Macrophage/Microglia-specific Protein Iba1 Enhances Membrane Ruffling and Rac Activation via Phospholipase C-\(\gamma\)-dependent Pathway*

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Iba1 is a macrophage/microglia-specific calcium-binding protein that is involved in RacGTPase-dependent membrane ruffling and phagocytosis. In this study, we introduced Iba1 into Swiss 3T3 fibroblasts and demonstrated the enhancement of platelet-derived growth factor (PDGF)-induced membrane ruffling and chemotaxis. Wortmannin treatment did not completely suppress this enhanced membrane ruffling in Iba1-expressing cells, whereas it did in Iba1-nonexpressing cells, suggesting that the enhancement is mediated through a phosphatidylinositol 3-kinase (PI3K)-independent signaling pathway. Porcine aorta endothelial cells transfected with expression constructs of Iba1 and PDGF receptor add-back mutants were used to analyze the signaling pathway responsible for the Iba1-induced enhancement of membrane ruffling. In the absence of Iba1 expression, PDGF did not induce membrane ruffling in cells expressing the Tyr-1021 receptor mutant, which is capable of activating phospholipase C-\(\gamma\) (PLC-\(\gamma\)) but not PI3K. In contrast, in the presence of Iba1 expression, membrane ruffling was formed in cells expressing the Tyr-1021 mutant. In addition, Rac was shown to be activated during membrane ruffling in cells expressing Iba1 and the Tyr-1021 mutant. Furthermore, dominant negative forms of PLC-\(\gamma\) completely suppressed PDGF-induced Iba1-dependent membrane ruffling and Rac activation. These results indicate the existence of a novel signaling pathway where PLC-\(\gamma\) activates Rac in a manner dependent on Iba1.

Cell motility is a dynamic process driven by structurally and functionally coordinated reorganization of the actin cytoskeleton (1, 2). Among various types of cells, macrophages are extremely motile to migrate rapidly to sites of infection or inflammation, suggesting that highly integrated systems should exist to regulate the actin cytoskeleton in macrophages (3, 4). In addition to circulating monocytes/macrophages, there are many types of tissue-resident macrophages, including Langerhans cells, Kupffer cells, dendritic cells, splenocytes, and microglia. In response to various pathological phenomena, macroglia are activated to exhibit drastic changes in shape and the abilities to become locomotive and to phagocytose (5, 6). These cellular reactions are also profoundly underlain by dynamic remodeling of the actin cytoskeleton.

The Rho family GTPases, Cdc42, Rac, and Rho, are known to be molecular switches that organize remodeling of the actin cytoskeleton (7). Among them, in fibroblasts, Rac is activated by receptor tyrosine kinases such as platelet-derived growth factor receptor (PDGFR), leading to the formation of lamellipodia and membrane ruffles (8). Dominant active RacV12 induces remarkable membrane ruffling, and dominant negative RacN17 completely inhibits peptide growth factor-induced membrane ruffling; therefore, Rac is recognized to be an essential component in this type of membrane ruffling (8). Some studies describe signaling molecules capable of interacting with Rac; however, the processes by which receptor tyrosine kinases activate Rac are not fully understood.

Previously, we identified a calcium-binding protein, Iba1, which is restrictedly expressed in macrophages/microglia (9), and showed that the expression of Iba1 is up-regulated in activated microglia following facial nerve axotomy (10). In our recent study, Iba1 was further characterized by using a microglial cell line MG5 (11) and loss of function Iba1 mutants, and it was demonstrated that mutant Iba1 effectively suppresses the membrane ruffling produced by stimulation with macrophage colony-stimulating factor (M-CSF) or by expression of dominant active RacV12 (12). These observations suggested that Iba1 was involved in the molecular basis of membrane ruffling of macrophages/microglia and interacted with the signaling of Rac, which is a key molecule in controlling membrane ruffling also in macrophages (13). Iba1 is therefore considered to be one of the candidate molecules underlying the extremely motile property of macrophages/microglia.

In this study, to address this hypothesis, we introduced Iba1 in Swiss 3T3 fibroblasts, porcine aorta endothelial (PAE) cells, and Chinese hamster ovary (CHO) cells, none of which expresses endogenous Iba1, and examined the formation of membrane ruffles, chemotaxis, and profiles of intracellular signaling molecules, including PDGFR, phosphatidylinositol-3 kinase (PI3K), phospholipase C-\(\gamma\) (PLC-\(\gamma\)), and Rac.

