MacMARCKS Is Not Essential for Phagocytosis in Macrophages*

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MacMARCKS (also known as myristoylated alanine-rich protein kinase C substrate (MARCKS)-related protein) is a member of the MARCKS family of protein kinase C substrates. MacMARCKS contains within it a basic effector domain that contains the serine residues that are phosphorylated by protein kinase C, as well as a calcium/calmodulin and actin-binding site. Two previous reports demonstrated that a macrophage cell line expressing a mutant form of MacMARCKS that lacks the effector domain is defective in phagocytosis and cell adhesion (Zhu, Z., Bao, Z., and Li, J. (1995) J. Biol. Chem. 270, 17652–17655; Li, J., Zhu, Z., and Bao, Z. (1996) J. Biol. Chem. 271, 12985–12990). We report here that macrophages from MacMARCKS null mice phagocytose and spread normally. Thus, although MacMARCKS is recruited to phagosomes, it is not absolutely required for phagocytosis.

MacMARCKS, also known as myristoylated alanine-rich protein kinase C substrate (MARCKS)-related protein, is a PKC substrate that binds calcium/calmodulin and actin (reviewed in Refs. 1–3). MacMARCKS plays a role in coordinating the actin cytoskeleton during such diverse processes as neural tube closure and synaptic transmission and has been proposed to play an important role in phagocytosis by macrophages (4–10). MacMARCKS and the closely related family member, MARCKS, are rod shaped proteins that can be divided into three domains based on sequence homology and function (4). The amino terminus consists of an amino-terminal glycine that is myristoylated followed by a short stretch of highly conserved amino acids with unknown function called the MH2 (MARCKS homology 2) domain (4). The MH2 domain is followed by a short stretch of basic amino acids, known as the effector domain, which binds calcium/calmodulin, actin, and acidic lipids and which contains the serines that are phosphorylated by PKC (4, 5, 11, 12). PKC-dependent phosphorylation of the effector domain of MARCKS regulates the binding of calcium/calmodulin and F-actin to it and also regulates the association of MARCKS with membranes (13–16). Much less is known about the effector domain of MacMARCKS; however, the domains are structurally very similar, and PKC also regulates the binding of calcium/calmodulin to the MacMARCKS effector domain (4, 5).

The biochemical data suggest that MacMARCKS, like MARCKS, plays a role in integrating the effects of PKC and calcium on actin dynamics.

MacMARCKS has recently been suggested to play a crucial role in regulating the actin cytoskeleton during phagocytosis in macrophages; it is rapidly recruited to the forming phagosome, and expression of a mutant form of the protein lacking an effector domain was reported to completely inhibit phagocytosis in a macrophage cell line (9). A subsequent report from the same group demonstrated that macrophages expressing this mutant protein were also incapable of spreading (10). In this report we demonstrate that although MacMARCKS associates with phagosomes, its presence is not required for phagocytosis; macrophages derived from MacMARCKS null mice phagocytose zymosan normally. Macrophages from MacMARCKS null mice also spread normally. In addition, we show that in our hands, the mutant form of MacMARCKS that lacks the effector domain does not associate with phagosomes, does not displace WT MacMARCKS from phagosomes, and does not inhibit phagocytosis when transfected into a macrophage cell line.

EXPERIMENTAL PROCEDURES

Materials—Unless indicated otherwise, all reagents were obtained from Sigma.

