Macrophage spreading requires the microtubule cytoskeleton and protein kinase C (PKC). The mechanism of involvement of the microtubules and PKC in this event is not fully understood. Dynamitin is a subunit of dynactin, which is important for linking the microtubule-dependent motor protein dynein to vesicle membranes. We report that dynamitin is a Ca\(^{2+}\)/calmodulin-binding protein and that dynamitin binds directly to macrophage-enriched myristoylated alanine-Rice C kinase substrate (MacMARCKS), a membrane-associated PKC substrate involved in macrophage spreading and integrin activation. Dynamitin was found to copurify with MacMARCKS both during MacMARCKS purification with conventional chromatography and during the immunosorption of MacMARCKS using anti-MacMARCKS antibody. Vice versa, MacMARCKS was also found to co-sediment with the 20 S dynactin complex. We determined that the effector domain of MacMARCKS is required to interact with the N-terminal domain of dynamitin. MacMARCKS and dynamitin also partially co-localized at peripheral regions of macrophages and in the cell-cell border of 293 epithelial cells. Treatment with phorbol esters abolished this co-localization. Disrupting the interaction with a short peptide derived from the MacMARCKS-binding domain of dynamitin caused macrophages to spread and flatten. These data suggest that the dynamitin-MacMARCKS interaction is involved in cell spreading. Furthermore, the regulation of this interaction by PKC and Ca\(^{2+}\)/calmodulin provides a possible regulatory mechanism for cell adhesion and spreading.

Cell adhesion and spreading are essential for many physiological processes, including immune cell activation, angiogenesis, and cell proliferation and differentiation (1–5). Many cytoskeletal proteins (6, 7), including the microtubules (8–10), are involved in these processes. Particularly in macrophages, depolymerization of microtubules with nocodazole causes the spreading macrophages to retract quickly into a spherical shape (11). In fibroblasts, cell spreading is impaired when microtubules are disassembled (8, 9). Kinesin, the plus-end-directed microtubule-dependent motor protein, has also been implicated in cell spreading, because anti-kinesin antibody decreases cell spreading when injected into cells (10). However, it is not clear whether the microtubule cytoskeleton provides merely a structural support or plays a more active role such as regulating focal adhesion assembly. Recently, studies have revealed that cell adhesion and spreading closely correlates with microtubule function; the integrity of microtubules can influence both the growth of actin stress fibers and the assembly of focal adhesion points (12, 13). The growing microtubule filaments are targeted toward the focal adhesion point (13). The effects of microtubules on cell adhesion and on actin cytoskeleton are mediated by the GTP-binding protein Rho (14). All the data suggest an active role for microtubules in cell adhesion and spreading.

We report here that dynamitin, a subunit of the dynactin complex, is involved in stimulating cell spreading. Dynactin complex (reviewed in Refs. 15–18) is an important regulator of dynein, a microtubule-dependent minus end-directed motor protein (19). Dynactin complex was copurified with dynein and was found to stimulate dynein-mediated organelle movements (20, 21). This complex consists of as many as 10 subunits: p150\(^{\text{glued}}\)/p135\(^{\text{glued}}\) doublet, p62, dynamitin (p50), Arp1 (actin-related protein 1), actin, actin-capping protein \(\alpha\) subunit, actin-capping protein \(\beta\) subunit, p27, and p24 (reviewed in Refs. 17 and 18). These 10 subunits of dynactin form two distinct structure domains. The Arp subunit is an actin-related protein and is the key component in the filamentous backbone (22). The p150\(^{\text{glued}}\)/p135\(^{\text{glued}}\) subunits, homologues of Drosophila Glued gene product (23), form the side arm that binds to dynein (24), Arp, and microtubules (25). Dynamitin, the 50-kDa subunit of dynactin (26), is localized to the should (22). The dynactin complex was proposed to be a “molecular bridge” connecting dynein to vesicle membranes (15, 27) and was also proposed to enhance the processivity of dynein (28). This bridging function is based on observations that the p150\(^{\text{glued}}\) subunits of dynactin bind to the dynein complex (24) and Arp (25), whereas the Arp subunit binds to the spectrin network (18, 22, 25, 29) that lies underneath the cargo membranes (30). Dynamitin, when overexpressed, disrupts Golgi and lysosome distribution (31) and spindle organization (32). Overexpression of wild type dynamitin (32) or addition of excess amounts of recombinant full-length dynamitin (33) breaks the dynactin complex, whereas overexpression of the conserved N-terminal of dynamitin is not sufficient to break the dynactin complex but is sufficient to disturb the Golgi and endosomes (34). Because dynamitin overexpression dissociated dynein from the mitotic kinetochore (32) and Golgi membrane (35), it has been proposed to link dynein to its cargo.
However, interactions between motor proteins and membranes are usually reversible, and one or more regulatory mechanisms may exist. Our data demonstrate a direct binding between dynamitin and MacMARCKS. This interaction may regulate the association of dynactin to membrane, because the association of MacMARCKS to the membrane is regulated by PKC (36–38). MacMARCKS, also known as MARCKS-related protein, is a member of the MARCKS family of PKC substrate (38–40). MacMARCKS was so named because of its enrichment in endothox-in-activated macrophages (38). This myristoylated calmodulin-binding protein associates with both plasma and vesicle membrane, and its calmodulin-binding and membrane association are regulated by PKC-mediated phosphorylation on the two serine residues in its effector domain (36–38).

