Journal of Undergraduate Research

Volume 9, Issue 3 - Spring 2008

Purification Methods of MARCKS from Bovine Brain

Trina Chakravarty

ABSTRACT

Myristoylated alanine-rich C kinase substrate (MARCKS) is a well-characterized, natively unfolded protein that is thought to play a prominent role in the regulation of actin dynamics. MARCKS binds both calmodulin and actin and has been implicated in processes that necessitate regulated rearrangement of the actin cytoskeleton[1]. MARCKS is also known to interact with several other ligands and promote the regulation of cellular motility as well as cellular contractile function. The MARCKS protein is redistributed from the cytoplasmic face of the plasma membrane to the cytosol upon phosphorylation by protein kinase C following the stimulation of several cell types due to hormones and other agonists[2].

The studies conducted over the past year have focused primarily on the isolation and purification of the MARCKS protein. Non-myristoylated MARCKS was isolated from bovine brain, and the protein was isolated and purified using various techniques including douncing, perchloric acid treatment (PCA), and heat, and the samples were concentrated via dialysis. The results were analyzed via gel electrophoresis using an SDS gel to separate the protein based on molecular weight and charge. The western blotting technique was also used to quantitatively identify the amount of MARCKS isolated post-treatment. Using native gels, a 2-D gel analysis was also used to verify the amount of MARCKS present in the bovine samples that were created. An anion exchange column was finally used to identify at which salt concentration MARCKS elutes.

An understanding of MARCKS structure, of its interactions with calmodulin, actin and PKC, and of its capacity of cycle between the membrane and the cytosol provides a conceptual framework for understanding the biological role of the protein.

INTRODUCTION

Stimulation of membrane polyphosphoinositol turnover is one of the most commonly employed signal transduction systems in normal physiology. The two second messengers generated by this reaction include 1,4,5-triphosphate and diacylglycerols, which can activate most isoforms of protein kinase C (PKC)[3]. The PKC family members are diacylglycerol-activated, calcium-dependent protein kinases that regulate a large number of cellular responses including synthesis of macromolecules, activation of transport systems, secretion of

hormones, contraction of muscles, and many others . However, the molecular steps between the activation of PKC and the resultant biological responses are largely unknown. One approach to elucidating these molecular pathways is to study direct cellular substrates for PKC in the hope that the knowledge gained will help to explain downstream events[5]. Myristoylated alanine-rich C kinase substrate (MARCKS) is a widely distributed, specific PKC substrate whose phosphorylation has been used as a marker of PKC activation in vivo. The MARCKS family is comprised of two groups of proteins. The members of the first group, MARCKS, are 30-35 kDa proteins encoded by the *macs* genes. The second group, referred to as the MARCKS related proteins (MRP) is composed of 20 kDa proteins encoded by the *mrp* genes.

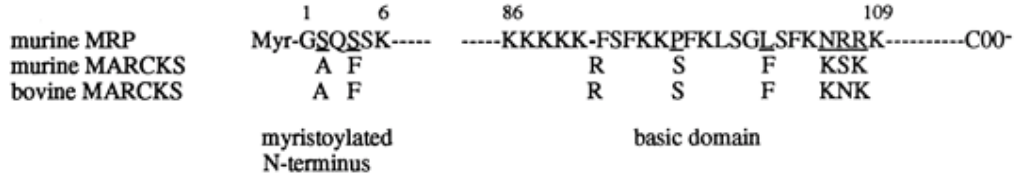

Figure 1. Structure of proteins of the MARCKS family. The amino acid sequence of the myristoylated N terminus, which contains the consensus sequence G*XXX*S recognized by NMT, as well as of the basic domain, which contains the serines phosphorylated by protein kinase C, are shown for murine MRP (*uppersequence*). Nonconserved residues (*underlined* in MRP sequence) are shown for murine and bovine MARCKS (*lowersequences*). The amino acid residues are numbered (*top*) according to the MRP sequence[6].

MARCKS is an acidic protein that is unusually rich in alanine, glycine, proline, and glutamic acid. The protein is a rod-shaped molecule containing three distinct domains: an N-terminal myristoylated domain that mediates binding to membranes, a highly conserved MH2 domain of unknown function, and a basic effector domain containing the PKC phosphorylation sites and the calmodulin and actin-binding sites. The effector domain (ED) is highly basic in contrast with the rest of the highly acid protein.

