p40 in the lysates. In contrast, no changes in the amounts of full-length MARCKS or p40 were detected in lysates prepared from control cells incubated in the presence of protease inhibitors at 30 °C (Fig. 5A, lanes 4–6). In fact, the disappearance of full-length MARCKS and the parallel increase in p40 occurred almost immediately in the absence of protease inhibitors. When the same analysis was performed on lysates prepared in the absence of protease inhibitors from cells treated with PMA, a decrease in full-length MARCKS and an increase in p40 were also seen (Fig. 5A, lanes 7–9). However, in contrast to the results from the control cell lysates, complete loss of full-length MARCKS did not occur. As seen with the control cell lysates, the protease inhibitors prevented the decrease in full-length MARCKS and the increase in p40 that occurred upon incubating the lysates from PMA-treated cells at 30 °C in the absence of protease inhibitors (Fig. 5A, lanes 10–12). Taken together, these data suggest that p40 is being generated from MARCKS by cellular protease action.

The incomplete loss of full-length MARCKS in lysates from PMA-treated cells suggested that PKC activation partially protected MARCKS from proteolysis either through MARCKS phosphorylation or inhibition of the responsible protease. To determine which of these possibilities pertained, we prepared lysates without protease inhibitors but with sodium fluoride, a serine/threonine phosphatase inhibitor. As seen in Fig. 5B, incubation of lysates from PMA-stimulated cells in the presence of sodium fluoride inhibited the normal loss of MARCKS and generation of p40. Sodium fluoride had no effect on the generation of p40 in lysates from control cells. These data further support the possibility that a PKC-dependent phosphorylation event inhibits the generation of p40 and the concurrent decrease in MARCKS, but still do not distinguish between phosphorylation of MARCKS or inhibition of the responsible protease.

The Activity Present in HFF Can Cleave Exogenously Added MARCKS and Generate p40—To further confirm that p40 was being generated from MARCKS, non-radiolabeled cell lysates were then assayed for their ability to produce p40 from an exogenous MARCKS substrate. For these experiments, full-length [35S]cysteine-labeled bovine MARCKS was translated in a reticulocyte lysate system, then incubated with cell lysates. When in vitro translated proteins were incubated with buffer alone and then subjected to SDS-PAGE, only one radiolabeled protein, full-length MARCKS, was detected (Fig. 6A). When in vitro translated MARCKS was incubated with non-radioactive lysates from either control or PMA-treated cells, a protein that co-migrated with p40 was generated (Fig. 6A). This generation of p40 occurred whether or not MARCKS was myristoylated during its translation, as demonstrated with a mutant MARCKS containing a Gly2→Ala2 change that prevents myristoylation (Fig. 6A). For this reason, the nonmyristoylated protein was used in some of the following experiments on in vitro translated proteins.

As an additional test of whether the activity responsible for cleaving MARCKS might itself be regulated by PKC-dependent phosphorylation, lysates were prepared from control or PMA-stimulated cells in the presence or absence of NaF, and then assayed for their ability to cleave in vitro translated MARCKS. NaF had no effect on the ability of the cell lysates to cleave MARCKS (data not shown). Taken together, the data suggest that the protease activity is not regulated by PKC-dependent phosphorylation.

Aspartate Replacement of Serines in the PSD of MARCKS Prevents the Generation of p40—The above experiments suggest that activation of PKC and phosphorylation of MARCKS can inhibit the cleavage of MARCKS and the generation of p40. To confirm that the phosphorylation of MARCKS could prevent this specific cleavage, in vitro translated MARCKS containing mutations in the PSD was incubated with cell lysates. Wild-type MARCKS, or mutants in which the four serines in the PSD were mutated to alanine, glycine, asparagine, or aspartate, were translated in vitro and then incubated with cell lysates prepared in the absence of both protease and phosphate inhibitors. Under these conditions, p40 was generated from wild-type MARCKS, and from the Ser→Ala, Ser→Gly, and Ser→Asn mutants, but not from the Ser→Asp mutant (Fig. 6B). The replacement of serine residues by aspartic acid results in a change charge similar to that seen with the addition of phosphate groups to the serines. Alanine and glycine mutations represent relatively conservative mutations, and asparagine is similar to aspartate in size but is uncharged. The marked resistance of only the aspartate mutant to cleavage is consistent with the idea that phosphorylation of MARCKS prevents its cleavage.

