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Myristoylation-dependent N-terminal cleavage of the myristoylated alanine-rich C kinase substrate (MARCKS) by cellular extracts

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Abstract — The myristoylated alanine-rich C kinase substrate (MARCKS) has been proposed to regulate the plasticity of the actin cytoskeleton at its site of attachment to membranes. In macrophages, MARCKS is implicated in various cellular events including motility, adhesion and phagocytosis. In this report we show that macrophage extracts contain a protease which specifically cleaves human MARCKS, expressed in a cell-free system or in *E. coli*, between Lys-6 and Thr-7. Cleavage of MARCKS decreases its affinity for macrophage membranes by ca. one order of magnitude, highlighting the contribution of the myristoyl moiety of MARCKS to membrane binding. Importantly, cleavage requires myristoylation of MARCKS. Furthermore, MARCKS-related protein (MRP), the second member of the MARCKS family, is not digested. Since Thr-7 is lacking in MRP this suggests that Thr-7 at the P1 position is important for the recognition of lipid-modified substrates. A different product is observed when MARCKS is incubated with a calf brain cytosolic extract. This product can be remyristoylated in the presence of myristoyl-CoA and N-myristoyl transferase, demonstrating that cycles of myristoylation/demyristoylation of MARCKS can be achieved in vitro. Although the physiological relevance of these enzymes still needs to be demonstrated, our results reveal the presence of a new class of cleaving enzymes recognizing lipid-modified protein substrates. © 2000 Société française de biochimie et biologie moléculaire / Éditions scientifiques et médicales Elsevier SAS

macrophages / MARCKS / membrane binding / MARCKS related protein / myristoylation / proteolysis

1. Introduction

The myristoylated alanine-rich C kinase substrate (MARCKS) family comprises two members: MARCKS (32 kDa) and MARCKS-related protein (MRP) (22 kDa) [1–4]. Gene disruption experiments in mice have shown that MARCKS proteins are required for normal brain development [5–7]. Aderem and colleagues have proposed that MARCKS proteins regulate the local plasticity of the actin cytoskeleton at membranes [8–13]. Evidence for other functions, including the regulation of free cytosolic calmodulin [14], membrane trafficking [12, 13] and phospholipase C activity [15], has also been reported.

The properties of MARCKS proteins can be regulated in several ways, with PKC-dependent phosphorylation of MARCKS as the best investigated short term regulatory mechanism [1, 3]. Early studies observed release of MARCKS from cellular membranes into the cytosol upon PKC activation [8, 11, 16, 17]. Recent studies, however, suggest that PKC-dependent phosphorylation regulates cycling of MARCKS proteins between different intracellular membranes rather than releasing these proteins into the cytoplasm [12, 13].

Changes in the expression levels of MARCKS proteins have also been widely reported and could provide the cell with long-term regulatory mechanisms [2, 18]. The expression of MARCKS is also regulated in a developmental manner with higher tissue levels found early in development [19]. In addition, MARCKS shows a specific tissue distribution with the concentrations of MRP being particularly high in the brain and reproductive tissues [20].

In addition to PKC-dependent phosphorylation and changes in the expression level, several reports suggest that a variety of mechanisms can potentially regulate the properties of MARCKS proteins: 1) since both PKC and calmodulin require calcium for their interactions with MARCKS proteins, calcium might regulate cross-talk between these signal transduction pathways by determin-

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Abbreviations: cPIC, concentrated protease inhibitor cocktail; MARCKS, myristoylated alanine-rich C kinase substrate; MRP, MARCKS-related protein; myr, myristoylated; NMT, myristoyl CoA:protein N-myristoyl transferase; P100, particulate fraction; RRL, rabbit reticulocyte lysate; RT, room temperature; S100, soluble fraction; unmyr, unmyristoylated

ing how PKC and calmodulin will compete for their common targets, namely the MARCKS proteins (for reviews see [11, 21]); 2) MARCKS is also phosphorylated by proline-directed protein kinases such as mitogen-activated protein kinase or Cdk5 kinase [22]; 3) poly(ADP-ribose) very effectively inhibits the interactions of purified MARCKS proteins with actin, calmodulin, PKC and membranes, indicating that MARCKS proteins could be targets of the poly(ADP-ribose) DNA damage signal pathway [23]; 4) a demyristoylation activity has been detected in brain synaptosomes [24]; 5) exposure of the 70-kDa PKC substrate, i.e., MARCKS, to the cytosol of ras-transformed fibroblasts, but not of normal fibroblasts, results in disappearance of the protein [25] possibly caused by cathepsin L; 6) cathepsin B also cleaves MARCKS in fibroblasts, possibly as a result of specific lysosomal targeting sequences within the MARCKS primary sequence [26, 27]; 7)finally, MRP is an in vitro substrate for the *Leishmania major* surface protease Leishmanolysin (gp63) [28], a phenomenon which might account for the cleavage of MRP in macrophages infected with this parasite [29].

Although the physiological relevance of the action of most, if not all, of these processes on MARCKS proteins has not yet been demonstrated, these findings suggest that cleavage of MARCKS proteins might be an important regulatory mechanism of their functions. In this report we show that the degradation product resulting from the incubation of myr MARCKS with a calf brain cytosolic extract can be enzymatically remyristoylated suggesting that the intracellular localization of MARCKS may also be regulated by demyristoylation/remyristoylation cycles. We also provide evidence for a protease in macrophage extracts which specifically cleaves the myristoylated N-terminus of MARCKS between residues 6 and 7.