PKC-mediated signal transduction pathway is one of the major pathways that regulate cell spreading (41–45) and integrin activation (reviewed in Ref. 46). Both MARCKS and MacMARCKS participate in PKC-dependent cell spreading (47, 48). Other studies have shown that the role of MacMARCKS in cell spreading may be mediated by its regulation of the activation of β2 integrin (49–51). MacMARCKS is also involved in vesicle redistribution (52). Therefore, the interaction between dynamitin and MacMARCKS may explain the involvement of microtubules in cell adhesion and spreading and may provide a regulatory mechanism for the link between dynactin and the membrane gauges.

EXPERIMENTAL PROCEDURES

Materials—J774 macrophage cells, 293 epithelial cells, and COS-7 cells were purchased from American Type Culture Collection. Human brain cDNA library inserted in pJG4-5 plasmid was kindly provided by Dr. R. Brent (Harvard University). Human brain cDNA library inserted in pcDNA I plasmid was purchased from Invitrogen. Calmodulin-Sepharose 4B and glutathione-Sepharose 4B were purchased from Amersham Pharmacia Biotech. Purified rat brain PKC was kindly provided by Dr. Y. Liu (University of Oklahoma Health Science Center). Rabbit anti-MacMARCKS antibody was generated in collaboration with Dr. S. Slivka (Tenabe Laboratory, La Jolla, CA) against His-MacMARCKS fusion protein and was affinity purified as described (53). Goat anti-dynamitin antiserum was generated using purified GST fusion protein of dynamitin in Ferrell Farms (Oklahoma City, OK). Other secondary antibodies were purchased from Jackson Immunological Laboratory (West Grove, PA). All other routine chemicals were purchased from Sigma.

Yeast Two-hybrid System and Dynamitin Cloning—Yeast two-hybrid system was constructed as described (54, 55). In brief, cDNA encoding full-length MacMARCKS was inserted in pEG202 in frame at the C-terminal of LexA using the 5′ EcoRI-BamHI 3′ sites. This construct was used as bait to screen an human brain cDNA library constructed in pJG4-5 plasmid. Using a previously described method (56), the bait and the library cDNA constructs were cotransformed into yeast EGY199 strain containing a reporter gene (plasmid pSH18-34). The same amounts of GST-dynamitin fusion proteins on the Sepharose were detected by immunoblotting with the anti-MacMARCKS antibody. The positive colonies from the second round selection were then isolated, and their pJG4-5 plasmids were sequenced. A positive clone (Clone C5, referred to in this paper as dynamitin-1), the new dynamitin isoform (Clone C19, referred to as dynamitin-2) (see Fig. 1A), and partial dynamitin (Clone C16, lacking N-terminal 71 amino acid residues).

### Materials

- J774 macrophage cells, 293 epithelial cells, and COS-7 cells from American Type Culture Collection
- Human brain cDNA library inserted in pJG4-5 plasmid from Dr. R. Brent
- Human brain cDNA library inserted in pcDNA I plasmid from Invitrogen
- Calmodulin-Sepharose 4B and glutathione-Sepharose 4B from Amersham Pharmacia Biotech
- Purified rat brain PKC from Dr. Y. Liu
- Rabbit anti-MacMARCKS antibody from Jackson Immunological Laboratory
- Goat anti-dynamitin antiserum

### Yeast Two-hybrid System and Dynamitin Cloning

- Yeast two-hybrid system was constructed as described
- cDNA encoding full-length MacMARCKS was inserted in pEG202 in frame
- The bait was used as bait to screen a human brain cDNA library
- The bait and the library cDNA constructs were cotransformed into yeast EGY199 strain
- Positive colonies were isolated, and their plasmids were sequenced

### RT-PCR of the Fragments of Dynamitin-1 and Dynamitin-2

RT-PCR was used to amplify the fragments from dynamitin-1 and dynamitin-2. The positive colonies from the second round selection were then sequenced.