Phosphorylation of MARCKS Decreases Cleavage of In Vitro Translated MARCKS—To further support the idea that phosphorylation of MARCKS prevents its cleavage by an activity present in cell lysates, in vitro translated MARCKS was subjected to phosphorylation by the catalytic fragment of PKC (PKM), followed by incubation with cell lysates. When in vitro translated MARCKS was phosphorylated by PKM prior to incubation with cell lysates, there was a decrease in the amount of p40 generated from full-length MARCKS. In a representative experiment, the percent of total radioactivity generated in p40 in the absence of PKM phosphorylation was 45 ± 2%; with prior PKM-mediated phosphorylation, the percent of total radioactivity generated in p40 was 12 ± 2% (numbers represent the mean ± S.D.; n = 3). Numbers were obtained by cutting out
bands from an SDS-PAGE gel and scintillation counting. This further supports the observation that phosphorylation of MARCKS prevents its cleavage.

A Cysteine Protease Is Responsible for MARCKS Cleavage and Generation of p40—As demonstrated above, protease inhibitors inhibited the generation of p40 from MARCKS in cell lysates. To begin to characterize the type of protease responsible for this activity, the protease inhibitors present in the mixture were analyzed individually. This was performed with MARCKS present in cell lysates as well as with in vitro translated MARCKS. When cell lysates were assayed for their ability to generate p40 from exogenous MARCKS in the presence of specific protease inhibitors, only leupeptin inhibited this protease activity (Fig. 7A). Similar results were obtained in experiments using endogenous MARCKS as a substrate (data not shown). Benzamidine-HCl, aprotinin, and phenylmethylsulfonyl fluoride inhibit serine proteases, whereas pepstatin is specific for acid proteases. Leupeptin inhibits serine proteases at concentrations of 200 \( \mu \)M, but inhibits cysteine proteases and not serine proteases at 200 nM (23). In the present experiments, leupeptin was effective at 2 \( \mu \)M, suggesting that it was probably inhibiting a cysteine protease.

To support the idea that this activity was due to a cysteine protease, the concentration dependencies of leupeptin and another cysteine protease inhibitor, antipain, were tested (Fig. 7B). Both of these protease inhibitors inhibited the p40 generating activity at concentrations compatible with the inhibition of a cysteine protease.

p40 and p40 Generating Activity Are Present in the Intact Animal—To test whether p40 exists in detectable concentrations in tissues, we performed Western blot analysis of rapidly frozen samples prepared from bovine spleen, liver, kidney, and brain using 2F12. This antibody cross-reacts with bovine MARCKS as well as human. It has previously been shown that spleen and brain express large amounts of MARCKS whereas kidney and liver express very little (17, 24). Full-length MARCKS was readily detectable in all four tissues, although to a much lesser extent in liver and kidney (Fig. 8). In addition, the spleen, liver, and kidney extracts contained an immunoreactive band migrating at approximately 40 kDa (Fig. 8). None of the other MARCKS-expressing tissues tested, including heart, testes, and muscle contained p40 (data not shown). It is important to note that four times more liver and kidney protein was analyzed than spleen and brain protein on this blot.

To establish the authenticity of p40 in adult bovine tissues, similar immunoblots were analyzed with a control monoclonal antibody; in addition, the 2F12 monoclonal was tested in the detection of full-length MARCKS and p40 in HFF extracts, and only the specific peptide competed with 2F12 in the detection of full-length MARCKS and p40. Finally, bona fide p40 that was immunoprecipitated from \(^{[35S]}\)cysteine-labeled HFF was included in the gels as a positive control. These experiments confirmed the authenticity of p40 in bovine tissue and showed that p40 in bovine tissues comigrates with p40 from immunoprecipitates of HFF (data not shown).

