Role of MacMARCKS in Integrin-dependent Macrophage Spreading and Tyrosine Phosphorylation of Paxillin*

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The cellular function of the MARCKS family of protein kinase C substrates is unknown. In this report, we present evidence that indicates a role for MacMARCKS, a member of the MARCKS family, in the integrin-dependent signal transduction pathways in macrophages. Using a dominant negative mutant of MacMARCKS, we showed that MacMARCKS participates in several integrin-dependent macrophage functions, including the phorbol ester-stimulated macrophage spreading, a process involving multiple integrins. The dominant negative mutant also blocks macrophage spreading on immune complex-coated surfaces, a process again requiring $\beta_2$ integrin. More direct evidence of the role of MacMARCKS in the integrin-dependent pathway is the ablation of macrophage binding to complement IC3b-coated sheep erythrocytes by MacMARCKS mutant, suggesting an effect of this mutant on the avidity of complement receptor 3, a member of the $\beta_2$ integrin family. To further evaluate the possible mechanism of MacMARCKS function, the integrin-dependent tyrosine phosphorylation of paxillin was examined. Concomitant with the inhibition of macrophage spreading and rosette formation, MacMARCKS mutant also inhibits integrin-dependent tyrosine phosphorylation of paxillin. Furthermore, immunofluorescent microscopy data showed that MacMARCKS and paxillin colocalize in the membrane ruffles at the leading edge of the spreading cells, providing a potential site and opportunity for MacMARCKS to participate in the regulation of integrin-dependent tyrosine phosphorylation of paxillin. Together, these data strongly suggest that MacMARCKS plays a role in integrin-dependent signal transduction pathways in macrophages.

Integrin-dependent adhesion and spreading are essential to leukocyte binding to endothelium and extravasation of the endothelial layer in response to chemoattractants, endotoxins, and cytokines (for review, see Refs. 1–5). Many other leukocyte functions also depend on integrin-mediated adhesion and spreading; examples include leukocyte binding and engulfing immune complex-coated particles (6, 7), leukotriene B$_4$ production (8), and tumor necrosis factor-induced responses including superoxide production (9) and degranulation (10). The importance of the integrin-mediated response is highlighted by a genetic deficient disease, leukocyte adhesion deficiency, in which patients are unable to express functional $\beta_2$ integrin (for review, see Ref. 11). Leukocytes from these patients fail to adhere to, spread on, and extravasate the endothelial layer to the site of infection.

A major intracellular event during integrin-dependent adhesion and spreading is the enhanced tyrosine phosphorylation that leads to cytoskeleton rearrangement. One of the major tyrosine-phosphorylated proteins among the potential cytoskeleton-associated targets is paxillin (12, 13). Paxillin is a protein that contains SH2 and SH3 domain binding motifs and localizes at focal adhesion (14), where it associates with vinculin (15), the Src family of tyrosine kinases (16, 17), and other SH2 and SH3 domain-containing proteins (18). In leukocytes, the tyrosine phosphorylation of paxillin is a $\beta_2$ integrin-dependent process and has been implicated in a number of leukocyte functions including adhesion (19), Fc receptor-mediated phagocytosis (20), and tumor necrosis factor-$\alpha$-induced leukocyte activation (19, 21). Leukocytes isolated from leukocyte adhesion deficiency patients fail to show the $\beta_2$ integrin-dependent tyrosine phosphorylation of paxillin (19, 21).

In integrin-dependent cell adhesion and spreading, the activation of protein kinase C (PKC) is an essential step (reviewed in Ref. 2). For example, upon activation of PKC with phorbol esters, such as phorbol 12-myristate 13-acetate (PMA), macrophages immediately spread out and flatten with the appearance of a fried egg (22, 23). This PMA-stimulated process is integrin-dependent (24–26). Activation of PKC is also a crucial step in most integrin-mediated cellular functions including leukocyte adhesion to the complement (27) and immune complexes (8), leukocyte extravasation (reviewed in Refs. 5 and 28), and T cell activation (28, 29). Activation of PKC with phorbol esters also promotes enhanced tyrosine phosphorylation of paxillin (19, 21), consistent with the fact that PKC is involved in establishing focal adhesion (30, 31). However, it is not clear how PKC is integrated into the integrin-dependent signal transduction pathway, nor do we know the candidates that are phosphorylated by PKC and transduce the phosphorylation signals.