Generation of MacMARCKS Null Macrophages—Heterozygous MacMARCKS+/- mice (7) were mated, and macrophages were prepared from fetal liver using a variation of a protocol developed by Moore and Williams (17). Briefly, fetuses were sacrificed at day 14.5 of gestation, and livers were harvested. Limbs and tails were collected at the same time for genotyping as described previously (7). The livers were dispersed by passage two times through a 26G needle, red blood cells were lysed, and the remaining cells were plated in 10-cm tissue culture dishes in Iscove’s modified Dulbecco’s medium (JRH Biosciences, Lenexa, KS) with 20% fetal calf serum (Hyclone, Logan, UT), 15% L cell conditioned medium, and supplemented with 1% L-glutamine, 100 units/ml penicillin G, and 100 μg/ml streptomycin. Cells were cultured at 37 °C in a 5% CO2 atmosphere, and half of the medium was changed every 3 days. After 7–10 days nonadherent cells were washed away, and the remaining cells (macrophages) were scraped off the plate in ice-cold phosphate-buffered saline (PBS) and plated for subsequent experiments.

Cell Culture—RAW 264.7 (ATCC TIB-71) were maintained at 37 °C in a 5% CO2 atmosphere in Hepes-buffered RPMI containing 10% fetal calf serum (Hyclone) supplemented with 1% L-glutamine, 100 units/ml penicillin G, and 100 μg/ml streptomycin. Cells were passaged by resuspension in PBS containing 1 mM EDTA.

Immunofluorescence Microscopy—Macrophages were processed for immunofluorescence microscopy as described previously (18). Briefly, cells were grown on glass coverslips, stimulated with 50 ng/ml lipopolysaccharide for 3 h, fixed in 10% neutral buffered formalin (Sigma), and permeabilized in acetone at −20 °C. Nonspecific sites were blocked by incubation in PAB (PBS, 0.5% bovine serum albumin, and 0.05% sodium azide) containing 10% horse serum, and primary antibodies diluted in PAB with horse serum were applied to the cells at 1 h at room temperature. The cells were washed in PAB, and the appropriate sec—

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Secondary antibodies diluted in PAB with horse serum were incubated with the cells for 1 h. After further washing in PAB, cells were mounted and observed by confocal microscopy using a MRC-1024 system (Bio-Rad) equipped with LaserSharp software and mounted on an Axiovert TV microscope (Carl Zeiss, Inc., Thornwood, NY) as described previously (18).

MARCKS and MacMARCKS were visualized using affinity purified rabbit antibodies as described previously (6, 19). F-actin was detected using FITC-phalloidin or tetramethylrhodamine isothiocyanate-phalloidin (Molecular Probes, Eugene, OR). Talin was detected using monoclonal antibody 8d4 (Sigma). The HA-tagged proteins were detected using a polyclonal rabbit anti-HA antibody (Babco, Berkeley, CA).

Phagocytosis Assays—Phagocytosis of zymosan by primary macrophages was measured as described previously (18). Zymosan particles (Sigma) in Hepes-buffered RPMI were centrifuged onto macrophage monolayers at 4 °C, the cells were warmed by addition of RPMI at 37 °C, and internalization was allowed to proceed. The cells were either fixed and processed for microscopy after 3 min of internalization as described above, or live cells were stained with 0.4% trypan blue in PBS after 90 min of internalization to facilitate counting of phagocytosed particles. Phagocytosis of zymosan by RAW cells was measured by centrifuging FITC-zymosan (Molecular Probes, Eugene, OR) onto macrophage monolayers as described above. After 1 h of internalization, the cells were washed, extracellular zymosan was dissolved by addition.
MacMARCKS and Phagocytosis

FIG. 3. Expression of AED-MacMARCKS has no effect on phagocytosis in macrophages. A, expression of MacMARCKS proteins in RAW 264 cells. Expression levels of endogenous MacMARCKS in control cells (lanes 1 and 4) was compared with that of HA-tagged wild type MacMARCKS (MM-WT) (clones AB12.1 and AC12.2 are shown in lanes 2 and 3, respectively), and HA-tagged MacMARCKS lacking the effector domain (MM-∆ED) (clones BF5.1 and BG9.1 are shown in lanes 5 and 6, respectively). Cell extracts were subjected to SDS-polyacrylamide gel electrophoresis and transferred to Immobilon, and HA-tagged proteins (HA, upper panels) and MacMARCKS proteins (MM, lower panels) were detected by immunoblotting with specific antibodies. B, localization of HA-tagged proteins to phagosomes. RAW 264 cells expressing either the HA-tagged wild type MacMARCKS (upper panels) or the HA-tagged MM-∆ED (lower panels) were allowed to ingest zymosan particles for 15 min, fixed, and processed for immunofluorescence microscopy as described under “Experimental Procedures.” Actin was localized with rhodamine-phalloidin (left panels), and HA-tagged MacMARCKS proteins were localized with an antibody to the HA epitope (right panels). The arrows indicate internalized particles.

