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IDENTIFICATION OF MYRISTOYLATED ALANINE-RICH C KINASE SUBSTRATE (MARCKS) IN ASTROCYTES

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1. ABSTRACT

We have characterized membrane-associated substrates of Ca^{2+}-dependent kinases in primary rat astrocytes by *in vitro* phosphorylation, 2-dimensional gel electrophoresis and autoradiography. The most prominent among these were three acidic, protein kinase C (PKC) substrates. These are important because they likely transduce cytokine and other neuro-immune modulatory signals mediated by PKC. We now show that one of these phosphoproteins is myristoylated alanine-rich PKC kinase substrate (MARCKS) or phosphomyristin C. The identity was corroborated by one- and 2- dimensional immunoblotting with an MARCKS-specific polyclonal antibody. Exposing primary astrocytes to phorbol 12-myristate 13-acetate stimulated phosphorylation of this protein. The level of MARCKS appeared inversely proportional to the proliferative potential of astrocytes because it was lower in spontaneously transformed as compared to passaged or confluent cells. These data are consistent with previous reports and indicate that one of three major acidic membrane-associated PKC substrates in astrocytes is MARCKS. Thus, MARCKS is likely near-proximal transducer of PKC-mediated signals in astrocytes.

2. INTRODUCTION

Protein kinase C (PKC) are a family of a Ca^{2+}- and lipid-dependent serine-threonine protein kinases, which play crucial roles in signal transduction, mitogenesis, cellular transformation and migration *(*1,2*)*. Twelve PKC isozymes are distinguished by tissue specificity and molecules that activate them *(1)*. PKCs referred here are those activated by Ca^{2+}, phosphatidyl serine and diacylglycerol and termed conventional PKC. PKC isozymes immunoreactivity has been detected in astrocytes of both human and rat brain *(*3-5*)*. In cultured astrocytes PKC translocation from the cytosol to the plasma membrane occurs upon stimulation with not only a phorbol ester *(6)* but also TGF-β1 *(7)*; and viruses *(*8*)*. PKC mediates in astrocytes (and other cell types) activation of NF-kappa-B *(*9*)* and subsequent induction of cyclooxygenase or prostaglandin E *(*10*)*, the signals elicited by IL-1 and TNFα *(*11*)*. Thus, PKC mediate in astrocytes transduction of signals elicited by cytokines. PKC also has important roles in astrocyte proliferation *(*12*)* and maturation *(*13*)*.

Membrane-associated PKC substrates are important because they are immediate proximal signal transducers. One of the most abundant, neuronal, membrane-associated PKC substrates, growth associated protein 43 (GAP-43) also is present in astrocytes *(*14,15*)* and oligodendrocytes *(*16*)*. GAP-43 expression in these cells is developmentally regulated in cell type-specific manner *(*16*)* suggesting a role for this protein in glial cells

in addition to neurons. We now show that the second major PKC substrate in astrocytes, in addition to GAP-43, is myristoylated alanine-rich C kinase substrate (MARCKS) also known as phosphomyristin C *(*17*)*. This is the first identification of MARCKS in astrocytes by combination of 2-dimensional polyacrylamide gel electrophoresis (PAGE) and Western blotting. MARCKS is important because it is phosphorylated within seconds of PKC activation and may, thus, be an essential and proximal mechanism for transducing a PKC-dependent signal *(*18*)*.

3. MATERIALS AND METHODS

3.1. Cell Culture

Astrocytes were isolated from neonatal rat cortex tissue and cultured as previously described *(*16,19*)*. Briefly, the tissue was mechanically disintegrated and the cells were cultured in 75 cm^2 flasks in DMEM/F12 (1:1 v/v) supplemented with 10% fetal calf serum for 7-10 days, i.e. until confluent. The cultures contained 95-98% astrocytes. Immunolabeling of astrocytic marker, GFAP, and other cell markers was as previously described *(*15,19*)*. Astrocytes were passaged only in experiment estimating MARCKS level as a function of proliferation. Spontaneously transformed rat astrocytes were a generous gift of M. Mersel *(*20*)*.

3.2. Isolation of plasma membranes

The cells were disintegrated and fractionated into cytosolic and microsomal fractions. The microsomal fraction, containing plasma and organelle membranes, was suspended with polytron in 57% (w/v) sucrose and placed at the bottom of a polyallomer ultracentrifuge tube. Thirty four % (w/v) and 9 % (w/v) sucrose solutions were layered, respectively, on top of each other on the microsomal suspension, and centrifuged at 80,000 x g for 5 hrs. Plasma membranes sedimented at the interface between 9% and 34% sucrose as shown previously *(*15*)* and were the source of "plasma membrane proteins."