One of the major PKC substrates that has been extensively studied in leukocytes is the myristoylated membrane-associated protein named MacMARCKS (also known as F52 or MRP) (32–34). MacMARCKS is a member of the MARCKS (myristoylated alanine-rich protein kinase C substrate) family of PKC substrates. MacMARCKS was so named because of its enrich-

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The abbreviations used are: SH2 and SH3, Src homology regions 2 and 3, respectively; BSA, bovine serum albumin; ED, effector domain deletion mutant of MacMARCKS; E$_{IC3b}$, IC3b-coated sheep erythrocytes; E$_{IgM}$, IgM-coated sheep erythrocytes; FM, full-length wild type MacMARCKS; PAGE, polyacrylamide gel electrophoresis; MARCKS, myristoylated alanine-rich protein kinase C substrate; PBS, phosphate-buffered saline; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate.
ment in endotoxin-activated macrophages (32). MacMARCKS is also a membrane-associated calmodulin-binding protein, and its calmodulin binding activity is regulated by PKC-mediated phosphorylation (32, 33, 35). Both the phosphorylation site and calmodulin-binding sites are localized in a basic domain that is shared by the MARCKS family. This basic domain is therefore called the "effector domain" (32, 33).

Despite much speculation, the intracellular function of MARCKS and MacMARCKS has yet to be demonstrated and remains a focus in the field. We recently reported that MacMARCKS is involved in macrophage phagocytosis (36). However, the mechanism of how MacMARCKS functions in macrophage phagocytosis is not clear. In this report, we will show for the first time evidence suggesting that MacMARCKS is an essential component in integrin-dependent cellular functions.

EXPERIMENTAL PROCEDURES

Materials—Hybridoma HB 226, which produces hamster anti-mouse β2 integrin (2E6); hybridoma HB197, which produces rat anti-mouse FcyR (2.4G2); and hybridoma TIB 112, which produces mouse IgM anti-sheep erythrocyte (S-S-3). J774.a1 cells were purchased from ATCC. A monoclonal anti-MHC I (M3/42) antibody was kindly provided by Dr. R. Steinman (Rockefeller University). Rabbit anti-BSA and goat anti-human serum albumin were purchased from Sigma. Mouse monoclonal anti-paxillin antibody was purchased from Transduction Laboratory. Mouse monoclonal anti-phosphotyrosine (4G10) was purchased from Upstate Biochemicals. Rabbit polyclonal anti-MacMARCKS anti-serum was affinity-purified as described (36). Sheep erythrocytes were purchased from Ferrell Farms (Oklahoma City, OK). All other routine chemicals including PMA and poly-L-lysine were purchased from Sigma. Despite much speculation, the intracellular function of MARCKS and MacMARCKS is not clear. In this report, we will show for the first time evidence suggesting that MacMARCKS is an essential component in integrin-dependent cellular functions.