In other experiments, extracts from mouse tissues were analyzed for their ability to generate p40 from in vitro translated MARCKS. Spleen, liver, and kidney all contained readily de-
MARCKS were incubated with lysis buffer (lanes 1 and 4) or detergent lysates from human fibroblasts which had been treated with Me2SO (lanes 2 and 5) or PMA (lanes 3 and 6). Lanes 1–3, in vitro translated wild-type MARCKS. Lanes 4–6, in vitro translated mutant nonmyristoylated MARCKS. Radiolabeled proteins were analyzed by SDS-PAGE followed by fluorography. The arrows indicate myristoylated (top) and nonmyristoylated MARCKS (second from top) and p40 (bottom). B, translated wild-type and mutant MARCKS were incubated with lysis buffer (lanes 1, 4, 7, and 10) or extracts from human fibroblasts which had been treated with Me2SO (lanes 2, 5, 8, and 11) or PMA (lanes 3, 6, 9, and 12). Lanes 1–3, MARCKS (tetra-Asp). Lanes 4–6, MARCKS (tetra-Asn). Lanes 7–9, MARCKS (tetra-Gly). Lanes 10–12, MARCKS (tetra-Ala). The radiolabeled proteins were analyzed by SDS-PAGE followed by fluorography. Arrows indicate MARCKS (top) and p40 (bottom).

![Fig. 6. Formation of p40 from in vitro translated wild-type and mutant MARCKS.](image)

Arrows indicate MARCKS (top) and p40 (bottom). Radiolabeled proteins were analyzed by SDS-PAGE followed by fluorography. The arrows indicate myristoylated (top) and nonmyristoylated MARCKS (second from top) and p40 (bottom). B, translated wild-type and mutant MARCKS were incubated with lysis buffer (lanes 1, 4, 7, and 10) or extracts from human fibroblasts which had been treated with Me2SO (lanes 2, 5, 8, and 11) or PMA (lanes 3, 6, 9, and 12). Lanes 1–3, MARCKS (tetra-Asp). Lanes 4–6, MARCKS (tetra-Asn). Lanes 7–9, MARCKS (tetra-Gly). Lanes 10–12, MARCKS (tetra-Ala). The radiolabeled proteins were analyzed by SDS-PAGE followed by fluorography. Arrows indicate MARCKS (top) and p40 (bottom).

![Fig. 7. The generation of p40 from MARCKS is inhibited by cysteine protease inhibitors.](image)

A, radiolabeled in vitro translated nonmyristoylated MARCKS was incubated at 30°C for 30 min with extracts from human fibroblasts in the absence or presence of specific protease inhibitors. Lane 1, no inhibitors; 2, 200 nM aprotinin; 3, 1 mM benzamidine-HCl; 4, 2 μM leupeptin; 5, 1 μM pepstatin; 6, 574 μM phenylmethylsulfonyl fluoride. Radiolabeled proteins were analyzed by SDS-PAGE followed by fluorography. Arrows indicate MARCKS (top) and p40 (bottom). B, radiolabeled in vitro translated nonmyristoylated MARCKS was incubated at 30°C for 30 min with extracts from human fibroblasts in the presence of increasing amounts of leupeptin (lane 1, 0.01 μM; 2, 0.05 μM; 3, 0.1 μM; 4, 0.5 μM; 5, 1 μM; 6, 2 μM) or antipain (7, 17 nM; 8, 170 nM; 9, 17 μM). Radiolabeled proteins were analyzed by SDS-PAGE followed by fluorography. Arrows indicate MARCKS (top) and p40 (bottom).