2. Materials and methods

2.1. Cell-free expression of MARCKS and MRP

Following linearisation with *Eco*RI, 2 µg of the plasmids pET3dF52M1 and pBSH80KM2 (kind gifts of Dr. Perry Blackshear, NIEHS, Research Triangle Park, USA) containing the genes coding for murine MRP and human MARCKS, respectively, were transcribed for 2 h at 37 °C in solutions containing 40 units T7 polymerase in Tris-HCl, pH 7.5, using the Riboprobe® transcription kit from Promega (final volume 100 µL). The DNA was then digested with 2 units of RQ1 DNAse for 15 min at 37 °C. The mRNA resulting from the transcription was precipitated with 50 µL 7.5 M ammonium acetate and resuspended in 25 µL RNAse-free water. 1.5–3 µL of each RNA solution was then translated for 90 min at 30 °C in 50 µL of a solution containing 33 µL rabbit reticulocyte lysate (Promega) and 2 µL of 1 mM amino acid cocktail (Promega). In addition, the solutions contained either 20 µCi [^{35}S]-Met (1000 Ci/mmol) for labeling of MRP at Met-130, 20 µCi [^{35}S]-Cys (1000 Ci/mmol) for labeling of MARCKS at Cys-278, Cys-309 and Cys-320 (note that, as the N-terminal Met residue of MARCKS protein is removed following synthesis, the myristoylated Gly residue is numbered as residue 1), or 50 µCi [^{3}H]-myristate (16.00 Ci/mmol) for labeling of MARCKS at the myristoylated N-terminus. All radioactive products were obtained from Amersham. Except when MARCKS was labeled with [^{3}H]-myristate, the translations were performed in the presence of 100 µM myristate to ensure complete myristoylation of MARCKS proteins [30].

2.2. Expression of MRP and MARCKS in E. coli

Recombinant murine myr- and unmyr MRP [31] and human unmyr MARCKS [32] were expressed in *E. coli* and purified following published procedures. For N-terminal sequencing of recombinant myr MARCKS cleaved by macrophage extracts, we used a human MARCKS [32] co-expressed with myristoyl CoA:protein N-myristoyl transferase (NMT) and subsequently purified as described for myr MRP [31].

2.3. Expression in E. coli and purification of His-tagged NMT

Human NMT was expressed as a histidine tagged fusion protein in the vector pET28c and purified on metal chelate columns as previously described [33, 34]. The enzyme was eluted from the columns using 100 mM imidazole and the eluate dialyzed against 50 mM Tris-HCl, pH 7.5. The dialyzed NMT was stored in aliquots at –70 °C, and was used directly for the experiments described here.

2.4. In vitro myristoylation of MARCKS

To provide large amounts of MARCKS labeled radioactively on the myristoyl moiety ([^{3}H]-MARCKS) for digestion experiments unmyr MARCKS (25 µg) was incubated with NMT (2.5 µg) and [^{3}H]-myristoyl-CoA (10 µM; specific activity 15 Ci/mmol) for 1 h at 30 °C, in a final volume of 250 µL 50 mM Tris-HCl, pH 7.5, containing 10 mM DTT. The [^{3}H]-myristoyl-CoA was prepared from [^{3}H]-myristic acid (30 Ci/mmol) as previously described [33] and adjusted with unlabeled myristoyl-CoA to the described specific activity. The entire reaction mix was then rotated with DEAE (200 µL; equilibrated with 10 mM Tris-HCl, pH 7.4) for 2 h at 4 °C and this then washed with 50 mM Tris-HCl, pH 7.4, containing 50 mM NaCl until the radioactivity in the eluate was less than 2000 dpm/mL. The bound MARCKS was then eluted with 50 mM Tris-HCl, pH 7.4, containing 300 mM NaCl and 200 µL fractions counted. The major radioactive peak

eluted in the second fraction which was used for all further experiments. Analysis of the fractions by SDS-polyacrylamide gel electrophoresis showed that about 50% of the MARCKS was labeled under these condition.

Elsewhere, except for the experiments in which MARCKS was analyzed by Western blot for which the amounts of MARCKS are given in the legend of the appropriate figures, 5 µg purified unmyr MARCKS was incubated for 1.5 h at 37 °C in a final volume of 25 µL in a solution containing 0.5 µg purified NMT, 20 µM myristoyl-CoA, Tris HCl, pH 7.4, 100 mM NaCl, 0.1 mM EGTA and 1 mM DTT. Myristoylation was almost complete, as monitored by Western blot and Coomassie blue staining (the apparent molecular mass of MARCKS increases from 70 to 83 kDa upon myristoylation).

2.5. Culture of macrophages

The murine macrophage cell lines RAW 267.4 and J774.4 were gifts of Dr. Joseph Pfeilschifter (University of Frankfurt, Germany). Cell were cultured at 37 °C in 80 cm^2 dishes using macrophage SFM-medium complemented with L-glutamine (Life Science) in the presence of 5 mg/500 mL streptomycin.

2.6. Preparation of macrophage extracts

2.6.1. High ionic strength protocol

The macrophage cultures were cultivated to a confluence of 80–90% and removed from the dishes in buffer A (0.25 M sucrose, 0.1 M $MgCl_2$, 10 mM Tris HCl, pH 7.4) using a rubber policeman. The macrophages were centrifuged at 5000 *g* (1000 rpm, Heraeus MiniFuge RF) and resuspended in buffer A containing a concentrated protease inhibitor cocktail (cPIC) composed of 0.1 mg/mL PMSF, 10 mg/mL aprotinin, 1 mg/mL chymostatin, and 1 mg/mL leupeptin. The cells were lysed with an homogenizer (50 strokes with a tight pestle A). The progress of lysis was controlled by inspection of the cells with a light microscope. The homogenate was centrifuged at 1000 *g* for 15 min at 4 °C (4000 rpm, Eppendorf centrifuge) to remove intact cells and nuclei. The cytosolic (S100) and particulate (P100) fractions of the macrophage extracts were separated by centrifugation at 100 000 *g* for 1 h at 4 °C (Beckman Optima TLX centrifuge, TLA 100.3 rotor, 55 000 rpm). P100 was resuspended in a buffer containing 10 mM MOPS, 0.1 mM EGTA, 100 mM NaCl, 1 mM DTT and 20% v/v glycerol.

2.6.2. Low ionic strength protocol

This protocol is essentially the same as described above except that buffer B (0.25 M sucrose, 10 mM Tris HCl, pH 7.4, 1 mM EDTA, 1 mM DTT) was used for the lysis instead of buffer A. For both protocols, P100 and S100 were either used directly or frozen in liquid nitrogen and stored, until use, at –70 °C.