3.3. Protein Phosphorylation

Phosphorylation of plasma membrane proteins *in vitro* by PKC was done as previously described (Paper I). The reaction mixture contained: plasma membrane proteins (200 μg from either primary astrocytes or brain cortex), crude PKC, 10 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 1.0 mM EGTA, 30 μM *(*^{32}P*)*ATP (specific activity 6,600 cpm/pmole), 1.1 mM $CaCl_2$ (approximate final concentration 100 μM $CaCl_2$) phosphatidyl L-serine (40 μg/ml) and diolein (1 μg/ml). The reaction lasted 1 min at 37 ºC and was stopped by freezing.

Phosphorylation in intact astrocytes was determined as follows. Astrocyte monolayers were washed twice with phosphate-free DMEM and then incubated with *(*^{32}P*)*-orthophosphate (50 μCi/ml; specific activity 285 Ci/mg Pi) for 4 hrs. At the end of the incubation, phorbol 12-myristate 13-acetate (PMA, 50 ng/ml) or solvent alone (control) were added directly to cultures to stimulate PKC. Washing the cells three times with cold phosphate-buffered saline (PBS) stopped the stimulation. The cells were then scraped into PBS at 4 ºC, mixed with carrier cells (from rat spleen) and rapidly homogenized. Plasma membranes were isolated from these cells as described above.

3.4. Electrophoresis

Proteins were resolved by one- and 2-dimensional polyacrylamide gel electrophoresis (PAGE) as previously described *(*14,15*)*. After electrophoresis, proteins were fixed, stained and autoradiographed as previously described *(*14,15*)*. The bands on one-dimensional autoradiograms were estimated by scanning densitometry with an ultrascan XL laser densitometer (LKB).

3.5. Western Blotting

Plasma membrane proteins (35μg) were resolved by one or 2-dimensional electrophoresis and transferred to nitrocellulose membrane (Schleicher and Schuell) by standard methods. The membranes were initially incubated with a solution of 5 % (v/v) goat serum, 3 % (wt/v) albumin, and 0.1 % (wt/v) Tween 20 for one hour at room temperature and then incubated with a 1/1000 dilution of anti-MARCKS peptide antiserum in 5 % (v/v) goat serum/TBS overnight at 4 °C. After incubation with primary antibody the membrane was rinsed three times in TBS/0.1% Tween 20 at 4 °C. The blot was incubated with biotin labeled goat anti-rabbit IgG (diluted 1/1000 v/v) in 5 % (v/v) goat serum/TBS for one-two hours at room temperature, washed three times with TBS, incubated with streptavidin-phosphatase (diluted 1/1000 v/v), washed three times with TBS and developed with BCIP/NBT phosphatase substrate system according to the manufacturers instructions.

3.6. Materials

PKC was partially purified from cerebral cortices of Sprague-Dawley rats as previously described *(*21*)*. A monospecific anti-MARCKS antiserum was raised in rabbits against a synthetic peptide (HN2-EAAEPEQPEQPEQPAA-COOH) coupled to keyhole limpet hemocyanin as previously described *(*22,23*)*. Goat anti-rabbit IgG, strepavidin-phosphatase and BCIP/NBT phosphatase substrates were from Kierkgaard and Perry, Inc. Tween 20 was from Fluka. All other reagents were from Sigma.

4. RESULTS

4.1. Acidic, Membrane-Associated PKC Substrates in Astrocytes and Brain are Similar to Each Other

To identify acidic PKC substrates, we isolated plasma membranes from rat neonatal cortex and rat neonatal astrocytes. The plasma membranes were incubated with partially purified PKC from rat brain and the resulting phosphoproteins were resolved by 2-dimensional PAGE in acidic pI range. Autoradiograms of these gels revealed a striking similarity of PKC substrates from astrocytes and cortex in the number, Mr, and pI of the resulting phosphoproteins (Figure 1). Based on Mr, pI, the broad phosphorylation conditions (Paper I), abundance and the partitioning of the protein upon fractionation of the cells (data not shown), the protein labeled pp1 appears similar, if not identical, to β-tubulin *(*24-26*)*. We suggest this with

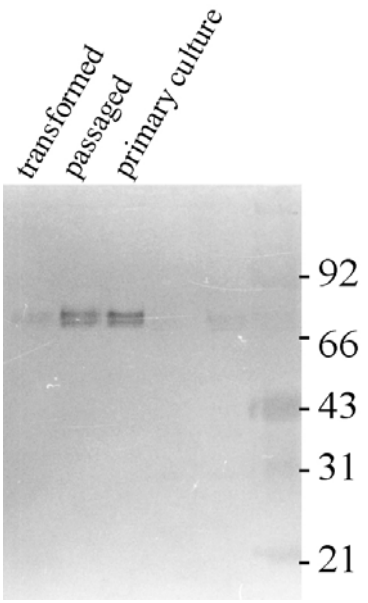

Figure 1. Acidic PKC substrates from cultured astrocytes (A, C) and rat brain (B, D) were similar to each other in number, Mr, and pI and contained MARCKS-like immunoreactivity. Plasma membrane proteins (40 µg) were phosphorylated by partially purified brain-derived PKC, resolved by pI in the acidic range and either autoradiographed (A, B) or immunoblotted for MARCKS (C, D). Note the broadening of the spots corresponding to pp2 and pp3 reflecting their microheterogeneity. The data are representative of three independent experiments.