![Fig. 8. p40 is present in rapidly frozen bovine tissues.](image)

One hundred μg (brain and spleen) or 400 μg (liver and kidney) of protein were separated by SDS-PAGE followed by transfer to nitrocellulose. The filters were probed with 2F12 monoclonal antibody followed by horseradish peroxidase-conjugated secondary antibody. Immunoreactive bands were visualized by enhanced chemiluminescence. Arrows indicate MARCKS (top) and p40 (bottom).
The importance of the PSD in regulating this interaction was further analyzed by testing the ability of synthetic peptides representing the PSD to inhibit the MARCKS protease. Wild-type peptide or peptides containing all four serines mutated to alanine or glycine partially inhibited this activity when added at concentrations of 100 μM to in vitro translated MARCKS. In contrast, similar concentrations of the peptide containing aspartic acid residues in place of the serines demonstrated no inhibitory effect on this activity, thus supporting the importance of the charged state of the PSD in regulating interactions with this protease (data not shown).

The alignment of MARCKS protein sequences in this region from different animal species is shown in Fig. 9. The sequence is identical among the species shown from Glu348 in the bovine sequence through the PSD. The human and bovine sequences, as well as that of Xenopus laevis, are identical at the cleavage site. However, the chicken, mouse, and rat sequences contain a serine in place of asparagine at the amino side of the cleavage site. Serine and asparagine are similar in that they are both polar, uncharged amino acids. It is therefore possible that they could substitute for each other at this cleavage site. The presence of the proteolytic activity in mouse tissues supports the possibility that mouse MARCKS is also a substrate for this activity. However, mutational analysis of the cleavage site will be necessary to establish whether Ser-Glu as well as Asn-Glu are recognized by this cellular protease.

**DISCUSSION**

This study shows that, in some cells and tissues, intact MARCKS co-exists with reasonably high concentrations of two cleavage products, each comprising about half of the intact protein sequence. The concentration of p40 ranges from undetectable in bovine brain to several times the full-length protein sequence. The concentration of p40 increases in bovine spleen, as determined by Western blotting. These cleavage products are formed by the action of a widely distributed, still uncharacterized cellular protease, apparently of the cysteine protease class. The rate of MARCKS cleavage by this protease activity is dependent upon its phosphorylation state, that is, phosphorylated MARCKS is a poor substrate for the protease whereas dephosphorylated MARCKS is a good substrate. This finding implied that the proteolytic cleavage site was at or near the PSD; direct amino acid sequencing of one of the cleavage products revealed a cleavage site between asparagine 147 and glutamate 148 of the bovine sequence, only three amino acids amino-terminal of the PSD. These data thus provide evidence for a novel means by which PKC can regulate MARCKS concentrations in the cell. They also suggest that MARCKS interacts physically with the responsible protease, and that the PSD represents an important component of the interaction.

That phosphorylation of a protein can affect its susceptibility to proteolytic cleavage has been described previously. For example, Elvira et al. (25) demonstrated that phosphorylation of connexin-32 by PKC, but not by the cAMP-dependent protein kinase, protected the protein from calpain-mediated proteolysis. Chen and Stracher (26) showed, both in intact cells and in a cell-free system, that actin-binding protein of human platelets in its phosphorylated form was a poorer substrate for calpain-mediated proteolysis than its dephosphorylated form.

Finally, protein kinase A-directed phosphorylation of the microtubule-associated proteins MAP-2 and tau protected these two proteins from calpain-mediated proteolysis, at least in a cell-free assay (27, 28). Phosphorylation may also make a protein more susceptible to proteolytic cleavage. For example, Warnen and Petryshn (29) demonstrated that phosphorylated nucleolin was more susceptible to a copurifying protease than the dephosphorylated form. Another example is IκB, the inhibitor of the transcription factor NF-κB (30). IκB in its unphosphorylated state binds to NF-κB and keeps it inactive and in the cytoplasm. Upon cellular stimulation, including PKC activation, IκB is phosphorylated and proteolized, releasing NF-κB and permitting its movement to the nucleus where it is active as a transcription factor (31–33).

The turnover of MARCKS with respect to PKC activation has been addressed previously. Brooks et al. (14, 15) demonstrated in Swiss 3T3 cells that long exposure (5 h) to phorbol ester and bombesin, both of which activate PKC in these cells, resulted in down-regulation of MARCKS mRNA and protein levels. At times less than 5 h, a modest increase in MARCKS protein levels was observed, compatible with the present findings. However, these authors also noted that the down-regulation of MARCKS occurred following activation of protein kinase A, implying a completely different mechanism. In another study, Lindner et al. (16) described a rapid decrease in MARCKS protein levels in Swiss 3T3 cells that occurred within 15 min of PMA treatment. We cannot account for the discrepancies between their data and those of Brooks et al. (14, 15), or between their data and those reported here, other than that human fibroblasts were used in the present studies rather than Swiss 3T3 cells.