of 100 units/ml lyticase in PBS for 10 min at room temperature, and cells were resuspended in PBS + 1 mM EDTA and analyzed by flow cytometry using a FACScan and CELLQuest software (Beckton Dickinson, San Jose, CA). Phagocytic indexes (number of particles/100 cells) were calculated by comparison of cellular fluorescence to fluorescence of single zymosan particles and expressed as phagocytic index ± S.E.

*cDNA Constructs—*The pcDNA 3 (Invitrogen, Carlsbad, CA) expression vectors encoding MacMARCKS and MacMARCKS ∆-effector domain fused to HA at their carboxyl termini have been described before (20). The wild type construct encodes all 200 amino acids of MacMARCKS, whereas the effector domain deletion mutant excludes amino acids 86–108. DNA for transfections was prepared using Qiagen Tip-500s according to the manufacturer’s instructions (Qiagen, Santa Clarita, CA).

*Macrophage Transfection—*RAW 264 cells were transfected using a variation on the method described by Stacey et al. (21). Five million cells in 210 μl of culture medium were placed in a 0.4-cm gap cuvette along with 5 μg of plasmid DNA in 40 μl of PBS. After 10 min at room temperature, the cells were electroporated using a Gene Pulser (BioRad) set at 960 microfarad and 300 V. The cells were immediately transferred to 5 ml of medium, pelleted by centrifugation at 600 × g, resuspended in fresh medium, and cultured for 24 h before addition of 400 μg/ml G418 (Life Technologies, Inc.). After 10 days of selection, cells were cloned twice by limiting dilution, and cells expressing the desired proteins were analyzed by immunoblotting.

*Immunoblotting—*Whole cell extracts of RAW cells were prepared by resuspending cells in solubilization buffer (1% Triton X-100, 150 mM NaCl, 2 mM EDTA, 50 mM Tris, pH 7.4, plus 0.5 mg/ml leupeptin, 0.09 trypsin inhibitory units aprotinin, and 1 mM phenylmethylsulfonyl fluoride) and incubating the mixture on ice for 20 min. Insoluble material was pelleted by centrifugation at 12,000 × g for 10 min in a microfuge, and the protein content of the supernatant was analyzed by the BCA method (Pierce). 20 μg of protein from each sample were resolved by 10% SDS-polyacrylamide gel electrophoresis and transferred to Immobilon-P (Millipore, Bedford, MA). The membranes were blocked overnight at 4 °C in blotto (PBS with 5% milk, 0.01% Tween 20, and 5 mM azide). Primary antibodies were diluted in blotto and applied to the membrane for 2 h at room temperature. The membranes were washed thoroughly with PBS ± 0.01% Tween 20 and incubated for 30 min with secondary horseradish peroxidase-conjugated antibodies (Amersham Pharmacia Biotech). The hemagglutinin tag was detected using a polyclonal rabbit anti-HA antibody (Babco, Berkeley, CA), and MacMARCKS was detected using a MacMARCKS-specific polyclonal antiserum (6).