### Expression of Fusion Proteins

- All cDNA constructs were inserted into yeast plasmid pSH18-34
- cDNA encoding deletion mutants were generated
- GST-free MB was generated
- The GST-dynamitin fusion proteins of dynamitin-2 and His 6 fusion proteins of dynamitin-2 were purified as described

### Calmodulin Binding Assay

- Purified GST-dynamitin-2 (5 μg) was mixed with glutathione-Sepharose 4B
- Fractions were collected and analyzed by SDS-PAGE

### Dynamitin Binding with MacMARCKS

- The same amount of His6 fusion proteins of MacMARCKS or its mutants (2 μg each) were incubated with glutathione-Sepharose (50 μl of 50% slurry) conjugated with GST fusion proteins of dynamitin-2 or its mutant
- C16, in 1 ml of phosphate-buffered saline in the Eppendorf tubes for 1 h at 4 °C
- The tubes were washed with five column volumes of loading buffer followed by five column volumes of the same buffer containing 200 μM NaCl
- Dynamitin-2 was eluted with elution buffer containing 20 μM Tris, pH 8.0, 200 mM NaCl, and 4 mM EDTA

### Dynamin-2 Interaction with MacMARCKS

- The interaction between dynactin and MacMARCKS may explain the involvement of microtubules in cell adhesion and spreading and may provide a regulatory mechanism for the link between dynactin and the membrane gauges.

1 The abbreviations used are, MARCKS, myristoylated alanine-rich C kinase substrate; MacMARCKS, macrophage-enriched MARCKS; GST, glutathione S-transferase; PKC, protein kinase C; PMA, phorbol myristate acetate; RT, reverse transcription; PCR, polymerase chain reaction; bp, base pair(s); PAGE, polyacrylamide gel electrophoresis; PIPES, 1,4-piperazinediethanesulfonic acid.
concentration of 50 μg/ml, 50 units of bovine thrombin were added, and the sample was digested at 37 °C for 15 min. The free GST and undigested GST-MB were removed by passing the sample through the glutathione-Sepharose columns. MacMARCKS (2 μg) was incubated with or without GST-free MB (8 μg) at 4 °C for 30 min and then allowed to bind to 50 μl of dynamitin-Sepharose as described above.

Coimmunoprecipitation of Dynamitin-2 with MacMARCKS—The affinity purified anti-MacMARCKS antibody, rabbit IgG, or bovine serum albumin was coupled to the Hi-Trap Sepharose column (1 ml; Amersham Pharmacia Biotech) as described by the manufacturer’s instructions. Human 293 cells (4 × 10⁶) were incubated with or without 100 μM PMA for 1 h. The cells were lysed in 2 ml of PEM lysis buffer (0.1 M PIPES, pH 6.9, 1% Nonidet P-40, 0.5 mM MgCl₂, and 1 mM EGTA). The lysate was centrifuged at 125,000 g for 10 min at 4 °C. The supernatant was then passed through the antibody column or control columns. After washing four times with 5 ml of phosphate-buffered saline, the column-bound proteins were eluted with 3 ml of 0.2M glycine at pH 3. The washing four times with 5 ml of phosphate-buffered saline, the column-bound proteins were eluted with 3 ml of 0.2M glycine at pH 3. The eluted proteins were concentrated to 100 μl of volume and subjected to SDS-PAGE followed by immunoblotting with anti-dynamitin antibody.

Copurification of Dynamitin with MacMARCKS—The purification procedure for MacMARCKS was described previously (38). In brief, approximately 3 ml of packed 293 cells were homogenized in 5 ml of lysis buffer containing 20 mM Tris-HCl at pH 7.6, 1 mM EDTA, 10 mM β-mecaptoethanol, 0.2% Triton X-100, and protease inhibitors as described (38). The lysate was centrifuged at 4 °C for 10 min, and the supernatant was passed through the antibody column or control columns. After washing four times with 5 ml of phosphate-buffered saline, the column-bound proteins were eluted with 3 ml of 0.2 M glycine at pH 3. The eluted proteins were concentrated to 100 μl of volume and subjected to SDS-PAGE followed by immunoblotting with anti-dynamitin antibody.

Codestination of MacMARCKS with Dynamitin—A 102-bp fragment for dynamitin-1 (Fig. 2) and a 93-bp DNA fragment for dynamitin-2 and a 58.5-bp fragment for dynamitin-3 were amplified from Jurkat, J774, and 293 cell RNA using the following primers: forward: 5’-TCCATTCTGATGCTGCCCTCT-3’ and reverse: 5’-CAGCAGTGGGATGTAGTTCC-3’. The existence of the two isoforms is not likely due to 5′-end polymorphism between individuals. To verify the existence of these two isoforms, we performed RT-PCR using the primers flanking the differing sequences of these two isoforms, which should give rise a 93-bp DNA fragment for dynamitin-2 and a 102-bp fragment for dynamitin-1 (Fig. 2). Using the RNAs from three cell lines including Jurkat, J774, and 293 cell, we amplified...
fied DNA bands corresponding to the dynamitin-2 (Fig. 2), whereas from human brain RNA, we amplified a band corresponding to the dynamitin-1 (Fig. 2). Sequence analysis confirmed that the amplified DNAs indeed coded for dynamitin-1 and dynamitin-2, respectively (data not shown). Based on these data, we believe that dynamitin-1 is the major form expressed in the brain, and dynamitin-2 is the major form expressed in our experimental cell lines. Therefore, these cell lines provide us with a useful tool to study the cellular functions of the interaction between MacMARCKS and dynamitin-2 without interference from dynamitin-1. Nevertheless, we should note that both forms of dynamitin interact with MacMARCKS equally in our in vitro binding assay.