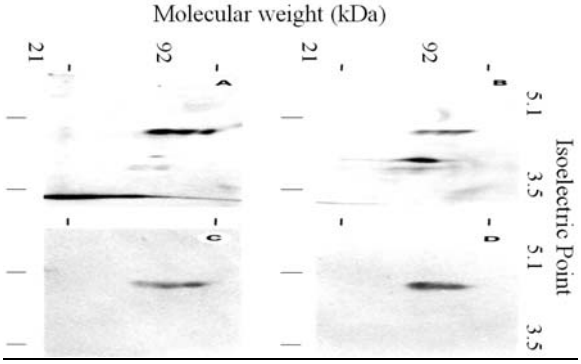

Figure 2. Phobol 12-myristate 13-acetate (PMA)-stimulated phosphorylation of proteins with the Mr similar to GAP-43 and MARCKS in cultured astrocytes. Astrocytes were cultured for 4 h in the presence of radioactive orthophosphate. Cell were either stimulated with PMA (50 ng/ml) for 15 min (A) or left alone (B). Plasma membrane proteins were isolated, resolved by one-dimensional gradient gel electrophoresis, autoradiographed (horizontal bars at the top of each panel), and the areas under the peaks were estimated by laser densitometry. In PMA-stimulated cells, the phosphorylation of proteins with the Mr similar to GAP-43 and MARCKS (arrows) increased by 25% and 215% over unstimulated controls, respectively. The results are representative of two independent experiments.

caution because more than one protein may be present in this area as judged by the shape of the spot (Paper I). The identity of pp3 was previously established by the use of five independent criteria as GAP-43 *(*15*)*. In contrast, pp2 in membranes from astrocytes was more intensely phosphorylated by PKC than its counterpart in membranes from rat brain (Figure 1). This was likely due to a lower basal phosphorylation state of pp2 rather than to a higher abundance in rat brain astrocytes compared to rat brain as a whole (data not shown). These data indicate that three acidic membrane-associated proteins in astrocytes are similar, if not identical to those in brain as a whole.

4.2. Anti-MARCKS Antibody Specifically Interacts with pp2 from Astrocytes

Phosphoprotein 2 migrated as a doublet on one- and 2 -dimensional polyacrylamide gels with an approximate molecular mass of 80 kDa and a pI of 3.9-4.0 (Figure 1, 3). This doublet was further resolved by acidic 2-dimensional gel analysis into three isoforms with distinct pIs between 3.6 and 4.1 (Figure 1). Pp2 from astrocytes and brain had identical Mr and pI, isoform composition, and were phosphorylated by PKC *in vitro* (Figure 1). Phosphorylation of astrocytic pp2 was inhibited by calmodulin (Paper I) as described for MARCKS *(*23*)*. Thus, the biochemical characteristics of astrocytic pp2 were similar to pp2 in plasma membranes from whole rat brain cortex and to a protein termed MARCKS *(*17*)*. To further test the similarity of pp2 and MARCKS we used one- and 2-dimensional immunoblotting of the acidic membrane proteins from astrocytes and cortex to determine whether or not pp2 interacts with an anti-MARCKS antibody. This antibody specifically precipitates MARCKS from bovine brain tissue (22). One-dimensional immunoblotting demonstrated that rat brain cortical astrocytes and immortalized cells derived therefrom contain an MARCKS-immunoreactive doublet of approximately 80 kDa. No other proteins interacted with the antibody suggesting that the interaction with pp2 was specific (Figure 3). Two-dimensional polyacrylamide gel separation of acidic membrane proteins from astrocytes and cortex followed by immunoblotting demonstrated a heterogeneous signal with several isoforms spanning the same pI range as phosphorylated pp2 (Figure. 1C and D). A similar profile of the MARCKS protein has been previously observed by others in immunocytes, fibroblasts, and tissues (22,27,28). Thus, one- and 2-dimensional immunoblotting with a monospecific antiserum demonstrated that rat cortical astrocyte membranes contain a heterogeneous population of MARCKS-immunoreactive proteins that are similar if not identical to those in the brain.