FIG. 1. Expression of MacMARCKS mutant. A, a diagram illustrating the full-length MacMARCKS (FM) and effector domain deletion mutant of MacMARCKS (ED). The amino acid sequence of the effector domain is shown in single-letter code. B, lysates from FM control cells and mutant J744 cells were resolved by SDS-PAGE and transferred to polyvinylidene difluoride membrane. The expression level of MacMARCKS protein was determined by immunoblotting with rabbit anti-MacMARCKS antiserum. C, lysate from ED mutant cells was subjected to two-dimensional isoelectric focussing and SDS-PAGE and transferred to polyvinylidene difluoride membrane. Endogenous MacMARCKS (Endo) and ED mutant proteins were detected by immunoblotting with anti-MacMARCKS antisera.

the coverslips or dishes were first soaked in 0.01% poly-L-lysine solution in PBS for 30 min at room temperature. After removing excess poly-L-lysine by three PBS washes, the coverslips or dishes were treated with 2.5% glutaraldehyde for 30 min followed by three PBS washes. BSA was cross-linked to poly-L-lysine by soaking the dishes with BSA (1 mg/ml in PBS) for 3 h. The unreacted active glutaraldehyde group was quenched by a 30-min treatment of BSA, 0.1 M glycine. After two washes with PBS, the coverslips or dishes were incubated with rabbit polyclonal anti-BSA IgG for 1 h at 37 °C and were ready to be used after three PBS washes.

Subcellular Localization of MacMARCKS on Immune Complex-coated Surface—The immune complex-coated surface was constructed as described above except human serum albumin and goat anti-human serum albumin were used instead. Cells on coverslips were fixed with 10% formalin in phosphate-buffered saline for 15 min at 4 °C. The cells were then permeabilized with acetone at 20°C for 5 min. The coverslips were washed twice with PBS and incubated with mouse anti-sheep erythrocyte IgG, 2 × 106 erythrocytes in EDTA/GVB (5 mM veronal buffer, pH 7.5, 75 mM NaCl, 2.5% dextrose, 0.05% gelatin, 0.5 mM MgCl2, 0.15 mM CaCl2). To obtain IgM-coated erythrocytes, mouse IgM-coated sheep erythrocytes were generated a J774 macrophage cell line expressing the effector domain deletion mutant of MacMARCKS (ED) (36) (Fig. 1), a dominant negative mutant that blocks macrophage phagocytosis of zymosan. To further evaluate the cellular function of MacMARCKS, we examined the effects of MacMARCKS mutation on integrin-dependent cellular functions because integrin activation is required for phagocytosis (38).

We first tested the effect of MacMARCKS mutant on PMA-stimulated macrophage spreading (22, 23), a process involving multiple integrins (24–26). Prior to adding PMA, ED mutant cells and control FM cells (J774 cells transfected with wild type MacMARCKS) adhered to the dishes and appeared rounded and refractive. Upon adding PMA, FM control cells spread out and became extremely flat within 10 min (Fig. 2A). However, the ED mutant cells remained rounded and refractive (Fig. 2A), suggesting that MacMARCKS plays a role in PMA-stimulated and integrin-dependent cell spreading. The degrees of spreading...
were measured from an average of 100 cells and presented in terms of cell-covered surface area. FM control cells covered an area approximately 7 times larger than did ED mutant cells or unstimulated control cells (Fig. 2B).

Dominant Negative Mutant of MacMARCKS Blocks Macrophage Spreading on Immune Complex-coated Surface—Fc receptor-mediated neutrophil spreading on immune complex-coated surface depends on β2 integrin (8, 19). Thus, we tested the effect of MacMARCKS mutation on J774 macrophage spreading on this surface. The FM control J774 cells adhered to and spread out on this surface within 15 min after its addition to this surface, showing a typical fried egg shape (Fig. 3A). But ED mutant cells failed to spread despite weakly adhering to this surface (Fig. 3A). The average cell-covered surface area of spread FM control cells was approximately 7 times larger than that of unspread ED mutant cells (Fig. 3B). Both anti-FcR antibody (2.4G2) and anti-β2 integrin antibody (2E6) blocked J774 cells spreading on this surface, indicating that it is indeed a FcR- and β2 integrin-dependent process (data not shown).