2.7. Isolation of the particulate and cytosolic fractions of calf brain

The low ionic strength protocol used for the isolation of macrophage extract was adapted to prepare calf brain extracts. Calf brain were obtained from animals killed at the local slaughterhouse. The brains were kept on ice, cut in smaller pieces, and prepared within 2 h or frozen in liquid nitrogen and stored, until use, at –70 °C. 20 g of calf brain tissue were homogenized (Polytron homogenizer, Tekmar) for 30 s in 100 mL ice-cold buffer B in the presence of cPIC. Unbroken cells were removed by centrifugation (Kontron centrifuge, SS34 rotor, 500 *g* for 30 min). Calf brain P100 and S100 were prepared by centrifugation at 4 °C and 100 000 *g* for 1 h (Kontron centrifuge, TFT 65.38, 65 000 rpm). S100 was concentrated by ultrafiltration using Vivaspin Concentrator (Vivascience Ltd, Lincoln, UK) with a cut off of 20 kDa. The fractions were either used directly or frozen in liquid nitrogen and stored, until use, at –70 °C.

2.8. Incubation of MARCKS proteins with cellular extracts

Unless indicated otherwise the following standard protocol was used for MARCKS proteins labeled with ^{35}S: a volume of cellular extract (macrophage P100 or S100; calf brain P100 or S100) containing 25 µg protein was incubated with 4 µL of a translation reaction containing ^{35}S-labeled MARCKS or MRP. These 4 µL were sufficient to allow detection of the proteins by SDS-PAGE followed by phosphor-imaging or fluorography (see below). MARCKS proteins and cellular extracts were co-incubated for 3 h at 25 °C in buffer B (final volume 20 µL). The incubation was performed in the presence of cPIC. The reaction was stopped by addition of 5 µL SDS sample buffer (1% SDS, 50% glycerol, 250 mM Tris, pH 6.8, 0.1% bromphenol blue, 5% mercaptoethanol) and heating at 95 °C for 3 min. For unlabeled MARCKS proteins purified from *E. coli*, the same protocol was used except that 10–30 ng proteins were used to allow their analysis by SDS-PAGE and Western blotting (see below).

2.9. Remyristoylation of [^{35}S]-MARCKS cleaved by macrophage or calf brain extracts

[^{35}S]-MARCKS was cleaved with macrophage P100 or calf brain S100 as described above (section 2.8.) except that the reaction volume was increased seven-fold (140 µL) to produce sufficient material for the incubation with NMT. At the end of the incubation, MARCKS products were purified from the other components of the reaction mixture by heating the solutions for 5 min at 95 °C in the presence of 0.4% Tx-100, followed by cooling on ice and centrifugation (14 000 *g*; 15 min). The supernatant of the samples incubated with macrophage

P100 was removed and incubated in the presence of NMT as described above (section 2.4.).

2.10. Analysis of proteins on polyacrylamide gels

SDS-PAGE was performed according to [35]. The proteins labeled with [^{35}S]-Cys or [^{35}S]-Met were visualized by screening the dried polyacrylamide gels in a Phosphor-Imager (Molecular Dynamics). Fluorography was used for detection of proteins labeled with [^{3}H]-myristate [36]. Unlabeled MARCKS or MRP were detected by Western blotting using polyclonal anti-MARCKS (a kind gift of Dr. Stéphane Manenti, INSERM, Toulouse, France) or anti-MRP as described [37].

2.11. Identification of the site of cleavage of MARCKS by the macrophage cytosolic extract

100 µg myr MARCKS, expressed in *E. coli* and purified, were incubated for 4.5 h at room temperature (RT) with macrophage S100 (13 mg protein) using the low ionic strength protocol (final volume: 350 µL). In order to stop the reaction and to remove proteins of the S100 extract, the solution was then heated for 10 min at 95 °C, kept on ice for 5 min, and the precipitated proteins were removed by centrifugation for 15 min at 13 000 rpm in an Eppendorf centrifuge. The extent of cleavage of MARCKS by S100 was estimated to be > 95%, as judged by visual inspection of Coomassie-blue stained polyacrylamide gels onto which a fraction of the solutions had been electrophoresed. The supernatant of the heated solution, which contained almost pure MARCKS, was used as is for sequencing by Edmann-degradation of the MARCKS product resulting from the incubation.

2.12. Phosphate analysis

The concentration of phospholipids present in macrophage P100 was determined by phosphate analysis [38].

2.13. Determination of protein concentration

Determination of protein concentrations was performed according to [39]. For particulate fractions, lipids were removed by precipitating the proteins with trichloroacetic acid (TCA) in the presence of sodium deoxycholate.

2.14. Binding of MARCKS to membranes of the macrophage particulate extract

Two µL of the translation reaction expressing [^{35}S]-MARCKS were titrated with increasing amounts of macrophage P100, the phospholipid concentration being used to estimate the 'relative amounts of membrane' present in the assay. The samples were incubated for 1 h at RT in buffer 10 mM MOPS, 0.1 mM EGTA, 100 mM NaCl, 1 mM DTT (final volume: 75 µL). As controls 25 µL were transferred to a new tube and incubated for additional 60 min at RT. The remaining solution was centrifuged at 100 000 *g* (55 000 rpm, 22 °C, 1 h, TLA 100.3 Rotor, Beckman centrifuge) to separate membranes from the aqueous solution. MARCKS remaining in the supernatant was analyzed by SDS-PAGE and phosphor-imaging. The relative amount of MARCKS present in the solution prior to centrifuging was estimated by analyzing the controls.