4.3. Stimulation of Astrocytes with PMA Leads to Increased Phosphorylation of Proteins with Mr Similar to GAP-43 and MARCKS

To determine whether or not these PKC substrates identified *in vitro*, function as such in intact astrocytes, we assayed their phosphorylation in cultured astrocytes stimulated with PMA. Intact astrocytes were labeled with *(*^{32}P*)*-orthophosphate and then exposed to either PMA or vehicle. Plasma membranes were isolated and their protein phosphorylation was estimated by a

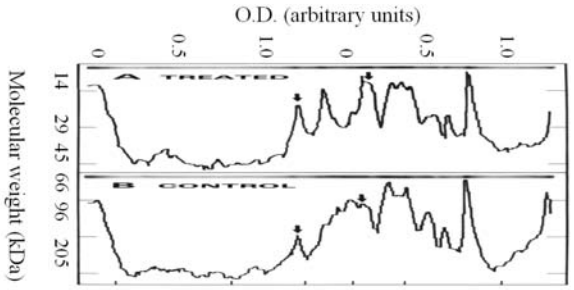

Figure 3. MARCKS levels decrease with the proliferative potential of rat astrocytes. The levels are estimated by western blotting of membrane-associated proteins (35 μg per lane) extracted from rat astrocytes that were spontaneously transformed, passaged or confluent primary culture (left to right). Note that the doublet of approximately 80 kDa is the only protein stained on this one-dimensional blot. Approximate positions of migration of molecular weight standard proteins are indicated on the x-axis.

combination of electrophoresis and autoradiography. PMA-stimulated phosphorylation of various proteins was easily discerned by comparing autoradiograms of gels containing proteins from stimulated and unstimulated astrocytes (Figure 2). PMA treatment increased by approximately 20% and 215% the phosphorylation of two proteins with molecular weights of 45 kDa and 80 kDa, respectively. We tentatively identify these proteins as GAP-43 and MARCKS, since 2-dimensional PAGE of *in vitro* phosphorylated proteins did not identify other phosphoproteins within this molecular weight region. The results are also consistent with the higher phosphorylation of MARCKS than GAP-43 *in vitro* (Figure 1 and Paper I). These results suggest that membrane-associated GAP-43 and MARCKS are PKC substrates in intact astrocytes.

4.4. Levels of MARCKS in Astrocytes as a Function of Proliferation

MARCKS has been reported be downregulated in transformed cells *(*29-31*)*. We, therefore, estimated level of MARCKS proteins by immunoblotting in spontaneously transformed rat astrocytes and compared it with the levels in astrocytes that had been passaged or in confluent culture. The level was lower in transformed astrocytes as compared to proliferating and confluent cells (Figure 3). This suggests, although it does not prove, that MARCKS expression may be inversely proportional to the proliferative potential in astrocytes.

5. DISCUSSION

The data presented here indicate that astrocytes contain three principal, acidic, membrane-associated PKC substrates present in the brain as a whole. One of these substrates is identified as MARCKS. This protein migrated on one- and 2-dimensional gels as a doublet of about 80 kDa, consisted of three differentially charged isoforms, was phosphorylated by PKC in the absence but not in the presence of CAM (Paper I), specifically interacted with a polyclonal antibody against MARCKS on one- and 2-dimensional Western blots partitioned between cytosol and membrane (data not shown). Based on these properties pp2 is similar, if not identical MARCKS *(*32,33*)*. This is the first unequivocal evidence that MARCKS is expressed in astrocytes and is consistent with immunocytochemical evidence suggesting that MARCKS protein is present in cytoplasm and plasma membranes of cells morphologically resembling astrocytes in rat brain *(*34*)*. This is also consistent with the report that oligodendrocytes express MARCKS in developmentally regulated manner *(*35*)*. We note that astrocytes and oligodendrocytes express GAP-43 also in a developmentally regulated manner *(*16,36*)*

Two species of MARCKS have been previously discovered by Western blotting and protein purification experiments *(*32,37*)*. The higher molecular weight form has been termed 80k-H and the lower molecular weight form as 80k-L These two forms are distinctly phosphorylated by PKC isozymes *(*37*)*. The present results support the notion that rat brain cortical astrocytes express both the high and low molecular weight isoforms of the MARCKS, that both of these isoforms contain the oligopeptide to which the antiserum was generated and are substrates for PKC in astrocytes. The precise function(s) of MARCKS in astrocytes is unknown. MARCKS protein expression is downregulated in transformed cells *(*29-31*)*. These observations suggest that a cell's proliferative potential may be inversely correlated with MARCKS protein expression and/or phosphorylation. Our results in astrocytes are consistent with this view. Spontaneously immortalized astrocytes *(*20*)* express the MARCKS protein at a much lower level than do passaged or confluent rat brain astrocytes. Thus, MARCKS may play a role in astrocytomas and astrocytosis, as previously suggested *(*12*)*.

6. ACKNOWLEDGEMENT

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