Three potential domains were identified in dynamitin. First is the N-terminal MacMARCKS binding domain (Fig. 1A, bold type) that will be discussed in detail in the following sections. Second, a Walker A motif and a Walker B ATPase motif (59, 60) were identified in dynamitin (Fig. 1A, underlined italic type). However, whether these motifs carry any ATPase activity and the potential function of this sequence are still under investigation. Third, a stretch of basic amino acid residues was detected in both forms of dynamitin between amino acid residues 58 and 80. This domain can be modeled into an amphipathic helix. In the helix, positively charged amino acid residues and hydrophobic amino acid residues each form a cluster adjacent to each other (Fig. 3A). This structure resembles a calmodulin-binding motif (61). Our experimental results show that both forms of dynamitin interact with MacMARCKS, equally in our in vitro binding assay.

FIG. 2. Amplification of dynamitin from three cell lines and human brain. The nucleotides and amino acid sequences represent the amplified dynamitin fragment by RT-PCR, which also shows the difference in sequence between dynamitin-1 and dynamitin-2. Gray boxes indicate the positions for primers. The graph of the acrylamide gel electrophoresis shows the presence of dynamitin-1 (a 102-bp fragment) in the brain and dynamitin-2 (a 93-bp fragment) in Jurkat, J774, and 293 cells. As negative control, RNA was omitted from the RT-PCR reaction. As positive control, the fragments were also amplified from plasmids containing dynamitin-1 or dynamitin-2.

interaction between MacMARCKS and dynamitin-2 by using purified fusion proteins. This evaluation would also allow us to determine whether there is a direct binding between these two proteins. In this experiment, His<sub>6</sub> fusion protein of MacMARCKS and a number of its mutants (see Fig. 1B for their structures) were incubated with GST-dynamitin-2-Sepharose beads (Fig. 4A) or with the control beads conjugated with GST or GST-VKC (an chimera of Rho and cdc42, gift from Dr. Y. Zheng, University of Tennessee). The binding assay showed that the full-length MacMARCKS (F), C-terminal 34-amino acid deletion mutant (CD), or N-terminal 18-amino acid deletion mutant (ND) was retained on the dynamitin-2-Sepharose
the anti-MacMARCKS antibody. mutants that bound to dynamitin were detected by immunoblotting with Sepharose beads conjugated with GST fusion proteins of dynamitin, as described under “Experimental Procedures.” MacMARCKS and its mutants that bound to dynamitin were detected by immunoblotting with the anti-MacMARCKS antibody. A, effector domain of MacMARCKS is required for its binding to dynamitin. Similar amounts of different MacMARCKS mutants (middle panel) were incubated with the same amount of dynamitin beads (bottom panel). Dynamitin-bound MacMARCKS was detected by immunoblotting with anti-MacMARCKS antibody (top panel). Tested MacMARCKS proteins include F, ND, CD, ED, SD, and SA. The negative controls are GST fusion protein of a chimera of cdc42 and Rho (VKC) and GST itself. The N-terminal deletion mutant of 71 amino acids (C16) of dynamitin-2 did not bind to MacMARCKS. The lower bands in the GST-dynamitin samples are the degradation product of GST-dynamitin-2 (Deg. Dynamitin-2). B, N terminus of dynamitin-2 binds to MacMARCKS. Glutathione-Sepharose beads conjugated with MB, CamD, and MBD was incubated with wild type MacMARCKS. The bound MacMARCKS were detected by immunoblotting. The antibody-bound proteins were then eluted and subjected to immunoblotting with antibodies against MacMARCKS and dynamitin. Rabbit total IgG and bovine serum albumin were conjugated to Sepharose-4B and used in a binding assay as a negative control. We found that dynamitin was coimmunoprecipitated with MacMARCKS when MacMARCKS was immunoprecipitated with anti-MacMARCKS antibody (Fig. 5A), whereas no dynamitin was absorbed by rabbit IgG-beads or bovine serum albumin-Sepharose beads. Although the anti-dynamitin antibody could not distinguish dynamitin-1 from dynamitin-2, what we observed here was likely to be dynamitin-2, because it is the major form expressed in 293 cells. Second, during the purification of MacMARCKS using the previously described procedure (38), dynamitin-2 was found in the final preparation of MacMARCKS (Fig. 5B). The entire purification procedure underwent heat precipitation, ion exchange, gel filtration, and calmodulin affinity purification. Although the calmodulin affinity column was irrelevant in this case because dynamitin is also a calmodulin-binding protein, the copurification after the first three steps suggested a very strong possibility of their interaction. Finally, sucrose gradient centrifugation of cell lysate was analyzed on SDS-PAGE followed by immunoblotting with anti-MacMARCKS and anti-dynamitin antibodies. MacMARCKS, which usually migrates on SDS-PAGE as a double band, was found to cosediment with the dynamitin at 20 S (Fig. 5C) in the sucrose gradient; this is a signature density of dynactin complex during the purification of dynactin complex from the brain tissue. The presence or absence of MacMARCKS in the 20 S complex seemed to have no effect on its integrity. In COS-7 cells, a cell line that does not express MacMARCKS (38), dynactin complex sediments at 20 S without MacMARCKS being detected (Fig. 5C). Because the 20 S complex represents intact dynactin complex, this result implies that MacMARCKS-associated dynamitin is bound with the dynactin complex. This result is also supported by the presence of p150(Claud during MacMARCKS purification (Fig. 5B). However, with or without MacMARCKS, the integrity of 20 S did not change; the 20 S dynactin complex from COS-7 cells appears intact, whereas these cells do not express MacMARCKS.
We also tested whether the *in vivo* association of dynamitin-2 and MacMARCKS is regulated by PKC-mediated phosphorylation. In the cells treated with PMA, in which MacMARCKS was heavily phosphorylated by PKC (38), we found that dynamitin-2 was no longer coimmunoprecipitated with MacMARCKS (Fig. 5A). The phosphorylation-mediated regulation is also confirmed by the *in vitro* binding assay. We found that phosphorylation of MacMARCKS by purified PKC abolished its binding with dynamitin-2 (Fig. 5D). This observation, along with other observations (62–65), provides evidence of the involvement of phosphorylation in microtubule-related functions.