**RESULTS AND DISCUSSION**

MacMARCKS null mice die at birth due to neural tube closure defects, so macrophages from adult MacMARCKS knockout mice could not be assessed for phagocytic competence (7). To study MacMARCKS null macrophages, we cultured fetal liver-derived macrophages from 14½ day MacMARCKS null embryos. Macrophages were recovered identically from wild type and null (MMKO) fetuses; cells were recovered in similar numbers and had similar morphologies (Fig. 1). Immunofluorescence microscopy and immunoblotting studies with antibodies to MARCKS and MacMARCKS confirmed that the MacMARCKS –/– macrophages had no detectable MacMARCKS, whereas the protein was easily detected in macrophages from wild type litter mates (7) (Fig. 1 and data not shown). The distribution and expression of MARCKS in the MacMARCKS null macrophages was unchanged; MARCKS was found diffusely distributed throughout the cells and on plasma membranes with occasional accumulation on plasma membrane ruffles (Fig. 1 and data not shown). MacMARCKS null macrophages spread normally in response to bacterial lipopolysaccharide and in response to phorbol esters (Fig. 1 and data not shown).

When wild type and MacMARCKS null macrophages were incubated with zymosan particles, no differences in phagocytic ability were observed. Wild type cells had a phagocytic index (particles/100 cells) of 444 ± 35, whereas MacMARCKS null macrophages had a phagocytic index of 397 ± 45. Because MacMARCKS was not required for internalization of zymosan, we examined more carefully the formation and maturation of the nascent phagosome. One of the earliest steps in phagocytosis is the polymerization of actin beneath a surface-bound particle. Localization of F-actin with FITC-phalloidin revealed that actin polymerized identically around nascent phagosomes in wild type and MacMARCKS null macrophages (Fig. 2) and

| cDNA          | Clone | Phagocytic index |
|---------------|-------|------------------|
| None          | Parental RAW 264 | 84 ± 1          |
| MM-WT         | AC11.2 | 72 ± 1          |
| AB12.1        |       | 77 ± 1          |
| MM-∆ED        | BG9.1  | 77 ± 1          |
| BF5.1         |       | 88 ± 1          |

**TABLE I**

Phagocytosis by macrophages expressing ∆ED-MacMARCKS

Untransfected RAW cells or two independent clones each expressing wild type or mutant forms of MacMARCKS were allowed to phagocytose FITC-zymosan. 10,000 cells were analyzed by flow cytometry, and the phagocytic index for each cell line was calculated as described under “Experimental Procedures.”
depolymerized after particle internalization (data not shown). Talin, an actin-binding protein that colocalizes with actin during phagocytosis of zymosan (18), was also found associated with phagosomes in MacMARCKS null macrophages. MARCKS also accumulated normally around nascent phagosomes, raising the possibility that MARCKS and MacMARCKS may have interchangeable functions during phagocytosis. 90 min after particle internalization, the lysosomal membrane marker, lamp-1, was found on zymosan-containing phagosomes in wild type and MacMARCKS null macrophages, suggesting that after particle internalization and subsequent actin depolymerization, phagosome maturation proceeded normally (data not shown).

A requirement for MacMARCKS in phagocytosis and macrophage spreading has been proposed by Li and co-workers (9, 10) based on experiments using the mouse macrophage cell line, J774. In their hands, expression of a mutant form of Mac-MARCKS lacking the effector domain in J774 cells inhibited phagocytosis of zymosan and prevented cell spreading (9, 10). Because these observations were at variance with our data using MacMARCKS null macrophages, we attempted to reproduce the inhibition of macrophage functions during phagocytosis. 90 min after particle internalization, the lysosomal membrane marker, lamp-1, was found on zymosan-containing phagosomes in wild type and MacMARCKS null macrophages, suggesting that after particle internalization and subsequent actin depolymerization, phagosome maturation proceeded normally (data not shown).

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Taken together, the above data demonstrate that although MacMARCKS localizes to phagosomes, it is not absolutely required for phagocytosis in macrophages. It is possible that MacMARCKS and MARCKS have identical or overlapping functions during phagocytosis and that in the absence of Mac-
MARCKS, MARCKS activity is sufficient. This possibility is currently under investigation.

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