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1 The abbreviations used are: PDGFR, platelet-derived growth factor receptor; M-CSF, macrophage colony-stimulating factor; PAE, porcine aorta endothelial; CHO, Chinese hamster ovary; PI3K, phosphatidylinositol-3 kinase; PLC, phospholipase C; FCS, fetal bovine serum; WT, wild type; GST, glutathione S-transferase; DME, Dulbecco’s modified Eagle’s medium; HRP, horseradish peroxidase; LPA, lysophosphatidic acid; PBS, phosphate-buffered saline; BSA, bovine serum albumin; FITC, fluorescein isothiocyanate; PKC, protein kinase C; [Ca\(^{2+}\)]\(_{i}\), intracellular calcium; PAK, p21-activated kinase.
EXPERIMENTAL PROCEDURES

Cell Culture and Transfection—Swiss 3T3 cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal calf serum (FCS). Mouse iba1 cDNA (9) was inserted into the tetracycline-regulated expression vector pTet-splice (Invitrogen) to construct pTet-iba1. The cells were transfected with pTet-iba1, transactivator pTet-itraK, and pHSV-neo by calcium phosphate coprecipitation, and stably transfected clones were isolated by selection with 400 μg/ml G418 (Invitrogen).

PAE cells (14), kindly provided by Dr. C.-H. Heldin (Ludwig Institute for Cancer Research, Sweden) and Dr. Koutaro Yokote (Chiba University, Japan) were cultured in Ham’s F12 medium (Invitrogen) supplemented with 10% FCS. pLXSN plasmids carrying wild type (WT) and a series of mutant human β-PDGFRs (15) were kindly provided by Dr. A. Kallaurkas (Skepsis Eye Research Institute, Harvard Medical School, Boston, MA). F5 mutant PDGFR, which was constructed by the substitution of phenylalanines for five tyrosine residues that are required for the binding of PI3K, RasGAP, SHP-2, and PLC-γ1, is unable to associate with any of these proteins. Add-back mutants of PDGFR were generated by restoring tyrosine residues at individual binding sites for each of the receptor-associated proteins (15). PAE cells were transfected with the tetracycline-regulated iba1-expressing system and cloned as described above. Subsequently, Iba1-expressing cells were transfected with WT PDGFR or the add-back series of PDGFR mutants by the FuGENE6 transfection reagent (Roche Molecular Biochemicals, Germany) and selected by 5 μg/ml of blasticidin S (Funakoshi, Japan).

PAE transfectants were incubated in Ham’s F12 containing 0.5% FCS for 8 h before microinjection of 0.6 μg/ml pFLAG-CMV2 carrying WT or mutant p-yc1 (pLXSN) DNA (12) were kindly provided by Dr. F.-G. Suh (16) (Pohang University of Science and Technology, Korea). Injected PAE cells were maintained for 3 h at 37 °C to induce protein expression. Expression plasmids for glutathione S-transferase (GST)-PLC-γ1-25SH2 and GST-P13-KSH2 (17) were kindly provided by Dr. T. Takenawa and Dr. K. Fukami (Institute of Medical Science, University of Tokyo, Japan). The purified GST fusion proteins were microinjected into cytoplasm and incubated for 10 min at 37 °C. The cells were then stimulated with PDGF (50 ng/ml) for 5 min.

CHO cells were maintained in RPMI 1640/Ham’s F12/DMEM (2:1:1) medium supplemented with 10% FCS. CHO cells were transiently transfectected using LipofectAMINE Plus reagent (Invitrogen) with pLXSN carrying WT PDGFR, pFLAG-CMV2 carrying WT or mutant p-yc1, and pEGFP-C1 (CLONTECH, Palo Alto, CA) carrying WT-iba1 or mutant iba1 (1–115) (12).

A microglial cell line, MG5, was maintained as described previously (12, 19).

Western Blotting—Cells were lysed in radioimmune precipitation buffer containing 20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 10% glycerol, 5 mM EDTA, 1% Triton-X100, 1% sodium deoxycholate, 0.1% SDS, 1 mM sodium vanadate, and 100 μM EDTA, 1% Triton-X100, 1% sodium deoxycholate, 0.1% SDS, 1 mM EDTA, 10 mM NaF, 2 mM sodium vanadate, 5 mM phenylmethylsulfonyl fluoride, 10 μM leupeptin, 10 μM pepstatin, and 0.5 mM dithiothreitol. The lysates were cleared by centrifugation, incubated with GST-PACK fusion protein (12, 19) and glutathione-Sepharose 4B (Amersham Biosciences, Inc.) for 30 min at 4 °C, and subsequently washed in Hepes-buffered saline. Bound activated Rac was visualized by Western blotting with an anti-Rac1 antibody (0.5 μg/ml) and HRP-conjugated anti-mouse goat IgG (1:1500 dilution) using the ECL system.

Immunoprecipitation—Serum-starved quiescent PAE and M-CSF-starved MG5 cells were stimulated with 50 ng/ml PDGF and 100 ng/ml mouse M-CSF (R&D Systems, Minneapolis, MN), respectively. The cells were lysed in radioimmune precipitation buffer at 4 °C for 20 min. Insoluble material was removed by centrifugation, and the cell lysates were normalized for protein concentration before immunoprecipitation. The lysates were incubated with 1.7 μg/ml anti-PLC-γ1 (UBI) or anti-PLC-γ2 (Santa Cruz Biotechnology, Santa Cruz, CA) antibody and with protein G-Sepharose beads (Amersham Biosciences, Inc.). The precipitates were then subjected to Western blotting with an anti-phosphotyrosine antibody, 4G10 (Seikagaku, Japan), and HRP-conjugated anti-mouse goat IgG (Amersham Biosciences, Inc.) using the ECL system.