3. Results and discussion

3.1. The particulate fraction of murine macrophages cleaves the N-terminus of human MARCKS

While investigating the binding of MARCKS to phagosomes isolated from macrophage cultures we found evidence for a specific modification of this protein, as judged by a shift in the apparent molecular mass on SDS polyacrylamide gels. This phenomenon was investigated in detail and is the subject of this report. We chose to express MARCKS using rabbit reticulocyte lysates (RRL). This cell-free system contains the enzymes and cofactors required to express a myristoylated form of MARCKS [30]. In addition, since MARCKS has three Cys residues at positions 278, 309 and 320, its C-terminus can be labeled radioactively by translating the mRNA in the presence of [^{35}S]-Cys. A labeled protein ([^{35}S]-MARCKS) with an apparent molecular mass of 83 kDa (p83) can accordingly be expressed and detected by autoradiography following SDS-PAGE of the translation reaction (*figure 1*, lane a). That p83 is myristoylated can be shown by translating the mRNA coding for MARCKS in the presence of [^{3}H]-myristate and analyzing the product of the reaction ([^{3}H]-MARCKS) by SDS-PAGE and fluorography (*figure 1*, lane c). Incubation of [^{35}S]-MARCKS with the particulate fraction (P100) of RAW macrophages results in a decrease in the intensity of p83 and in the appearance of a second band with a lower apparent molecular mass (p65) (*figure 1*, lane b). The relatively small shift in apparent molecular mass of MARCKS produced by the incubation suggests a site of cleavage close to either the N- or the C-terminus. Since incubation of [^{3}H]-MARCKS with macrophage P100 results in a decrease in the intensity of p83 without an increase in the intensity of p65 (*figure 1*, lane d) we conclude that macrophage P100 contains an activity which removes the myristoylated N-terminus of MARCKS. This activity will be referred as to the 'macrophage cleaving activity' throughout the text. Note the presence of a 38 kDa myristoylated band which is also processed by macrophage P100 and which, due to its absence in the [^{35}S]-Cys-labeled reaction, is likely to be a N-terminal translation product of MARCKS mRNA (*figure 1*, lanes c and d).

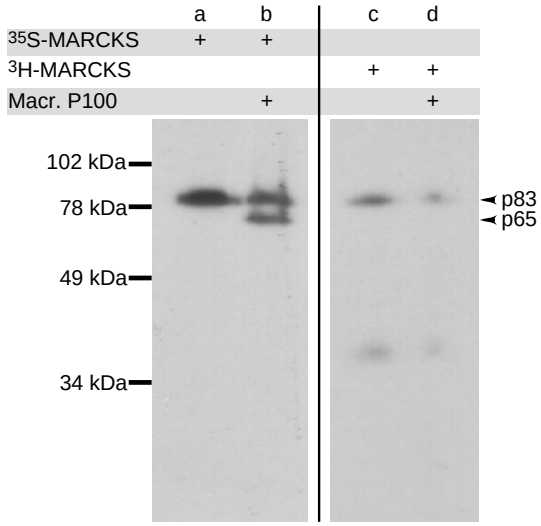

Figure 1. N-terminal MARCKS-cleaving activity of the particulate fraction of murine macrophages. [^{35}S]-MARCKS (a, b) and [^{3}H]-MARCKS (c, d) were incubated for 3 h at RT in the absence (a, c) or presence (b, d) of macrophage P100 (25 µg protein). The reaction was stopped by the addition of SDS-sample buffer followed by heating at 95 °C for 3 min. The MARCKS polypeptides present at the incubation (p83, p65) were analyzed by SDS-PAGE and detected by autoradiography ([^{35}S]-MARCKS) or fluorography ([^{3}H]-MARCKS).

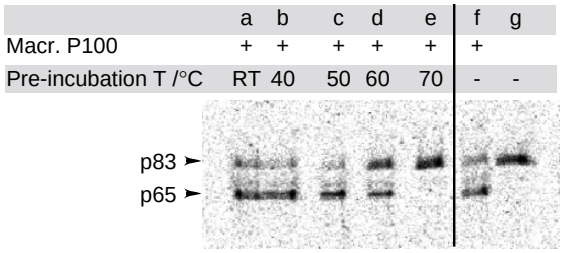

Figure 2. Effect of temperature on the MARCKS cleaving activity of the particulate fraction of macrophages. Macrophage P100 aliquots (25 µg protein) were pre-incubated for 2 h at increasing temperatures (a, RT; b, 40 °C; c, 50 °C; d, 60 °C; e, 70 °C). The cleaving activity of these extracts was subsequently measured by incubation with [^{35}S]-MARCKS for 3 h at RT. The extent of processing of p83 to p65 was analyzed by SDS-PAGE followed by autoradiography. As controls [^{35}S]-MARCKS was incubated in the presence (lane f) or in the absence (lane g) of a macrophage P100 aliquot which had not been pre-incubated.

3.2. Characterization of the MARCKS cleaving activity of macrophage extracts

Since myristoylation is a chemically stable lipid modification [40], cleaving of MARCKS by macrophage P100 is most likely due to an enzymatic activity. To confirm this assumption, we have pre-incubated macrophage P100 for 2 h at different temperatures and subsequently tested the cleaving activity of this extract. *Figure 2* shows a clear temperature-dependent loss of enzyme activity. An almost complete inhibition is observed when the extract is pre-incubated at 70 °C. These data strongly suggest that the macrophage cleaving activity has an enzymatic origin.

Figure 3 documents the time-dependence of the cleavage of MARCKS by macrophage P100. Under the conditions used in this assay 50% MARCKS is cleaved within 75 min as determined by an exponential fit. This experiment suggests that the enzyme-catalyzed character of the reaction could be investigated by measuring the initial rate of cleavage as a function of the substrate concentration. However, in order to allow cleavage and the analysis of an appropriate range of substrate concentrations, sufficient [^{35}S]-MARCKS should be purified from the in vitro translation reaction mix and/or from the incubation mix, which was not attempted. Note that P100 from a J774 macrophage culture also processes MARCKS to p65, showing that this activity is not restricted to extracts of a specific cell line (data not shown).