Dominant Negative Mutant of MacMARCKS Blocks J774 Cell Rosette with Complement iC3b-coated Sheep Erythrocytes—More direct evidence that MacMARCKS participates in integrin-dependent macrophage function was obtained by examining integrin interaction with its ligand. Macrophages form rosettes with complement iC3b-coated sheep erythrocytes (EiC3b) through its complement receptor 3 (αmβ2, CR3), a member of the β2 integrin family. To determine the effect of MacMARCKS mutation on integrin-mediated ligand binding, EiC3b were incubated with FM control and ED mutant cells for 30 min. No rosettes formed in the absence of PMA (Fig. 4A). Upon adding PMA for 25 min, all FM control cells were heavily rosetted with EiC3b (Fig. 4A). In contrast, ED mutant cells showed minimum binding to EiC3b even with PMA stimulation (Fig. 4A). Control J774 cells showed a binding index (bound EiC3b/100 J774 cells) of 12 times higher than that of ED mutant cells (Fig. 4B), suggesting integrin αmβ2 function is defect. Thus, MacMARCKS is likely to an essential component in integrin activation.

MacMARCKS Mutation Blocked Integrin-dependent Tyrosine Phosphorylation of Paxillin—One of the major signal transduction events of integrin activation is the enhanced tyrosine phosphorylation (1, 2). In neutrophils, integrin activation leads to an enhanced tyrosine phosphorylation of paxillin in a β2 integrin-dependent manner (19, 21). To further evaluate the possible mechanism of MacMARCKS function in integrin-dependent cellular events, we examined whether the integrin-dependent tyrosine phosphorylation of paxillin was disrupted by MacMARCKS mutation and whether it correlates with the physiological phenotype described above.

Paxillin was immunoprecipitated with anti-paxillin antibody from cells treated with the assays described above. The tyrosine phosphorylation of paxillin was detected with anti-phosphotyrosine antibody. While PMA stimulated macrophage spreading, it also stimulated an enhanced tyrosine phosphorylation of paxillin in FM control cells, whereas only a trace amount of tyrosine phosphorylation of paxillin was detected in ED mutant cells after PMA stimulation (Fig. 5A), consistent with its incapability of spreading. Furthermore, when J774 cells-EiC3b rosettes were formed upon PMA treatment, an enhanced tyrosine phosphorylation of paxillin was also detected in FM control cells (Fig. 5B). Without PMA, the presence of EiC3b alone was not sufficient to induce the enhanced tyrosine phosphorylation of paxillin (Fig. 5B) or the rosette formation.
Previous reports have shown that tyrosine phosphorylation of paxillin induced by immune complex-coated surface is a \( \beta_2 \) integrin-dependent process in neutrophils \((19, 21)\). Our experiments showed that it is also true in J774 macrophages, because antibody against \( \beta_4 \) integrin (2E6) can block J774 cells from spreading on immune complex-coated surface (data not shown) and paxillin phosphorylation (Fig. 5C). In addition, our experiments showed that inhibiting PKC with staurosporin also blocked the spreading on the immune complex-coated surface (data not shown) as well as tyrosine phosphorylation of paxillin (Fig. 5C), further suggesting involvement of PKC in macrophage response to immune complex as previously suggested \((39)\). Similarly, expression of the ED mutant of MacMARCKS also abolished the tyrosine phosphorylation of paxillin induced by immune complex-coated surface (Fig. 5C), again correlating with its incapability of spreading on this surface (Fig. 3A).

MacMARCKS Colocalizes with Paxillin at the Leading Edge of Membrane Ruffles—Using immunofluorescent microscopy, we then examined the subcellular localization of MacMARCKS and paxillin during cell spreading. In J774 cells spreading on the immune complex-coated surface, both MacMARCKS and paxillin were concentrated and colocalized at the membrane ruffles at the leading edge of spreading cells (Fig. 6). Such a colocalization provides MacMARCKS a potential opportunity and subcellular site to participate in the regulation of paxillin phosphorylation. Staining of both proteins was also seen in vesicular structures at the perinuclear regions, which may represent a prestored or recycled population. Interestingly, once paxillin appeared in the typical podosome structure, MacMARCKS no longer colocalized with paxillin in these structures (Fig. 6). The J774 cells on BSA-coated control surface remained round, and no specific subcellular localization of either proteins was seen with confocal microscopy (data not shown).