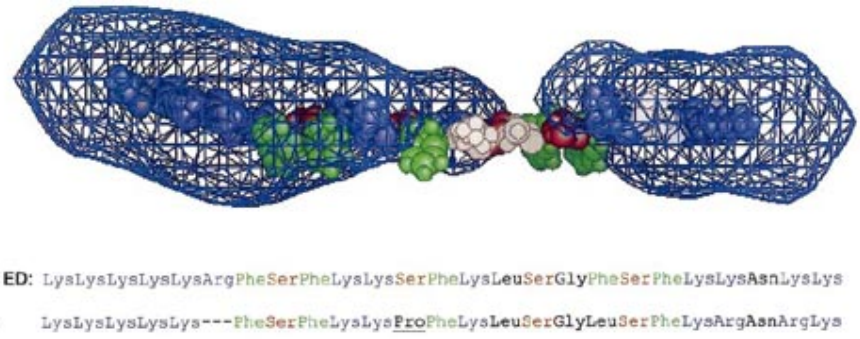

Figure 2. Molecular model of the ED. An atomic model of the MARCKS ED, built in agreement with all known structural data, shows the molecule in an extended conformation. This peptide model was built using the Insight-II/Biopolymer package (MSI) and was provided by Dr. Diana Murray of Cornell University. The 13 basic lysine and arginine residues are colored blue, the five aromatic phenylalanine residues are colored green and the four serine residues containing the

PKC-phosphorylated sites are colored brown. Shown as a blue mesh is the +25 mV equipotential surface, as calculated in a 100mM univalent salt solution using GRASP. Note the strongly positive field around the N- and C-terminal lobes of the ED. Below the primary sequences of the bovine MARCKS and MRP EDs are aligned using the same color code. The proline residue of MRP that replaces the second serine residue of MARCKS is underlined[7].

MARCKS is known to be involved in brain development, post-natal survival, cellular migration, adhesion, and neurosecretion. MARCKS is also reported to be involved in the etiology of diseases such as Alzheimer′s, chronic bronchitis, asthma, cystic fibrosis, and rheumatoid arthritis[8]. Several in vivo studies have implicated MARCKS in an actin-regulating function, as MARCKS co-localizes with F-actin, and the dissociation of this complex is regarded as a change in actin dynamics[9].

In order to elucidate the function of MARCKS and understand its function in vitro, purification techniques that avoid excessive denaturing steps were performed.

MATERIALS AND METHODS

Makeup of bovine brain homogenate samples (PCA treatment)

538 µL of homogenized bovine brain was combined with 12 µL diisopropylfluorophosphate (DFP),12 µL PMSF, 2000 µL 0.5M PCA and was left for five minutes on ice. The sample was then neutralized with 500 µL 2M tris base and 30.62 µL of the detergent Triton X (10%) was added. The sample was then dounced and an additional 40 µL of DFP was added and spun in a TL 100.4 rotor centrifuge at 65,000 rpm for 15 minutes at 4°C.

Makeup of bovine brain homogenate samples (PCA and heat treatment)

In an attempt to further purify MARCKS, a combination of Triton-X, PCA, and heat is used. 538 µL of homogenized bovine brain was combined with 12 µL diisopropylfluorophosphate (DFP),12 µL phenylmethylfulfonyl fluoride (PMSF), 2000 µL 0.5M PCA and was left for five minutes on ice. The sample was then neutralized with 500 µL 2M tris base and 30.62 µL of the detergent Triton X (10%) was added. The sample was then dounced and an additional 40 µL of DFP was added and spun in a TL 100.4 rotor centrifuge at 65,000 rpm for 15 minutes at 4°C. The sample was then heated for 10 minutes at 80°C and spun once more in the TL 100.4 rotor at 65,000 rpm for another 15 minutes at 4°C.

Testing PCA vs. PCA and heat treatment of bovine brain in the purification of MARCKS

Both treated samples of bovine brain homogenate were compared on an 8% SDS gel. A western blot analysis was performed to determine the different techniques of purification of MARCKS.

2-D gel of heat and PCA-treated bovine brain

A 6% native gel was run using 30 µL of the PCA and heat-treated bovine brain to determine the level of purity of MARCKS. The gel was run at 80 volts for 2 hours. The lane from the native gel containing the sample was then run on a 10% SDS gel, which was again run at 80 volts for 2 hours.

Dialysis

The dialysis buffer used consisted of 10 mM tris-HCl, 100 µM EGTA, 5 mM β-mercaptoethanol (ME), 2 mM EDTA, 100 µM PMSF, and 0.6 mM DFP. For the dialysis, a Spectrum #1 1.0 cm dialysis tubing was used. The membrane was soaked in deionized water for 10 minutes and 13 mL of the sample of PCA and heat-treated bovine brain homogenate was dialyzed for 12 hours. After 12 hours, the sample was spun in a 50.2 Ti rotor at 36,000 rpm for 15 minutes at 4°C.