3.3. Comparison of the N-terminal MARCKS cleaving activities of murine macrophage and calf brain extract

Cleaving of the N-terminus MARCKS by macrophage P100 leads us to ask whether this activity is similar to the

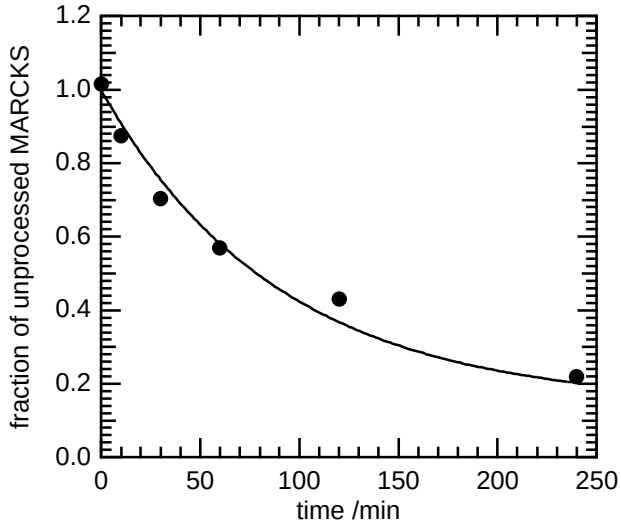

Figure 3. Kinetics of MARCKS cleavage by a murine macrophage particulate extract. [^{35}S]-MARCKS was cleaved at RT by a RAW macrophage P100 preparations (25 µg protein). The products of the reaction were analyzed by SDS-PAGE followed by autoradiography and scanning of the intensities of the p83 and p65 bands. The graph shows cleavage (expressed as the ratio of the intensity of p83 to the sum of the intensities of p83 and p63) as a function of time.

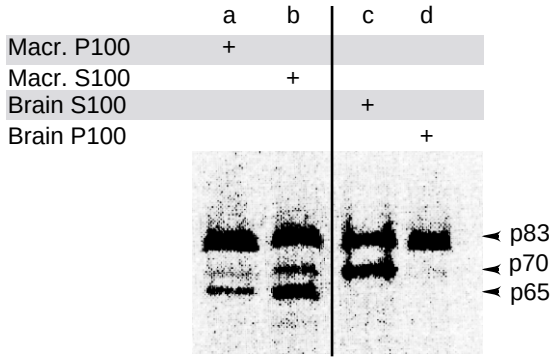

Figure 4. Comparison of the N-terminal MARCKS cleaving activities of calf brain and murine macrophage extracts. [^{35}S]-MARCKS (p83) was incubated at RT for 3 h in the presence of cellular extracts (a, macrophage P100; b, macrophage S100; c, calf brain S100; d, calf brain P100; 25 µg total protein). The reaction was stopped and the products (p70 and p65) were analyzed by SDS-PAGE followed by autoradiography. In order to separate p70 from p65 the electrophoresis was conducted for 80 min instead of the 40 min required to let the bromphenol blue dye migrate out of the gel.

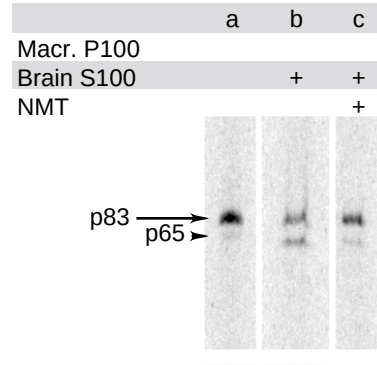

Figure 5. Remyristoylation of MARCKS cleaved by the calf brain cytosolic extract. [^{35}S]-MARCKS (p83) (lane a) was cleaved by calf brain S100 to p70 (lane b). Remyristoylation of p70 (lane c) was assessed by incubating the protein in the presence of myristoyl-CoA and NMT for 2 h at 30 °C. The samples were analyzed by SDS-PAGE followed by autoradiography.

demyristoylation activity previously reported in calf brain [24]. A comparison of the distribution of the macrophage and brain activities shows that the brain activity is strictly cytosolic *(figure 4)* (compare the incubation with calf brain S100 in lane c with the incubation with calf brain P100 in lane d) whereas the macrophage activity is present in both macrophage P100 (*figure 4*, lane a) and S100 (*figure 4*, lane b). More importantly, macrophage P100 and S100 process p83 to p65 whereas calf brain S100 processes p83 to p70, a protein with a slightly higher apparent molecular mass than p65. Thus, one has to conclude that the cleaving activities observed in these extracts are different. Interestingly, the brain extracts cannot process p83 to p65. Note also that the putative demyristoylation activity of calf brain S100 is also present in the macrophage fractions, in particular in S100. Preliminary studies show that the relative cleaving activities of macrophage S100 and P100 strongly depend on the fractionation protocol (ionic strength, volume of the buffer used for lysis and centrifugation) suggesting that a peripheral membrane protein might be responsible for the activity of the extracts (data not shown).

It should be noted that 0.5% Triton X-100 completely inhibits cleavage of MARCKS by both macrophage and calf brain S100 (data not shown). The mechanism of this inactivation is unclear: Triton X-100 could possibly bind to the N-terminus of MARCKS, in particular to the myristoyl moiety, and consequently hinder the interaction of the cleaving enzymes with MARCKS. Triton X-100 could also modify the quaternary structure of MARCKS; this mechanism is however unlikely since MARCKS can be cleaved either in solution by S100 *(figure 4)* or when membrane-bound in the presence of P100 *(figure 10)*. Alternately, Triton X-100 could directly inhibit the activity of the cleaving enzymes.

Using NMT and myristoyl-CoA we have previously shown that unmyr MARCKS proteins can be myristoylated in vitro [41]. We have taken advantage of this property to characterize the N-terminus of the MARCKS polypeptides produced by calf brain S100 (p70). [^{35}S]-MARCKS was cleaved by this extract and the reaction was stopped by heating at 95 °C (MARCKS is heat-soluble). The supernatants containing MARCKS were then incubated with NMT in the presence of myristoyl-CoA. *Figure 5* shows that p70, i.e., MARCKS cleaved by calf brain S100, can be myristoylated (*figure 5*, compare lanes b and c). Interestingly, incubation of brain cytosols with ATP and [^{3}H]-myristic acid resulted in the myristoylation of MARCKS by the radioactive fatty acid [42]. Thus, the presence of the demyristoylation activity, NMT and an active acyl-CoA synthetase in the cytosol, would provide the means for an acylation/de-acylation cycle to be established. In contrast p65, i.e., MARCKS cleaved by macrophage P100, cannot be remyristoylated (data not shown). To exclude the possibility that the lack of myristoylation of p65 could be due to the presence of inhibitors of NMT in macrophage P100, we have also incubated p65 in the presence of 1 µg unmyr MARCKS which had previously been expressed in *E. coli* and purified. Again, radioactively-labeled p65 could not be myristoylated whereas purified unmyr MARCKS could, as judged by a shift from p70 to p83 observed in a Coomassie blue-stained polyacrylamide gel (data not shown). Thus we conclude that, although the NMT is active, p65 cannot be myristoylated by NMT.