To investigate the *in vivo* function of the dynamitin-MacMARCKS interaction in macrophages, we examined the subcellular localization of these two proteins in macrophages. The centrosomal staining of dynamitin was not visible in the J774 macrophages. In addition to the fine punctate staining, dense patches of dynamitin were also observed in the membrane edges of these cells where it colocalized with MacMARCKS protein (Fig. 6, J774). MacMARCKS has been shown to localize both on the plasma membrane and on the vesicle membranes (47, 52). The localization pattern of dynamitin in the plasma region is subject to PKC regulation; adding PMA abolished dynamitin from the membrane edge in macrophages, whereas some of MacMARCKS protein remained at the edge (Fig. 6, J774 + PMA).
Dynamitin-MacMARCKS Interaction Is Involved in Cell Spreading—To explore the potential function of the dynamitin-MacMARCKS interaction in vivo, MacMARCKS-binding peptide of dynamitin-2 (MB) was introduced into the cells to competitively disrupt this interaction. The peptide was shown to inhibit MacMARCKS-dynamitin interaction (Fig. 4C). Although our current report focuses on the effect of this peptide on cell spreading, its effect on mitosis and lysosomal distribution was also observed to be similar to that reported by Echeverri et al. (32), which will be studied further.

We studied the short-term effects (in the minute range) of the disruption of this interaction. Instead of transfecting the cDNA construct, which usually takes 10–20 h to accumulate enough mutant protein, GST-MB fusion peptide was introduced into J774 macrophage cells by microinjection. As a control, we also injected GST alone and a GST fusion peptide of the C-terminal 48 amino acids of dynamitin-2 (amino acids 288–335, GST-C), which is not involved in dynamitin-MacMARCKS interaction. Although the un.injected and control injected cells mostly remained spherical, GST-MB injected cells became flattened within a time range between 5 and 20 min (Fig. 7, GST-MB). In five separate experiments, we counted 219 cells in which 96% (210) of cells spread after injection. However, only 21% (105 of 500 cells of five experiments) of uninjected cells and 20% (61 of 300 cells of three experiments) of GST injected cells spread spontaneously. It is important to note that the C-terminal domain peptide of dynamitin did not show any effects (Fig. 7, GST-C), and only 19% (47 of 250 cells of three experiments) spread spontaneously. To be sure that the effect of MB was not a result of unknown contamination in the sample, we also passed the MB solution through the anti-dynamitin antibody column. The MB-depleted solution was also injected in the cells, and no effect was observed (data not shown). This result strongly suggests that dynamitin-MacMARCKS interaction is involved in regulating cell spreading. This phenotype of dynamitin-MacMARCKS interaction agrees with the observation that MacMARCKS is involved in cell spreading (47).

