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Identification and Characterization of MARCKS in *Xenopus laevis*[1]

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MARCKS proteins are widely distributed in mammalian cells and subserve an important role as probes in the examination of signal transduction processes because they are specific endogenous phosphoreceptors for activated protein kinase C. Experiments were performed to determine whether MARCKS proteins are present in amphibia and to show their usefulness as substrates for stimulated PKC activation, using cultured renal epithelial cells (A6) derived from *Xenopus laevis* as an experimental model. © 1997 Academic Press

MARCKS was identified in the A6 cell as a soluble 80kDa protein in 40% acetic acid which exhibited increased phosphorylation after exposure to PMA and yielded characteristic 'fingerprint' phosphopeptides after exposure to S. aureus protease. In addition, a protein with the same characteristics was immunoprecipitated with antisera raised against bovine MARCKS. Further, phosphorylation of this MARCKS protein correlated with endogenous changes in PKC activation induced with a physiological agonist. These results demonstrate that MARCKS is present in amphibia and suggest that it may also exist in other lower vertebrates used for experimental studies.

Myristoylated, alanine-rich C-kinase substrate (MARCKS) is widely distributed among mammalian cells and plays a central role in the regulation of the cytoskeletal dependent processes, such as locomotion, secretion and phagocytosis. When bound to calmodulin, MARCKS tethers long filaments of F-actin to plasma membrane, while after phosphorylation by protein kinase C (PKC) the binding of calmodulin is disrupted and MARCKS translocates to a cystolic site releasing bound F-actin filaments. This functional property which links the calcium-calmodulin and the PKC signal transduction pathways, permits the regulation of dynamic changes in the cytoskeleton (1).

MARCKS also is a specific substrate for all classes of PKC isoforms, with the exception of the ζ-isoform (2). It therefore, serves as an important experimental probe to identify endogenous activation of PKC, as well as in in-vitro assays (3). Its usefulness in the identification of PKC activation, however, has been confined to mammalian cells and the fowl (1), despite the extensive use of lower vertebrates and non-vertebrates as models for biochemical processes. The present study was performed to examine whether MARCKS is present in renal epithelial cells derived from Xenopus laevis. This amphibian species has important implications in many types of experimentation, such as expression of various proteins in the oocyte to study functional properties and use of cultured renal epithelial cells as an experimental model for electrogenic sodium transport.

EXPERIMENTAL PROCEDURES

Materials. Phorbol 12-myristate 13-acetate (PMA), vasopressin dimethyl sulfoxide (DMSO), protein A Sepharose-CL4B and protease inhibitors were obtained from Sigma (St. Louis, MO). ^{32}[P]-orthophosphoric acid was supplied by Amersham Inc. (Arlington Heights, IL). Rabbit antiserum raised against a purified bovine brain MARCKS protein was kindly provided by Dr. Angus Nairn, Rockefeller University. All other reagents were of highest grade available.

Cell culture. Experiments were performed on a clone of A6 cells (A6S2) derived from *Xenopus laevis.* The methods employed for cell culture have been described elsewhere (4,5). Briefly, experiments were performed on cells grown to confluence on porous Falcon membranes (Beckton-Dickenson, Bedford, MA) with an active surface area of 4.5 cm^2 after 10-14 days of subculture at 26°C, when these cells form a monolayer of fully differentiated epithelial cells with maximum electrical resistance.

^{32}P-labeling and incubations. Cells were washed twice with a phosphate free amphibian Kreb's Ringer buffer (110 mM NaCl, 3 mM KCl, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$, 5 mM glucose and 10 mM HEPES, pH 7.4) and incubated with 100 μCi/ml $^{32}P_i$ (specific activity, ~200 mCi/mmol) for 3 h at 26°C. Previous studies have shown that

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^{32}P, added in trace amounts, achieves equilibrium with the cellular pool of ATP within 2 hr (6).