3.4. N-terminal sequencing of p65

The results presented so far show that macrophage extracts contain a cleaving activity which removes the N-terminus of MARCKS, resulting in a polypeptide which cannot be remyristoylated by NMT. p65 might either be a demyristoylated form of MARCKS whose N-terminal Gly residue is blocked (the consensus sequence for myristoylation is Gly-X-X-X-Ser) or p65 might result from a proteolytic cleavage close to the N-terminus of MARCKS. To characterize the N-terminus of p65 we have expressed myr MARCKS in *E. coli* and purified the protein. Myr MARCKS was then incubated with macrophage S100. As for the protein expressed in the cell-free system, the extract processes myr MARCKS to p65. The reaction was stopped by heating the solution at 95 °C and the precipitated macrophage proteins were removed by centrifugation. The supernatant was subjected to N-terminal Edman degradation. An analysis of the amino acid released by the reaction reveals the presence of the following sequence: Thr-Ala-Ala-Lys-Gly-Glu. Since the N-terminal sequence of human MARCKS is: myristoyl-Gly-Ala-Gln-Phe-Ser-Lys-Thr-Ala-Ala-Lys-Gly-Glu-...

we conclude that macrophage P100 cleaves MARCKS between Lys6 and Thr7.

3.5. Mechanism of N-terminal cleavage of MARCKS by macrophage extracts

The coexistence of MARCKS demyristoylating- and N-terminal proteolytic activities in macrophage fractions suggests two pathways by which p65 could be produced. Myr MARCKS could be directly cleaved by a protease to yield p65 (pathway a). Alternately, production of p65 could only take place following demyristoylation of MARCKS (pathway b). We have reasoned that NMT could inhibit pathway b by competing with the putative protease whereas it should not in pathway a. To investigate the mechanisms responsible for the production of p65, we have therefore incubated MARCKS with macrophage P100 or calf brain S100 in the presence or absence of NMT plus myristoyl-CoA. *Figure 6* reveals that NMT does not compete with the macrophage cleaving activity (the ratio p83/p65 remains similar in the presence of NMT; compare lanes b and c) whereas it inhibits the activity of the brain extract (the ratio p83/p70 is increased by the presence of NMT; compare lanes d and e). These results apparently support pathway a in which myristoylated MARCKS is directly processed to p65 by the macrophage extract. Similar results would also be obtained if an enzyme processes p70 to p65, according to pathway b, was so effective that remyristoylation by NMT could not take place.

To further differentiate between these pathways we have then asked whether unmyr MARCKS (p70) can be processed to p65 by macrophage S100. To this end we first

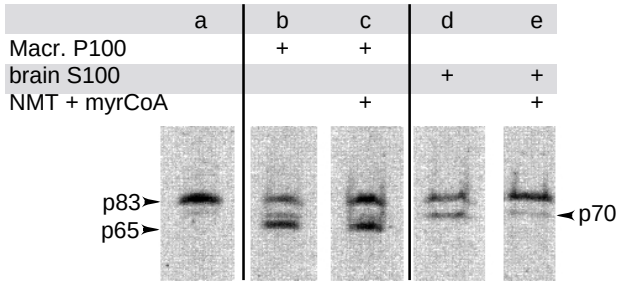

Figure 6. N-terminal cleavage of MARCKS by cellular extracts in the presence of NMT. [^{35}S]-MARCKS was incubated with macrophage P100 (lanes b and c) or calf brain S100 (lanes d and e) (25 µg protein) in the absence (lanes b and d) or presence (lanes c and e) of NMT (20 ng/mL) and 25 µM myristoyl-CoA. After 2 h at RT the reaction was stopped and the reaction products (p83, p70 and p65) analyzed by SDS-PAGE followed by autoradiography.

needed to obtain unmyr MARCKS. An unmyristoylated protein can be produced in a cell-free system by expressing MARCKS in the presence of an inhibitor of NMT or by altering the consensus sequence for myristoylation by site-directed mutagenesis (usually Gly to Ala) [30]. In our hands DL-β-hydroxymiristic acid, an inhibitor of NMT, was not efficient (data not shown). We have also reasoned that a mutation at the N-terminus might alter the recognition of MARCKS by the putative protease producing p65 in macrophage extracts. We have therefore chosen to express unmyr MARCKS in *E. coli* (*E. coli* does not possess an endogenous NMT activity) and to purify the protein. Unlike myr MARCKS, which could be obtained in sufficient qualities and quantities for the sequencing experiments described above, purified unmyr MARCKS was contaminated with a series of degradation products which appear as a smear on the Western blots and whose largest apparent molecular mass, denoted *, was slightly lower than 65 kDa (*figure 7*, lane b). The same degradation products were present after in vitro myristoylation by NMT (*figure 7*, lane a), suggesting that the N-terminus of unmyr MARCKS is exposed to *E. coli* proteases. After addition of macrophage S100 to MARCKS and heating, the relative reactivity of the MARCKS antibody for the bands detected in lanes a and b is decreased and/or modified such that the contribution of the degradation products to the Western blot signal is limited to the upper band labeled * for unmyr MARCKS (compare *figure 7*, lanes b and f) and almost no longer apparent for myr MARCKS (compare *figure 7*, lanes a and c). These fortunate observations allow a confident interpretation of the results obtained in this experiment. Unlike myr MARCKS which is processed to p65 by macrophage S100 (*figure 7*, lanes c–e), unmyr MARCKS (p70) remains constant throughout the incubation (*figure 7*, lanes f–h). As discussed above, * (*figure 7*, lanes f–h) should not be