Our data show that when equal amounts of trichloroacetic acid precipitable counts were immunoprecipitated from boiled lysates from Me5SO- or PMA-stimulated cells (see for example, Figs. 2B, 3, and 4, and data not shown), the amount of full-length MARCKS in the immunoprecipitates was always slightly greater in lysates from PMA-stimulated cells compared to control lysates. This observation is consistent with phosphorylation of MARCKS preventing its ongoing cleavage, so that the level of full-length MARCKS is greater in stimulated cells than in unstimulated cells. The question remains, what happens to the p40 and its amino-terminal counterpart that are present in the cells prior to PKC activation and disappear within 15 s of PKC activation? There are at least three potential explanations for the rapid disappearance of these fragments. First, activation of PKC might cause the tight association of these fragments with an Nonidet P-40-insoluble cytoskeletal fraction. To address this possibility, cell lysates were prepared and centrifuged without boiling. The insoluble material was then solubilized in 0.5 M NaCl, followed by dilution to salt and detergent conditions appropriate for immunoprecipitation. Neither MARCKS nor p40 was detected in the immunoprecipitates from this fraction, from either control or PMA-stimulated cells (data not shown). Second, activation of PKC might stimulate the secretion of these fragments. To address this, media from [35S]cysteine-labeled HFF stimulated with Me5SO or PMA were subjected to immunoprecipitation. Antibody 2F12 detected a secreted protein that comigrated with p40 and was competed for antibody binding by the epitope.

---

3 Y. Shi and P. J. Blackshear, unpublished observations.
peptide. However, this protein does not appear to be a fragment of MARCKS because 1) its presence in the medium was not increased by PMA treatment of the cells, and 2) it was also detected in medium from cells lacking MARCKS. Finally, p40 and its amino-terminal counterpart might be subjected to rapid proteolysis following PKC activation. We addressed this issue by analyzing lysates prepared from cells stimulated for less than 1 min with PMA or serum, in which lysates from both [35S]cysteine- and [3H]alanine-labeled cells were immunoprecipitated with 2F12 or the amino-terminal-specific antibody. Even when the immunoprecipitates were analyzed on 15% acrylamide gels, no lower molecular weight immunoreactive fragments were detected. However, it is possible that smaller fragments would remain undetected if rapid proteolysis were occurring following PKC activation. We favor, but cannot prove, this final explanation for the rapid disappearance of p40 and the amino-terminal fragment that occurs following PKC activation. To address this question, it will be necessary to perform studies in which p40 is expressed in cells totally lacking MARCKS, or lacking the forms that interact with the 2F12 antibody; the fate of p40 in response to PMA can then be studied independently of the rate of its formation from intact MARCKS.

GAP-43 (also known as B-50 and neuromodulin), a neuron-specific protein that is highly enriched in growth cones and nerve terminals, resembles MARCKS in that it is a heat-stable substrate for PKC, it is fatty acylated, and it binds calmodulin in a PKC-regulated fashion (34). Coggins and Zwiers (35) have described the susceptibility of this protein to specific proteolytic cleavage at or around the single PKC target serine, Ser41. They demonstrated that GAP-43, in either its phosphorylated or dephosphorylated form, can be cleaved by α-chymotrypsin, and this suggested that this cleavage occurred at Phe32. They also demonstrated the existence of a protease that co-purifies with GAP-43, which presumably cleaves the protein at Ser41. In a previous paper (36) they suggested that the co-purifying protease was sensitive to cysteine-type inhibitors. These authors also demonstrated that binding of calmodulin to the unphosphorylated GAP-43 protects it from cleavage by the copurifying protease (36). To date, we have seen no effect of calmodulin to inhibit the proteolytic cleavage of MARCKS. The facts that the co-purifying protease cleaves GAP-43 in either its phosphorylated or unphosphorylated forms, and that this is inhibited by calmodulin, suggest a completely different level of regulation from that observed with the MARCKS protease described here.