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Anion exchange column analysis

To further purify MARCKS without denaturing the protein, an anion exchange column was performed to determine at what salt concentration the protein elutes. The start buffer contained a 20 mM tris-HCl solution and the finish buffer contained a mixture of 20 mM tris-HCl and 1.0 M NaCl. A HiTrap® 1 mL MonoQ column was used to elute MARCKS.

Western blot

After the SDS gel was run, the gel, filter paper, and fiber pads were soaked in a 20% MES/MeOH transfer buffer for 10 minutes. The PVDF membrane was equilibrated for 10 minutes as well. The gel sandwich cassette was run at 25 volts overnight. The next day, the membrane was incubated in blocking buffer (BSA A7030 5%, 100 mM L-lysine, diluted 1:2.5 in 1X PBS) for 20 minutes. A 3-hour incubation of the membrane was performed with a primary Abcam® goat anti-MARCKS antibody (IgG, AHP695) in a 1:1000 dilution. An hour-long incubation with the secondary antibody (Sigma® A5420, HRP conjugate) was performed in a dilution of 1:50,000. To develop the blot, equal parts of Supersignal West Pico Luminol Enchancer solution and peroxide solution were mixed and the membrane was incubated in the mixture for five minutes. In the darkroom, the blot was developed for 5, 10, and 15 seconds.

RESULTS

Western blot of PCA vs. PCA and heat treatment of bovine brain homogenate

Following a western blot assay of bovine brain treated with both PCA and heat and PCA treatment alone, it was observed that the inclusion of heat treatment does not further degrade MARCKS (Figure 3). There was an overall estimated 25% recovery of MARCKS following both heat and PCA and heat treatments of the bovine brain homogenate samples.

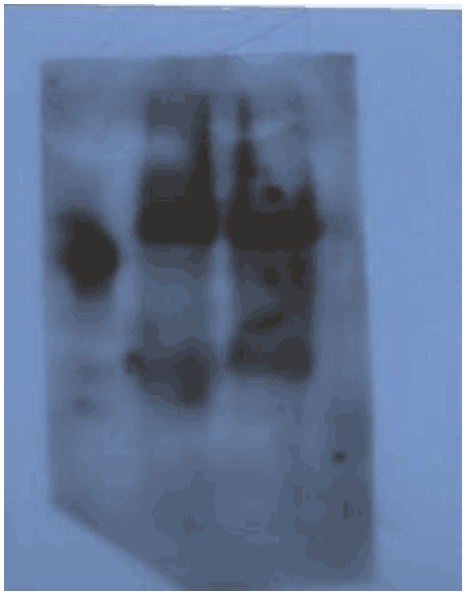

Figure 3. Bovine brain homogenate sample treated with PCA and PCA/heat treatments. Reading from left to right, Lane 1 is 2 µL of MARCKS control (50 µL MARCKS62 scan 1069, GFC and 450 µL MARCKS buffer). Lane 2 is 40 µL of PCA-treated bovine brain homogenate, and Lane 3 is 40 µL of PCA- and heat-treated bovine brain homogenate. Lanes 2 and 3 qualitatively show approximately equal amounts of myristoylated MARCKS.

2-D gel of heat- and PCA-treated bovine brain

The gel was very light but a band which may have corresponded to MARCKS was detected.

Anion exchange column analysis

It is known that MARCKS elutes at a salt concentration of approximately 40%. According to Figure 4, it was presumed that MARCKS eluted around fraction 8.