In addition, we injected GST-MB into J774 macrophages expressing the effector domain deletion mutant of MacMARCKS (47). Expression of this dominant negative mutant in J774 macrophage cells blocks phorbol ester-stimulated or immune complex-stimulated cell spreading. Using these mutant cells allows us to determine whether MacMARCKS is required for the effect of GST-MB. If dynamitin functions independently from MacMARCKS or if it functions down stream of MacMARCKS, then mutation of MacMARCKS should not change the effect of injecting GST-MB. One would expect cell spreading after injection of this peptide. If these cells did not spread, it would suggest that the role of dynamitin in cell spreading depends on a functional MacMARCKS or dynamitin functions upstream from MacMARCKS. The experiment showed that injection of GST-MB into these MacMARCKS mutant cells did not cause cell spreading as it did in normal J774 cells (Fig. 7). Therefore, this experiment suggests that MacMARCKS is required for this MB-induced effect, which could be either due to the fact that MacMARCKS is downstream of dynamitin or due to the fact that MacMARCKS and dynamitin collaborate in one function.

FIG. 7. Dynamitin-MacMARCKS interaction is involved in cell spreading and the formation of tubular lysosome. J774 wild type cells injected with GST-MB (MacMARCKS-binding peptide of dynamitin-2, amino acids 1–83) became flat and spread out (differential interference contrast). Injection of GST or GST-C (C-terminal peptide of dynamitin not involved in MacMARCKS binding, amino acids 291–335) into the same type of cells did not cause cells to spread (bottom panel). Injection of GST-MB into J774 cells expressing dominant negative mutant of MacMARCKS (ED, see Fig. 1 and Ref. 47) did not induce spreading, indicating that a functional MacMARCKS is required for the effects of the MB peptide. The injected cells are indicated by immunostaining for GST with the anti-GST antibody (blue).

DISCUSSION

The data presented here show that dynamitin-2 binds to the PKC substrate MacMARCKS under both in vitro and in vivo conditions and that this binding is regulated by PKC-mediated phosphorylation of MacMARCKS. In addition to the in vitro binding assay using purified fusion proteins, the in vitro interaction between dynamitin and MacMARCKS was demonstrated in three different ways including coimmunoprecipitation and copurification in both directions. The N-terminal domain of dynamitin, very well conserved among different species (34), is required in this interaction. The effector domain of MacMARCKS, which contains the phosphorylation sites (serines 93 and 104), is also required for the interaction.

The PKC-mediated phosphorylation of MacMARCKS regulates its binding to dynamitin both in vitro and in vivo. When MacMARCKS was phosphorylated in vitro with purified PKC,
it no longer binds to the dynamitin. In PMA-treated cells, MacMARCKS was heavily phosphorylated by PKC (38). In this case, dynamitin was no longer coimmunoprecipitated with MacMARCKS. Therefore, we concluded that phosphorylation of MacMARCKS by PKC is an important regulation of dynamitin-MacMARCKS interaction.

PKC is known to phosphorylate serines 93 and 104 of MacMARCKS (38), consistent with our finding that the effector domain mediates the interaction with dynamitin. As expected, the substitution of aspartic acid at these two sites to mimic phosphorylation resulted in decreased binding to dynamitin; however, substitution of alanine also resulted in decreased binding, suggesting that serines 93 and 104 may represent critical residues for the interaction between MacMARCKS and dynamitin. Currently, the affinity and stoichiometry of the MacMARCKS-dynamitin interaction are unknown, but future research in this area may shed light on the involvement of the serine residues.

Experiments in vivo further suggest that the MacMARCKS-dynamitin interaction may be involved in cell spreading. Microinjection of the N-terminal MacMARCKS-binding peptide of dynamitin (MB) caused macrophages to spread, whereas injection of the C-terminal peptide of dynamitin showed no such effect. In addition, a functional MacMARCKS is required for this effect of MB, because injection of this peptide into cells expressing MacMARCKS mutant showed no effect. These data agree with the reported function of MacMARCKS in regulating cell spreading (47) and suggest one of the possible functions of MacMARCKS-dynamitin interaction.

It is not surprising that dynamitin, a protein involved in microtubule function, participates in cell spreading. The involvement of the microtubule cytoskeleton in cell adhesion and spreading has been well documented, and such an involvement is more prominent in macrophages than in fibroblasts. Recent reports suggest that microtubules do not merely provide a structural reinforcement during cell spreading but also are involved in regulating focal adhesion (12, 13). Recently, the p62 subunit of dynactin was shown to be involved in cell adhesion (66). Because dynamitin is an important regulator of the dynactin and microtubule function, it is feasible for dynamitin to be involved in cell spreading. Our data show that injecting the MacMARCKS-binding peptide of dynamitin disrupts MacMARCKS-dynamitin interaction and promotes macrophage spreading. These observations suggest that the binding of dynamitin with MacMARCKS inhibits cell spreading. This suggestion is also reasonable because MacMARCKS is involved in PMA-stimulated macrophage spreading (47) through its regulation of the activation of $\beta_2$ integrin (49, 50). Our recent study shows that MacMARCKS acts on the cytoskeletal constraint on the mobility of integrin molecules (51).