**DISCUSSION**

Despite much speculation, the cellular function of the MARCKS family of PKC substrate remains unknown. In this report, we present data for the first time that place MacMARCKS in the integrin-dependent signal transduction pathways in macrophages. Using a dominant negative mutant of MacMARCKS, we show that MacMARCKS participates in several integrin-dependent macrophage functions. Examples include the inhibition of PMA-stimulated macrophage spreading, a process involving multiple integrins \((24–26)\). It also blocks macrophage spreading on immune complex-coated surface, a process requiring \( \beta_3 \) integrin \((8)\). More direct evidence is the ablation of macrophage binding to \( \text{EiC}3b \), showing MacMARCKS mutation affects CR3 avidity, a member of \( \beta_2 \) integrin family. To further investigate the mechanism of how MacMARCKS participates in integrin-dependent cellular functions, we showed that integrin-dependent tyrosine phosphorylation of paxillin is also abolished by the MacMARCKS mutant. Thus, our results suggest that MacMARCKS is involved in the signal transduction pathway that results in integrin activation and paxillin tyrosine phosphorylation. The effect of MacMARCKS on integrin-dependent cell spreading is...
consistent with our previous report that MacMARCKS is required for phagocytosis (36), because the activation of integrin and the tyrosine phosphorylation of paxillin are both involved in phagocytosis as well (19, 20).

The close relationship between MacMARCKS and paxillin was further illuminated by the observation that MacMARCKS colocalizes with paxillin in the membrane ruffles at the leading edge of spreading macrophages, providing an opportunity and a potential subcellular site for MacMARCKS to participate in the regulation of paxillin phosphorylation. Interestingly, MacMARCKS colocalized with paxillin only in the membrane ruffles, not in the established podosome structure where paxillin appears in punctate structure, suggesting that MacMARCKS may only transiently colocalize with paxillin during initial formation of focal contacts. Unfortunately, we do not yet know the subcellular localization of mutant MacMARCKS because of the lack of mutant-specific antibody.

An obvious question raised by these results is whether MacMARCKS affects integrin activation first, which then ultimately affects tyrosine phosphorylation of paxillin and cytoskeleton rearrangement such as spreading (outside-in signaling), or whether MacMARCKS affects tyrosine phosphorylation of paxillin first, which then regulates cytoskeleton and ultimately affects the receptor avidity on the surface (inside-out signaling). Currently, we do not have evidence to support either possibility. We favor the hypothesis that MacMARCKS may affect the cytoskeleton first and then the integrin avidity because both MARCKS (40) and MacMARCKS (32) are cytoskeleton-associated proteins.

One of the mechanisms by which cytoskeleton may affect receptor activation is the receptor clustering, which is often required for receptor activation. One well illustrated example is the acetylcholine receptor for which one of the required components for its clustering on the surface of the cells was shown to be a 43-kDa myristoylated cytoskeletal protein. Coexpression of this 43-kDa protein and acetylcholine receptor reconstitutes the formation of receptor patches (41). In the case of β1 integrins, clustering is also an essential process for the full activation of the integrin itself and aggregation of cytoskeleton components (42). Integrin activation is a cytoskeleton-dependent process (43), and the cytoskeleton is suggested to affect the clustering of surface integrins, essential for the full activation of integrins (for review see Ref. 3). MacMARCKS is required in PMA-stimulated tyrosine phosphorylation of paxillin in macrophages. We therefore favor the hypothesis that MacMARCKS may regulate paxillin tyrosine phosphorylation through which MacMARCKS affects the clustering and avidity of the surface integrin to interact with matrix and thus affects cell spreading.

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