Following labeling, cells were washed with serum-free culture medium three times and incubated with PMA or agonists as indicated in the figure legends. Reactions were terminated by replacing the medium with 0.8 ml ice-cold lysis buffer (10 mM Tris, 10 mM NaCl, 20 mM NaF, 20 mM $NaPP_i$, 1 mM $ZnSO_4$, 0.5% Triton X100, 0.5 mg/ml saponin, pH 7.4) containing 2 mM AEBSF, 125 μM leupeptin, 100 μM chymostatin, and 25 μg/ml aprotinin. Cells were kept on ice for 5 min, scraped off with a rubber policeman and centrifuged for 1 min in a microcentrifuge. Supernatants were used to extract MARCKS protein either by acetic acid extraction or by immunoprecipitation with anti-MARCKS antibody.

Acid extraction of the MARCKS protein. MARCKS protein from A6 cell lysates was extracted by a slight modification of an acetic acid extraction procedure (7). Briefly, cell lysates were freed of nuclei and cell debris by centrifugation and boiled for 5 min, and total proteins were then extracted by the chloroform and methanol precipitation procedure of Wessel and Flugge (8). Total proteins were dissolved in SDS sample buffer (100 mM Tris-HCl, pH 6.8, 2% SDS, 25 mM 2-mercaptoethanol, 10% glycerol and 0.07% bromophenol blue) and boiled again for 5 min. Samples were cooled down to room temperature and an equal volume of 80% ice-cold acetic acid was added to each sample to obtain a final concentration of 40%. After 30 min on ice, acid soluble proteins were separated by centrifugation at 4°C (12,000g, 2 min). Acetic acid was removed by 3-4 cycles of drying and resuspension in H_2O in a speed vacuum evaporator until the original bromophenol blue color reappeared. Samples were then boiled again and subjected to SDS-PAGE and autoradiography.

Immunoprecipitation of the MARCKS protein. Aliquots (300 μl) of A6 cell lysates were pre-cleared by incubation with 50 μl of a 10% suspension of Pansorbin cells (Calbiochem) for 1 hr at 4°C. Pansorbin cells were removed by centrifugation. Lysates were then incubated overnight at 4°C with a 1:25 dilution of anti-MARCKS antiserum, raised to the bovine brain form of the protein (9). Protein A conjugated Sepharose-CL4B beads (prewashed, 2 mg/ 300 μl) were then added and incubations were continued for an additional hour. After aspiration of unbound protein, pelleted beads were washed at 4°C as follows; 1× 1 ml antibody incubation buffer; 2× 1 ml PBS, pH 8.0, containing 300 mM NaCl; 2 × 1 ml PBS, pH 8.0. Finally, beads were suspended in SDS sample buffer and boiled for 5 min. Samples were centrifuged and supernatant was used for SDS-PAGE and autoradiography.

SDS-PAGE, immunoblotting, and autoradiography. Slab gel electrophoresis was performed at constant current (30 mA) for approximately 5h using a Laemmli buffer system. Gels were stained with either Coomassie blue or silver stain, dried and autoradiographed to visualize ^{32}P-labeled proteins. For immunoblotting, gels were electrophoretically transferred onto nitrocellulose membranes using BioRad wet transfer apparatus and Towbin buffer system. Nitrocellulose membranes were stained with Ponceau Red to visualize the transferred proteins. This stain was washed off by excessive washings with distilled water and did not interfere with the subsequent immunostaining procedure. For immunostaining with MARCKS antibody, blots were incubated as follows: 5% skimmed milk in PBST (10 mM phosphate buffered saline, pH 7.4 and 0.1% Tween 20), 1h; MARCKS protein antiserum (1 : 200 dilution) in 1% skimmed milk and PBST, 3h; PBST 3 × 15 min: HRP conjugated goat anti-rabbit IgG (H+L), 1: 3000 dilution in 1% skimmed milk and PBST 1h; PBST 5 × 15 min. All immunoblots were developed by ECL reagent.