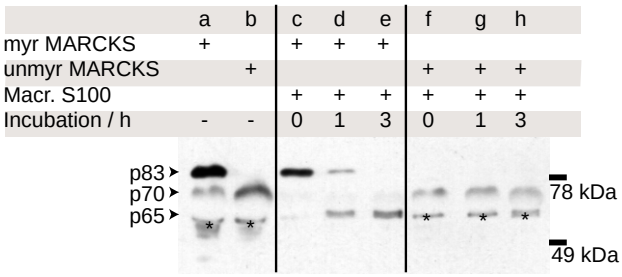

Figure 7. Lack of cleavage of unmyr MARCKS by macrophage extracts. Unmyr MARCKS was expressed in *E. coli* and purified (lane b). An aliquot of this protein was subsequently myristoylated by NMT in the presence of myristoyl-CoA (lane a). Approximately 30 ng myr- (lanes c–e) or unmyr- (lanes f–h) MARCKS were incubated at RT for 0 (lanes c and f), 1 (lanes d and g) or 3 (lanes e and h) h with macrophage S100 (25 µg protein). The products resulting from these incubations (half of the reaction volume) were analyzed by Western blotting using a polyclonal antibody recognizing human MARCKS. The band denoted with an asterisk (*) is a degradation product produced during the purification of unmyr MARCKS from *E. coli* (see text).

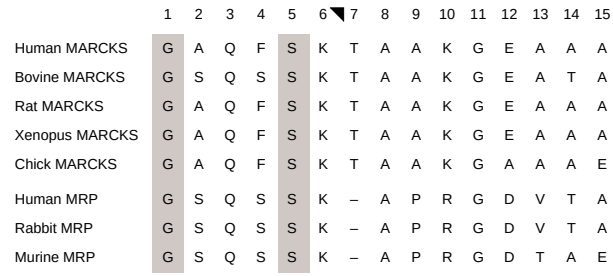

Figure 8. N-terminal sequences of different MARCKS and MRP proteins. The first 15 N-terminal amino acid residues of MARCKS (14 for MRP) are shown. The consensus sequence for myristoylation, Gly-X-X-X-Ser, is shaded. The site of cleavage of MARCKS by the macrophage extracts is indicated by an arrowhead between residues 6 and 7.

mistaken with p65 since it is a degradation product originating from the purification of unmyr MARCKS from *E. coli*. An appropriate way to appreciate the lack of cleavage of unmyr MARCKS by the macrophage extract is therefore to compare the time-dependent decrease in the p85/p65 ratio (*figure 7*, lanes c–e) to the constant p70/* ratio (*figure 7*, lanes f–h). Note that myr and unmyr MARCKS are stable when incubated in the absence of macrophage S100 (data not shown).

An analysis of the radioactive fragments produced during incubation of MARCKS, labeled by addition of [^{3}H]-myristoyl-CoA during its biosynthesis, reveals the presence of unidentified myristoylated products suggesting that the N-terminal myristoylated peptide primarily produced by the macrophage extracts, i.e., myr-Gly-Ala-Gln-Phe-Ser-Lys, could be further cleaved to eventually lead to myristate (data not shown).

Taken together our results show that macrophage extracts contain a proteolytic activity which specifically cleaves the N-terminus of MARCKS between Lys6 and Thr7 and which requires myristoylation of MARCKS (pathway a).

3.6. Processing of MARCKS-related protein (MRP) by macrophage extracts

Although MRP, the second member of the MARCKS protein family, is significantly smaller than MARCKS (22 kDa for MRP versus 32 kDa for MARCKS) both proteins share important properties including an acidic pI, a conserved effector domain and N-terminal myristoylation. In this respect, both proteins interact with calmodulin, protein kinase C, actin, and membranes [1, 4]. Although MARCKS proteins contain the consensus sequence for myristoylation an inspection of their N-terminal sequences reveals two important differences *(figure 8)*: 1) residue 7 which is a threonine in MARCKS is missing in MRP; and 2) the alanine at position 9 in MARCKS is replaced by a proline in MRP. It was therefore interesting to investigate whether macrophage extracts can cleave MRP. In analogy to the experiments reported in *figure 7*, unmyr and myr MRP were expressed in *E. coli*, purified and incubated with macrophage S100 *(figure 9)*. The resulting MRP products were analyzed by Western blotting using a polyclonal antibody directed against MRP. Note that both myr MRP (apparent molecular mass 42 kDa, lane a) and unmyr MRP (apparent molecular mass 38 kDa, lane d) appear as a doublet on polyacrylamide gels and can be nicely separated by running the electrophoresis for twice the time required to let the bromphenol blue dye migrate out of the 15% polyacrylamide SDS gels (Vergères G., unpublished work). The double band corresponding to myr MRP is cleaved to a lower doublet whose apparent molecular mass cannot be distinguished from unmyr MRP (*figure 9*, compare lanes a–c with lane d), suggesting that MRP is demyristoylated rather than being cleaved by the protease that cleaves MARCKS. Note also that unmyr MRP is apparently not cleaved as judged by the lack of shift in apparent molecular mass (*figure 9*, lanes d–f). That myr MRP is demyristoylated is demonstrated in two additional experiments: 1) in contrast to the results obtained with MARCKS (see *figure 6*), adding NMT and myristoyl-CoA to the macrophage extract completely prevents myr MRP from being cleaved (*figure 9*, lanes g–i); 2) MRP which has been cleaved by the macrophage extract can subsequently be remyristoylated by NMT (*figure 9*, lanes j–l). Note that myr and unmyr MRP are stable when incubated

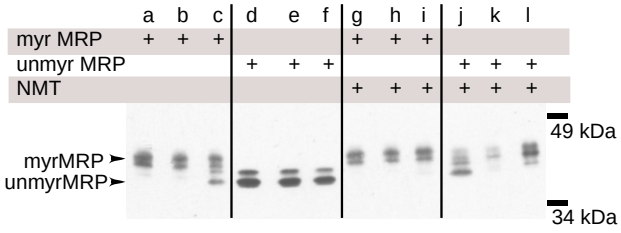

Figure 9. Processing of MRP by murine macrophage extracts. Myr and unmyr murine MRP were expressed in *E. coli* and purified. 50 ng myr- (lanes a–c) or unmyr- (lanes d–f) MRP were incubated at RT for 0 (lanes a and d), 1 (lanes b and e) or 3 (lanes c and f) hours with macrophage S100 (25 µg protein). Myr MRP was also incubated with macrophage S100 in the presence of NMT (0.02 µg/mL) and myristoyl-CoA (25 µM) (lanes g–i). As a control, 50 ng unmyr MRP was myristoylated by NMT (0.02 µg/mL) for 0 (lane j), 1 (lane k) and 3 (lane l) h at RT in the presence of myristoyl-CoA (25 µM). The products resulting from these experiments (half of the reaction volume) were electrophoresed on a 15% polyacrylamide gel and analyzed by Western blotting using a polyclonal antibody recognizing murine MRP.