Not only is it feasible that dynamitin may be involved in macrophage spreading, it is also feasible that the role of dynamitin in cell spreading occurs through its interaction with MacMARCKS, whose role in macrophage spreading is known through its regulation of the $\beta_2$ integrin (47, 49–51). An important question regarding the dynamitin/dynactin complex is how this complex connects dynein motor protein to cargo membrane (15, 27). Although a bridge between spectrum and Arp is proposed, a regulatory mechanism is still needed. Therefore, it is reasonable to propose that the dynamitin-MacMARCKS interaction may be part of the proposed bridge and may provide a regulation to this complex through PKC-mediated phosphorylation of MacMARCKS. Many microtubule-related functions are regulated by phosphorylation. For example, dynein-dependent vesicle transportation is regulated by phosphorylation (63, 64). Phosphorylation also regulates kinesis activity (62). MacMARCKS is a PKC substrate, and it not only is required for integrin activation at cell surface but also associates with intracellular membrane compartments such as secretary vesicles of neuronal cells and phagosomes of macrophages (52, 57). In addition, dynamitin-MacMARCKS interaction is regulated by PKC. Therefore, its interaction with dynamitin would be an ideal candidate to regulate the dynactin-membrane association.

The key issue is how dynamitin-MacMARCKS interaction may be involved in cell spreading. We believe that MacMARCKS may hold the key. MacMARCKS is required for PMA-stimulated cell spreading through its regulation of $\beta_2$ integrin (47, 49, 50), an adhesion molecule essential for cell spreading. The first step of integrin activation is release of cytoskeletal constraint on the integrin molecules so that integrin diffuses faster on the cell membrane and has a better chance of binding a ligand (67). Our recent data suggest that MacMARCKS, after being phosphorylated by PKC, is responsible for breaking the cytoskeleton complex that restrains the integrin molecules (51). MacMARCKS might also participate in establishing focal adhesion, which is a later stage of integrin activation. It is possible that dynamitin-MacMARCKS interaction might be part of the cytoskeletal complex that restrains integrin molecules. When MacMARCKS is phosphorylated by PKC, it no longer binds to dynamitin, and concomitantly, the cytoskeletal constraint on integrin is released. It is important to note that PMA and GST MB both caused the dissociation of dynamitin-2 from MacMARCKS and that both treatments stimulated cell spreading, which raises the possibility that PKC-regulated dissociation of MacMARCKS from dynamitin-2 may be an important step during cell spreading. This possibility remains to be tested.

Because dynamitin is involved in dynactin/dynein function, our data also suggest the potential involvement of dynactin/dynein in cell spreading in addition to its known function in vesicle and spindle organization. This speculation is in agreement with the finding that the p62 subunit of dynactin is also involved in cell adhesion (66). Kinesin, another microtubule-dependent motor protein, has already been shown to be involved in cell spreading and lysosomal distribution (10). It is interesting to note the opposite effects of blocking kinesin function and interfering with dynamitin-2 function. Although blocking kinesin inhibits cell spreading (10) and the formation of tubule lysosome (68), interfering with dynamitin-MacMARCKS interaction promotes cell spreading and formation of tubular lysosomes (data not shown).

As a working hypothesis, we propose that by interacting with MacMARCKS, which associates with plasma membranes, dynamitin may bridge the microtubule motor proteins to the plasma membranes. Therefore, dynamitin/microtubule may be a part of the unknown cytoskeletal complex that constrains integrin molecules at the resting stage to keep cells round. PKC is likely to regulate this bridge, because the interaction between dynamitin and MacMARCKS is regulated by PKC-mediated phosphorylation. As a consequence of MacMARCKS phosphorylation, dynamitin-MacMARCKS interaction is disrupted, freeing integrin to bind its ligand and mediate cell spreading.

If this hypothesis is true, how is it that depolymerization of microtubules causes macrophages to become round rather than to spread? A possible explanation is that integrin activation and cell spreading involve first breaking of the cytoskeleton and then reestablishing the cytoskeletal link later at focal adhesion points. Therefore, a total breakdown of microtubules by nocodazol causes cells to become round because the drug may further affect the integrity of focal adhesion point.
Dynamitin and MacMARCKS in Cell Spreading