RESULTS

The pI of MARCKS proteins, ranging from 4.1-4.5, renders them relatively acid soluble, and this property has recently been exploited to identify MARCKS proteins by virtue of their solubility in 40% acetic acid (9). In our experiments, ATP pools of intact A6 cells were labeled with $^{32}[P_i]$ and cells were stimulated with PMA, as a positive control. Analysis of total proteins by SDS-PAGE and autoradiography yielded a number of observable phosphorylated proteins including proteins at approximately 80 kDa (Fig 1A). Phosphorylation at the 80 kDa region was apparently increased upon stimulation with 300 nM PMA, but was not very distinct in total cell homogenates, perhaps due to the presence of multiple phosphoproteins in this region. However, when total phosphorylated proteins were extracted with 40% acetic acid and the acid soluble portions were analyzed by SDS-PAGE, a major protein band was recovered at 80 kDa, corresponding to the known molecular size of at least one MARCKS protein. The phosphorylation of this 80 kDa protein was rapidly increased by PMA, suggesting that it might be a MARCKS protein (Fig 1B).

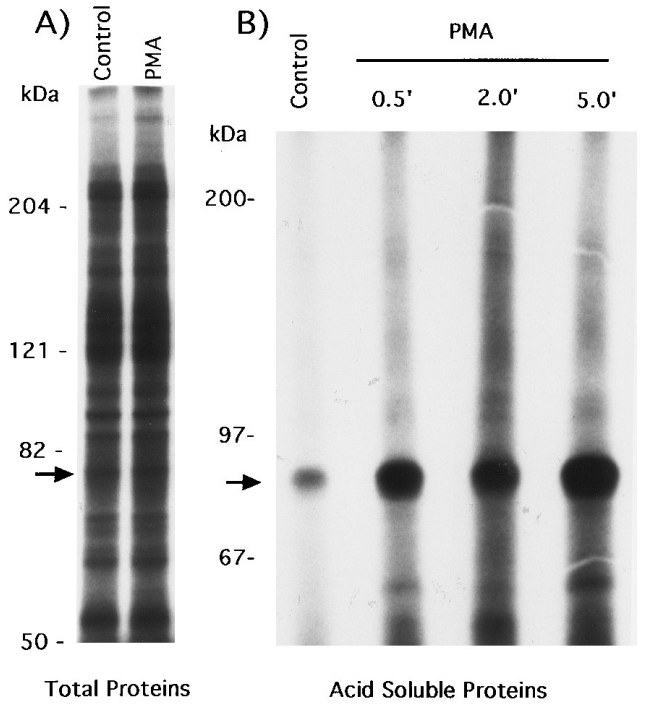

FIG. 1. Isolation of MARCKS protein by acetic acid extraction. (A) Autoradiograph of total phosphorylated protein obtained from $^{32}[P_i]$ labelled A6 cells and resolved by SDS-PAGE (7.5%). (B) A similar autoradiograph of acid soluble A6 cell proteins obtained as described in 'Experimental Procedures'. Arrow represents the position of a 80 kDa protein which is phosphorylated upon stimulation with 300 nM PMA at indicated times. The data presented are representative of 5 experiments.

To further characterize this protein as possible MARCKS, we probed it with an antibody raised against purified bovine brain MARCKS protein. This antibody is known to immunoprecipitate the 87 and 80 kDa