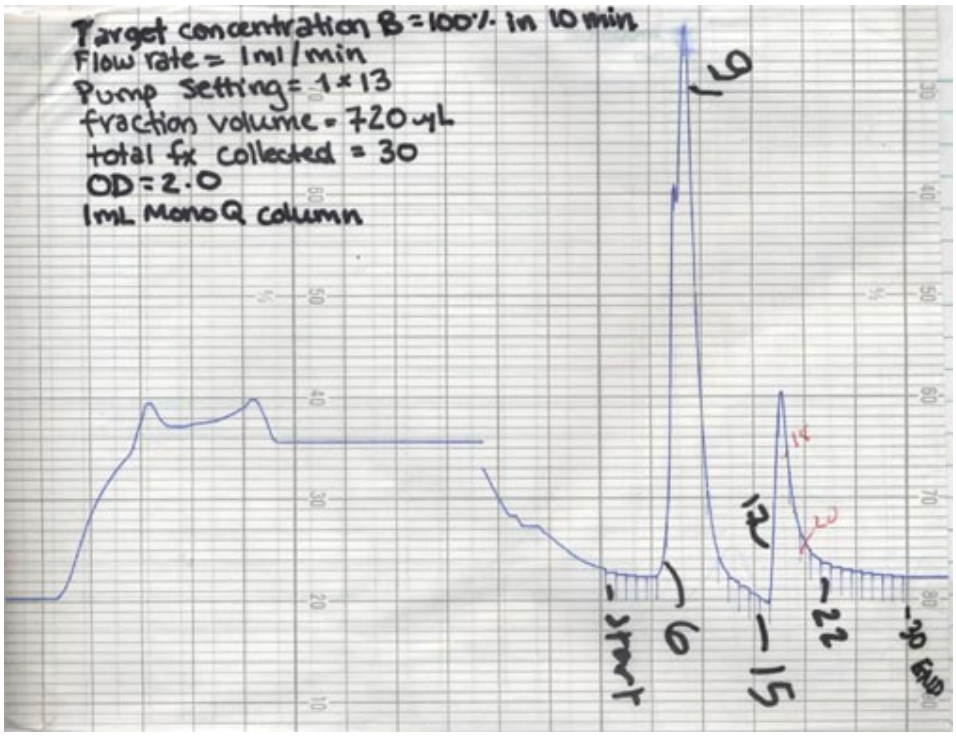

Figure 4. Anion exchange column. Fractions 6 through 30 were collected. 100% elution was assumed to have occurred by fraction 19. 40% elution was assumed to be at fraction 8 (not marked on graph). Since MARCKS shows no visible absorption peak when eluted, an SDS gel was run to determine whether MARCKS was present in anion exchange column fraction 8 (see Figure 5).

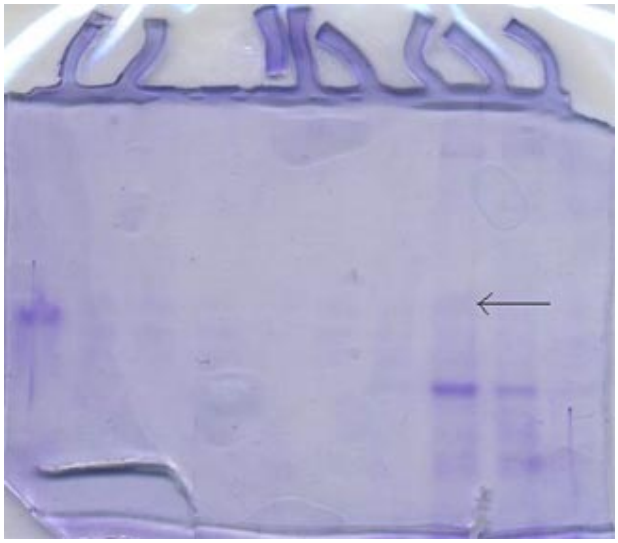

Figure 5. SDS gel analysis of anion exchange column fractions 5-11. All lanes have 30 µL material loaded. Reading from left to right, Lane 1 is the MARCKS control (50 µL MARCKS62 scan 1069, GFC, and 450 µL MARCKS buffer). Lane 2 is the flow through from the column. Lane 3 is the pre-loaded anion exchange column sample. Lanes 4-10 are fractions 5, 6, 7, 8, 9, 10, and 11, respectively. There is very light visible staining at the arrowhead, indicating the possible presence of myristoylated MARCKS. This would suggest that MARCKS eluted at fraction 9.

DISCUSSION

The findings in this study support the hypothesis that PCA and heat treatments of bovine brain homogenate do not detrimentally reduce the recovery of MARCKS. In agreement with previous studies, MARCKS is a heat-stable protein and will elute only in the absence of calcium.[10] While excessively stringent detergent usage can severely alter the amount of MARCKS in a bovine brain homogenate sample, the techniques listed above aided in proposing reasonable purification methods of MARCKS.

However, since MARCKS function is greatly regulated via posttranslational modifications including phosphorylation and myristoylation, it is vital to further investigate these posttranslational modifications in detail to understand their physiological functions[11]. More importantly, the mechanism of PKC-dependent cellular responses could be understood to a greater extent. Although the MARCKS PSD peptide is a substrate for PKC as well as conventional species, almost nothing is known about its comparative behavior as a substrate for the 10 or more PKC isoenzymes described to date, and thus a study in this direction would be vital in understanding its interactions with other isoenzymes.

Another area of further interest is MARCKS myristoylation. MARCKS was first found to be myristoylated by Aderem et al[12] in mouse macrophages. Initial suggestions that the protein might be myristoylated posttranslationally in an agonist-dependent manner were not supported by James and Olson[13], who demonstrated in other cells that the protein was myristoylated co-translationally. Another recent study[14] showed, however, that there exists a pool of non-myristoylated MARCKS in rat brain, which conceivably could be myristoylated posttranslationally. The

question of possible posttranslational myristoylation and demyristoylation, perhaps stimulated by agonists, has therefore not been completely resolved and is another interesting field to explore.

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