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REFERENCES

1. Hynes, R. O. (1992) Cell 69, 11–25
2. Stewart, M., Thiel, M., and Hogg, N. (1995) Curr. Opin. Cell Biol. 7, 690–696
3. Weber, C., Alon, R., Moser, B., and Springer, T. A. (1996) J. Cell Biol. 134, 1063–1073
4. Rosales, C., and Juliano, R. L. (1995) J. Leukoc. Biol. 57, 189–198
5. Friedlander, M., Brooks, P. C., Shaffer, R. W., Kincaid, C. M., Varner, J. A., and Luttrell, L. M. (1992) Nature 355, 579–583
6. Danilov, Y. N., and Juliano, R. L. (1992) J. Cell Biol. 115, 1634–1638
7. Allan, V. (1996) Curr. Biol. 6, 630–633
8. Pastel, B. M., Olmsted, S., and Vallee, R. B. (1998) J. Cell Biol. 137, 1057–1069
9. Vergeres, G., Manenti, S., Weber, T., and Sturzinger, C. (1995) J. Biol. Chem. 270, 18979–19007
10. Li, J., and Adem, A. (1992) Cell 70, 791–801
11. Blackshear, P. J., Verghese, G. M., Johnson, J. D., Hauk, D. M., and Stumpo, D. J. (1998) J. Biol. Chem. 273, 13540–13546
12. Umekage, T., and Kato, K. (1991) FEBS Lett. 266, 147–151
13. Kaverina, I., Rottner, K., and Small, J. V. (1998) J. Cell Biol. 141, 671–683
14. Vaughan, K. T., and Vallee, R. B. (1995) J. Biol. Chem. 270, 13540–13546
15. Vallee, R. B., and Sheetz, M. P. (1996) J. Biol. Chem. 271, 2167–2174
16. Allan, V., Keynes, R. J., Thelen, M., Nairn, A. C., and Aderem, A. (1990) J. Exp. Med. 172, 1211–1215
17. Gietz, D., St, Woods, R. A., and Schiestl, R. H. (1992) Mol. Cell. Biol. 12, 403–412
18. Giguere, V., and Juliano, R. L. (1992) J. Biol. Chem. 267, 1815–1829
19. Paschal, B. M., Holzbaur, E. L., Meyer, D. I., and Vallee, R. B. (1999) Mol. Biol. Cell 10, 4107–4129
20. Gill, S. R., Schroer, T. A., Szilak, I., Steuer, E. R., Sheetz, M. P., and Cleveland, D. W. (1991) J. Cell Biol. 115, 1639–1650
21. MacLennan, D. H., and Vallee, R. B. (1989) J. Cell Biol. 113, 1354–1357
22. Gundersen, G. G., and Vallee, R. B. (1989) J. Cell Biol. 116, 899–908
23. Allan, V. (1996) Curr. Biol. 6, 630–633
24. Pastel, B. M., Olmsted, S., and Vallee, R. B. (1998) J. Cell Biol. 137, 1057–1069
25. Pastel, B. M., Saito, T., and Vallee, R. B. (1998) J. Biol. Chem. 273, 2129–2136
26. Pastel, B. M., and Vallee, R. B. (1999) J. Biol. Chem. 274, 21217–21222
27. Chang, S., Hemmings, H. C., Jr., and Adem, A. (1996) J. Cell Biol. 137, 1174–1178
28. Pastel, B. M., Keenan, K. F., Thelen, M., Nairn, A. C., and Adem, A. (1998) J. Biol. Chem. 273, 13540–13546
29. Pastel, B. M., and Vallee, R. B. (1998) J. Biol. Chem. 273, 2129–2136
30. Beck, K. A., Buchanan, J. A., Malhotra, V., and Nelson, W. J. (1994) J. Cell Biol. 127, 707–723
31. Beilharz, J. K., Echeverri, C. J., Nilsson, T., and Vallee, B. R. (1997) J. Cell Biol. 139, 469–484
32. Echeverri, C. J., Paschal, B. M., Vaughan, K. T., and Vallee, R. B. (1996) J. Cell Biol. 132, 617–633
33. Echeverri, C. J., Shpetner, H. S., and Vallee, R. B. (1991) J. Cell Biol. 113, 1354–1357
34. Weis, M. W., and Vallee, R. B. (1988) J. Biol. Chem. 263, 9208–9214
35. Murphy, P. M., and Nathans, D. (1987) Cell 51, 783–792
36. Casteels, K., and Vallee, R. B. (1987) J. Biol. Chem. 262, 10776–10782
37. Paschal, B. M., and Vallee, R. B. (1998) J. Cell Biol. 139, 1057–1069
38. Paschal, B. M., Holzbaur, E. L., Pfister, K. K., Clark, S., Vasiliev, J. M., and Vallee, R. B. (1999) Cell 97, 2139–2144
39. Hellenbeck, P. J., and Swanson, J. A. (1990) Nature 346, 864–866
40. Paschal, B. M., and Vallee, R. B. (1998) J. Cell Biol. 141, 1057–1069
41. Schekman, R., and Emr, S. D. (1988) J. Cell Biol. 110, 181–190