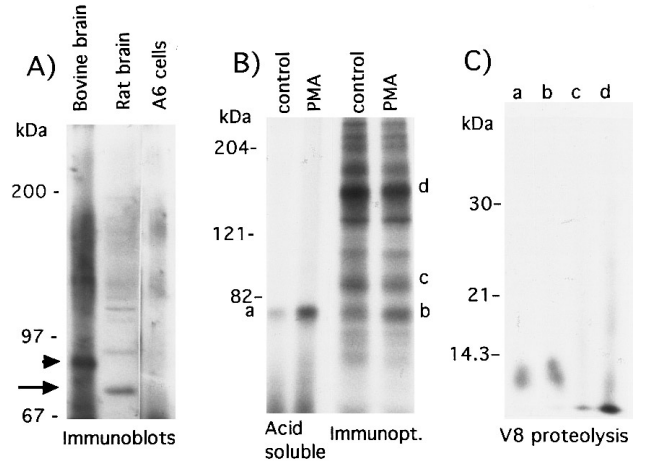

FIG. 2. Identification of MARCKS protein by a MARCKS specific antibody and its characterization. (A) Immunoblot probed with an antibody raised in rabbits against the 87 kDa bovine brain MARCKS, visualized with ECL reagent. This antibody recognizes a 87 kDa (arrow head) and 80 kDa (arrow) protein in bovine and rat brain tissues, respectively; no such protein was detectable in A6 cells. (B) Comparision of MARCKS proteins isolated by acid extraction and immunoprecipitation by bovine MARCKS antibody from $^{32}P_i$ labeled A6 cell lysates. Note that proteins at positions (a) (acid soluble) and (b) (immunoprecipitation) have similar electrophoretic mobilities and that phosphorylation at both positions increased with 300 nM PMA. The results shown in A and B are representative of 5 experiments. (C) S. aureus V8 proteolysis of the bands excised from the gels in B (marked as a,b,c and d) and separated on a 15 % gel. Both acid soluble (a) and immunoprecipitated (b) bands yielded an identical proteolytic pattern of a doublet of 13 and 9 kDa polypeptides, a characteristic of MARCKS protein, whereas bands c and d (other proteins immunoprecipitated with MARCKS antibody) provided a very different pattern. Similar results were obtained in 2 additional experiments.

MARCKS proteins from bovine and rat brain extracts, respectively (9). Fig 2A shows that this antibody also reacted positively on immunoblots with a bovine 87 and a rat 80 kDa protein. However, it did not recognize a similar protein of either size in A6 cell extracts. Therefore, we used immunoprecipitation to determine whether this antibody could recognize MARCKS in $^{32}[P_i]$ labeled A6 cell extracts. As shown in Fig 2B, the antibody indeed immunoprecipitated a 80 kDa protein from A6 cells, and the protein closely migrated with the acid soluble A6 protein. Preimmune serum did not immunoprecipitate this 80 kDa protein (results not shown). Phosphorylation of the immunoprecipitated 80 kDa band was specifically increased by PMA, as in the case of the acid soluble protein, shown for comparison of molecular size. These results, together, strongly argue that the 80 kDa protein was MARCKS.

To confirm the identity of both the acid soluble and the immunoprecipitated 80 kDa proteins as A6 cell MARCKS, we excised and analyzed each of the two 80 kDa bands by limited proteolysis. When the excised bands were reprocessed on 15% SDS-PAGE in the presence of *S. aurens* V8 protease, the acid soluble (lane a) and the immunoprecipated (lane b) 80 kDa bands each yielded an identical phosphopeptide pattern, a doublet of 13 and 9 kDa bands (Fig 2C) characteristic of authentic MARCKS proteins (9-11). However, the proteolytic pattern of two other protein bands that co-immunoprecipitated with MARCKS (lanes c and d), exhibited a very different pattern. Therefore, we conclude that both acid extraction and immunoprecipitation procedures identify an 80 kDa MARCKS protein in A6 cells.

After identification of MARCKS in A6 cells, we sought to determine whether this protein was capable of detecting endogenous activation of PKC when cells were exposed to a physiological agonist. When ATP pools of A6 cell monolayers were labeled with $^{32}[P_i]$, cells were exposed to 1μM vasopressin (AVP). PMA, a pharmocologic activator of PKC, was also used at 300nM as a positive control. MARCKS was then extracted in 40% acetic acid and analyzed by SDS-PAGE and autoradiography. As shown in Fig. 3, phosphorylation of the 80kDa protein was increased, compared to control, both by AVP and PMA, consistent with agonist-induced activation of MARCKS. To examine whether MARCKS was capable of detecting variations in PKC activation, A6 cells were preincubated for 30 min with the specific PKC antagonist chelerythrine in a concentration of 5 μM. Previous studies showed that this concentration resulted in nearly complete inhibition of vasopressin stimulated sodium transport in A6 cells (Ki for isolated enzyme $\sim$ 1μM) (5). Under this condi-