Recently, Allen and Aderem (37) described the PKC-mediated cycling of MARCKS between the plasma membrane and lysosomes in mouse fibroblasts. Studies from several laboratories have established that dephosphorylated MARCKS is associated with the plasma membrane through both the PSD and the myristoylated amino terminus (19, 38–43); upon PKC-mediated phosphorylation, membrane association through the PSD is inhibited (42, 44–47). In this paper (37), the authors demonstrate co-localization of phosphorylated MARCKS with a lysosome-specific marker following PMA stimulation of mouse embryo fibroblasts. Preliminary evaluation of the protease described here suggests that it is associated with the particulate fraction of the cell and is active at acidic pH, both characteristics of a lysosomal enzyme. However, our studies indicate that phosphorylated MARCKS is not a substrate for this protease. Therefore, movement of phosphorylated MARCKS to the lysosome may represent an alternative mechanism of MARCKS regulation to the proteolytic cleavage we describe here; alternatively, movement to the lysosomes may be followed first by dephosphorylation and then by the specific proteolytic cleavage described here.

The presence of a proteolytic activity in extracts of multiple tissues from mouse, human, and cow that is capable of generating p40 from MARCKS demonstrates that this protease is widely distributed. However, there are significant, tissue-specific differences in the apparent activity of this protease. For example, both the protease activity and p40 are essentially absent in brain, a tissue that expresses the highest levels of MARCKS (17, 24). On the other hand, liver and kidney, which express low levels of MARCKS (17, 24), express the protease and contain readily detectable amounts of p40. Whether the protease is involved in regulating MARCKS levels, or whether MARCKS is involved in regulating the activity of the protease, are questions that remain to be answered.

Preliminary characterization of the MARCKS protease suggests that it is a cysteine protease. One well known cysteine protease that is important in PKC regulation is calpain (48–50). Calpain is a cytosolic protein that can be translocated to the membrane upon activation of PKC (51). It has an absolute requirement for calcium and consists of two forms distinguished by their micromolar or millimolar calcium requirements. Calpain is also under the regulation of an endogenous protein inhibitor, calpastatin. In addition, many calpain substrates also appear to be calmodulin binding proteins (52). For these reasons, we considered that calpain might be a good candidate for the MARCKS protease described here. However, this activity shows no calcium dependence, and thus is unlikely to be calpain. Further studies are in progress to characterize and identify this protease.

Amino-terminal sequencing of p40 from bovine spleen established the site of cleavage to be between an asparagine and glutamate, three amino acids upstream of the PSD. These residues are identical in the human and Xenopus MARCKS sequence and contain a serine in place of the asparagine in the rat, chicken, and mouse sequences. Although a search of the literature did not reveal a previously identified cellular cysteine protease with specificity for the dipeptide Asn/Ser-Glu, there are a few examples of asparagine-specific proteases. These include an asparagine-specific cysteine protease that is involved in the post-translational modification of the plant storage protein, glycamin. This protease recognizes an Asn-Gly linkage (53). In addition, the metalloproteinase stromelysin recognizes an Asp-Ph linkage in pig cartilage proteoglycan. Human and rat proteoglycan also contain this sequence, but the bovine sequence contains a serine in place of the asparagine (54). This suggests that, like the protease described here, stromelysin may be able to accept an Asn→Ser change and still cleave its substrate. Experiments are in progress to analyze the ability of the MARCKS protease to recognize a Ser-Glu site in place of the Asn-Glu site.

Acknowledgments—We thank Dr. Deborah J. Stumpo and Sharon Swierczynski for stable LM/TK MARCKS-expressing clones and bovine cDNA constructs, Judith Phelps for peptide sequence analysis, and Jay Murray for bovine tissue retrieval. We are grateful to Elizabeth Kennington for help with the p40 purification, and to all members of the Blackshear laboratory for helpful discussions.