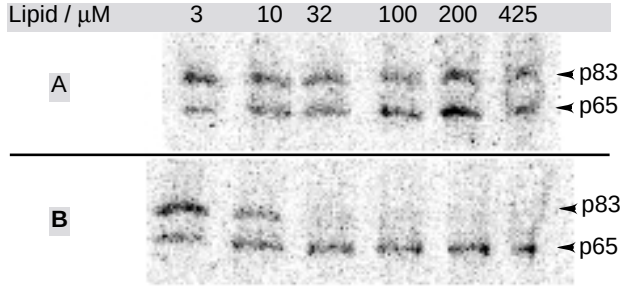

Figure 10. Regulation of MARCKS membrane association by the N-terminal cleaving activity of macrophage extracts. [^{35}S]-MARCKS was incubated for 1 h at RT with increasing amounts of macrophage P100 (measured as the concentrations of membrane phospholipids: 3–425 µM). **A.** At the end of the incubation one-third of the volume in the samples was analyzed by SDS PAGE followed by autoradiography. **B.** The remaining two-thirds were centrifuged at 100 000 *g* to pellet the membrane fraction and half of the supernatant was analyzed as in **A**.

in the absence of macrophage S100 (data not shown). Taken together, these results indicate that macrophage S100 can demyristoylate MRP but that the enzyme responsible for the N-terminal cleavage of MARCKS is not active on MRP.

3.7. Effect of the cleavage of MARCKS on its binding to macrophage membranes

Does cleavage of the myristoylated N-terminus of MARCKS modify its binding to membranes? To investigate this aspect we have produced p65 by incubating [^{35}S]-MARCKS (p83) with increasing amounts of macrophage P100 (the amounts of membranes present in the macrophage P100 aliquots were quantified by measuring their phospholipid concentrations). *Figure 10A* shows that the extract processes p83 to p65. The samples were then centrifuged at 100 000 *g* to separate the soluble from the particulate fractions, and the soluble fraction was analyzed for the presence of p83 and p65. *Figure 10B* shows that p65 remains in the supernatant, even in the presence of 425 µM phospholipids, whereas p83 already disappears from the supernatant in the presence of 10 µM phospholipids, as a consequence of its binding to membranes. These data therefore show that removing the N-terminus decreases the affinity of MARCKS for macrophage membranes by at least one order of magnitude.

4. Conclusions

In this report we have shown that the MARCKS product produced during the incubation of the protein with a cytosolic brain extract can be enzymatically remyristoylated. Our results also demonstrate that macrophage extracts contain a specific proteolytic activity which cleaves the N-terminus of MARCKS between Lys7 and Thr8. The specificity of this activity is expressed in two different manners: i) unnmyr MARCKS cannot be cleaved by the macrophage extracts, suggesting that myristoylation could be part of the recognition motif of the protease; Interestingly, myr MARCKS is cleaved by cytosolic extracts (i.e., in aqueous solution) as by particulate extracts (i.e., membrane-bound) indicating that the quaternary structure of the protein (i.e., aggregation state) is unlikely to be of relevance for its recognition by the protease; and ii) Myr MRP cannot be cleaved by the macrophage extracts, suggesting that the protease does not recognize every myristoylated protein. The absence of Thr7 at the N-terminus of MRP or the replacement of alanine at position 9 in MARCKS by a proline in MRP likely explains the lack of cleavage of this protein by the macrophage extracts. Noteworthy, other endoproteases have been identified that cleave peptide bonds at the level of hydroxy amino acid residues. For example, the bacterial pathogens *Streptococcus sanguis*, *Neisseria gonorrhoeae* and *Haemophilus influenza* produce proteases which cleave Pro-Thr peptide bonds in human immunoglobulin A1 [43]. An endopeptidase was also purified from *Archachatina ventricosa* which hydrolyzes only the amide bonds of peptide substrates having a threonine residue at the P1' position [44].

What might be the function of cellular enzymes cleaving MARCKS proteins? Several publications indicate that enzymatic cleavage of MARCKS could modulate the association of MARCKS proteins with membranes: 1) the soluble fraction of calf brain can demyristoylate

MARCKS [24] and MRP [31]. Whereas myr MARCKS mostly associates which the particulate fraction of calf brain [45], the non-myristoylated form has been purified from the soluble fraction [46]; 2) recently Spizz and Blackshear have reported that cathepsin B produces p40 by cleaving MARCKS between Asn197 and Glu148 on the N-terminal side of the effector domain [26, 27]. p40 was also detected in calf brain [47]. Since the affinity of a peptide corresponding to the effector domain of MARCKS for negatively-charged phospholipid vesicles is significantly higher than the affinity of the intact protein [48, 49] p40 could, due to the exposure of its effector domain, have a much higher affinity for membranes compared to unmyr MARCKS. Thus the affinity of MARCKS for intracellular membranes may depend on the degree to which it has undergone cleavage.

With the exception of the cleavage of MARCKS by cathepsin B [27] the enzymatic activities discussed in this report, including the cleavage of MARCKS by macrophages, have been reported in extracts but not in intact cells. An estimation of their in vivo relevance therefore awaits characterization in intact cells. Nevertheless, the fundamentally important finding of this report is that, calf brain cytosolic and the macrophage extracts contain a new class of enzymes whose recognition motifs involve lipids (i.e., myristate) covalently associated with a protein.

Acknowledgments

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