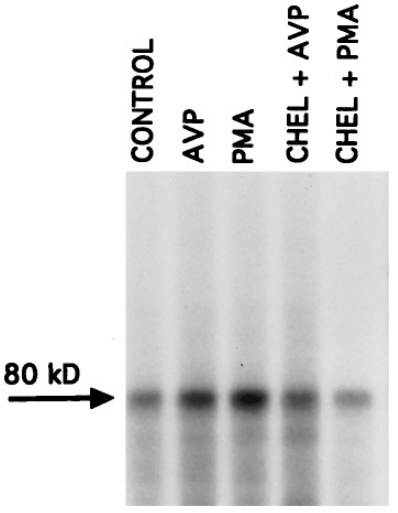

FIG. 3. Demonstration of the effect of a physiological agonist on MARCKS phosphorylation. $^{32}[P_i]$ labeled A6 cells were stimulated with either AVP (1μM, 10 min) or PMA (300nM, 10 min). Cell lysates were extracted in 40% acetic acid and visualized by autoradiography following SDS-PAGE. Cells were exposed to 5μM chelerythrine (CHEL), a specific inhibitor of PKC for 30 min before addition of agonists. Arrow shows the position of the 80 kDa protein that is phosphorylated in response to either AVP or PMA. Data are representative of 3 experiments.

tion, phosphorylation of MARCKS was reduced when cells were exposed to AVP and PMA, compared to the effect observed in the absence of chelerythrine. Taken together, these results indicate that MARCKS can serve to recognize endogenous activation of PKC in A6 cells.

DISCUSSION

MARCKS proteins are identified by combined physical and biological characteristics. They are recognized as a PKC-phosphoreceptor on immunoblots with a relative molecular size of approximately 80 to 90 kDa after extraction of cell lysates with 40% acetic acid, a method that exploits the property of resistance to precipitation in an acidic solution (9). Alternatively, they can be recognized on autoradiographs of 2-dimensional polyacrylamide electropheresis gels as having a pI of 4.1 to 4.5 and which exhibit increased phosphorylation when cells are exposed to PKC agonists, such as phorbol esters (9). An additional method to confirm that a protein is authentically MARCKS is the generation of doublet phosphopeptides with a relative molecular size of 9 and 13 kDa after exposure to S. aureus protease (9-11).

This study demonstrates that a MARCKS protein with a molecular size of 80 kDa is present in A6 renal epithelial cells, derived from Xenopus laevis, because this protein was soluble in 40% acetic acid, exhibited increased phosphorylation after exposure of intact cells to the phorbol ester PMA and yielded the characteristic 'fingerprint' phosphopeptides after exposure to S. aureus V8 protease. In addition, a protein of similar size with comparable characteristics was immunoprecipitated with antisera previously shown to recognize MARCKS present in bovine brain (9). This finding represents the first demonstration, to our knowledge, that MARCKS exists in lower vertebrates and, in particular, in Xenopus laevis which is commonly used to assess the biological properties of expressed proteins. This study also shows that dynamic endogenous changes in PKC activation, induced by agonists and antagonists, can be monitored by detectable changes in MARCKS phosphorylation.

Since the A6 cell is a major experimental model in the study of electrogenic sodium transport, our findings indicate that MARCKS may be used as a specific probe to assess the endogenous activation of PKC by physiological agonists. This method has important theoretical advantages compared to the traditional in-vitro assay because MARCKS is a unique substrate for PKC (12), while other commonly employed substrates such as histones are not specific (13), and because the concentrations of calcium and phospholipid substrates used in in-vitro assays may not reflect physiological intracellular conditions. In addition, since previous studies suggest that alterations in the cytoskeleton may regulate basal and hormonal upregulation of sodium transport (14), the opportunity to determine changes in the phosphorylation and location of MARCKS may provide important insights into the correlation of dynamic changes in the cytoskeleton with sodium transport.

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