REFERENCES

1. Aderem, A. (1992) Cell 71, 713–716
2. Blackshear, P. J. (1993) J. Biol. Chem. 268, 1501–1504
3. Graff, J. M., Young, T. N., Johnson, J. D., and Blackshear, P. J. (1989) J. Biol. Chem. 264, 21818–21823
4. Hartwig, J. H., Thelen, M., Rosen, A., Janney, P. A., Nairn, A. C., and Aderem, A. (1992) Nature 356, 618–622
5. Stumpo, D. J., Bock, C. B., Tuttle, J. S., and Blackshear, P. J. (1995) Proc. Natl. Acad. Sci. U.S.A. 92, 944–948
6. Thelen, M., Rosen, A., Nairn, A. C., and Aderem, A. (1990) Proc. Natl. Acad. Sci. U.S.A. 87, 5603–5607
7. Seykora, J. T., Ravetch, J. V., and Aderem, A. (1991) Proc. Natl. Acad. Sci. U.S.A. 88, 2505–2509
8. Harlan, D. M., Graft, J. M., Stumpo, D. J., Eddy, R. L., Jones, T. B., Boyle, J. M., and Blackshear, P. J. (1991) J. Biol. Chem. 266, 14399–14405
9. Wolfman, A., Wingrove, T. G., Blackshear, P. J., and Macara, I. G. (1987) J. Biol. Chem. 262, 16546–16552.
10. Reed, J. C., Rapp, U., and Cuddy, M. P. (1991) Cell. Signalling 3, 569–576.
11. Joseph, C. K., Oureshi, S. A., Wallace, D. J., and Foster, D. A. (1992) J. Biol. Chem. 267, 1327–1330.
12. Wojtaszek, P. A., Stumpo, D. J., Blackshear, P. J., and Macara, I. G. (1993) Oncogene 8, 735–760.
13. Otsuka, M., and Yang, H. C. (1991) Biochem. Biophys. Res. Commun. 178, 494–500.
14. Brooks, S. F., Herget, T., Erusalimsky, J. D., and Rozengurt, E. (1991) EMBO J. 10, 2497–2505.
15. Brooks, S. F., Herget, T., Broad, S., and Rozengurt, E. (1992) J. Biol. Chem. 267, 14212–14218.
16. Lindner, D., Gschwendt, M., and Marks, F. (1992) J. Biol. Chem. 267, 24–26.
17. Stumpo, D. J., Graff, J. M., Albert, K. A., Greengard, P., and Blackshear, P. J. (1989) Proc. Natl. Acad. Sci. U. S. A. 86, 4012–4016.
18. Gunning, P., Leavitt, J., Muscat, G., Ng, S. Y., and Kedes, L. (1987) Proc. Natl. Acad. Sci. U. S. A. 84, 4831–4835.
19. Swierczynski, S. L., and Blackshear, P. J. (1995) J. Biol. Chem. 270, 13436–13445.
20. Graff, J. M., Rajan, R. R., Randall, R. R., Nairn, A. C., and Blackshear, P. J. (1991) J. Biol. Chem. 266, 14390–14398.
21. Verghese, G. M., Johnson, J. D., Vasulka, C., Haupt, D. M., Stumpo, D. J., and Blackshear, P. J. (1994) J. Biol. Chem. 269, 9361–9367.
22. Lobaugh, L. A., and Blackshear, P. J. (1989) J. Biol. Chem. 265, 15277–15284.
23. Johnson, G. V. W., and Foley, V. G. (1993) J. Neurosci. Res. 34, 642–647.
24. Litersky, J. M., and Johnson, G. V. W. (1992) J. Biol. Chem. 267, 1563–1568.
25. Warrener, P., and Petryshyn, R. (1991) Biochem. Biophys. Res. Commun. 180, 716–723.
26. Baeuerle, P. A., and Baltimore, D. (1988) Science 242, 540–546.
27. Grimm, S., and Baeuerle, P. A. (1993) Biochem. J. 290, 297–308.