RESULTS

Enhancement of PDGF-induced Membrane Ruffling in Iba1-expressing Swiss 3T3 Cells—In this study, to analyze the functions of intact Iba1, we transfected a tetracycline-inducible Iba1-expression construct into Swiss 3T3, a fibroblast cell line expresssing no endogenous Iba1. As a result, we established five clones of stable transfectants exhibiting inducible Iba1 expression. Immunoblotting with the anti-Iba1 antibody demonstrated that the expression of Iba1 was tightly inhibited under the presence of tetracycline whereas 13 or 22 h after the removal of tetracycline, strong expression of Iba1 was induced (Fig. 1A).

In Swiss 3T3 cells, bradykinin, LPA, and PDGF are known to specifically activate Cdc42, Rho, and Rac, respectively, and lead the cells to form filopodia, stress fibers, and membrane ruffles (7). To examine the effects of Iba1 on these structures, the Iba1-inducible cells were serum-starved, stimulated with bradykinin, LPA, and PDGF, and stained with phalloidin to visualize the actin cytoskeleton. When Iba1 expression was suppressed in the presence of tetracycline, the cells formed filopodia, stress fibers, and membrane ruffles in response to bradykinin, LPA, and PDGF, respectively (Fig. 1B), as reported for parent Swiss 3T3 cells (8, 19). When Iba1 expression was induced by tetracycline removal, the Iba1-inducible cells formed filopodia and stress fibers and reduced ruffling as shown in the absence of Iba1 expression after stimulation with bradykinin and LPA (Fig. 1B). By contrast, in response to PDGF, the Iba1-expressing cells formed apparently enhanced membrane ruffles in comparison with the Iba1-noneexpressing cells (Fig. 1B). When the cells were doubly stained with phalloidin and the anti-Iba1 antibody after PDGF stimulation, Iba1 was
shown to be localized at the sites of membrane ruffles, together with F-actin (Fig. 1C), but Iba1 did not colocalize with F-actin in filopodia or stress fibers induced by bradykinin or LPA stimulation (data not shown). All other clones of the transfectants exhibited similar enhanced membrane ruffling (data not shown), indicating that Iba1 definitely enhances PDGF-dependent membrane ruffling in Swiss 3T3 transfectants.

Enhanced Chemotaxis of Iba1-expressing Swiss 3T3 Cells—Because membrane ruffling is considered to be related to cell motility (23), we determined the chemotaxis of Iba1-expressing cells by the Boyden chamber method (18) using PDGF as a chemoattractant. As shown in Fig. 2, Swiss 3T3 parent cells and the Iba1-nonexpressing transfectants showed similar motile responses toward PDGF in a dose-dependent manner, whereas the Iba1-expressing cells exhibited about a 2-fold increase in chemotactic response. Similar results were obtained in all clones of Iba1 transfectants. Tetracycline itself had no effect on PDGF-induced migration in Swiss 3T3 cells (data not shown). These results indicate that Iba1 is also able to enhance the chemotaxis of Swiss 3T3 cells.

PI3K-independent Membrane Ruffling of Iba1-expressing Swiss 3T3 Cells—The PI3K signaling pathway is reported to be necessary for PDGF-induced membrane ruffling of Swiss 3T3 cells (24). To investigate whether this pathway is also required for the Iba1-dependent enhancement of membrane ruffling, the effect of PI3K inhibitors, wortmannin and LY294002, on PDGF-induced membrane ruffling was examined in both Iba1-nonexpressing and -expressing cells. Without treatment with the inhibitors, both transfectants formed membrane ruffles as a result of PDGF stimulation, but the extent of ruffle formation was greater in Iba1-expressing cells than in Iba1-nonexpressing cells (Fig. 3). With the wortmannin treatment, membrane ruffling of Iba1-nonexpressing transfectants was completely abolished, indicating that the formation of membrane ruffles of Iba1-nonexpressing Swiss 3T3 cells depends totally on the PI3K signaling pathway. By contrast, the Iba1-expressing cells formed obvious membrane ruffles even after wortmannin treatment, indicating that membrane ruffling of Iba1-expressing cells does depend on a certain signaling pathway in addition to PI3K. The same results were obtained using another PI3K inhibitor, LY294002 (Fig. 3). These observations led us to speculate that the enhanced membrane ruffling associated with Iba1 is transduced by a PI3K-independent pathway.

Involvement of PLC-γ in Membrane Ruffling of Iba1-expressing PAE Cells—To elucidate the possibility that Iba1 is involved in the PI3K-independent signaling pathway, we utilized the add-back mutants of PDGFR, which were transfected into PAE cells lacking endogenous PDGFR (14). PDGFR associates with various signaling molecules via its autophosphorylated tyrosines. PI3K selectively targets tyrosine at amino acid positions 740 (Tyr-740) and Tyr-751, whereas RasGAP, SHP-2, and PLC-γ recognize Tyr-771, Tyr-1009, and Tyr-1021, respectively. These signaling molecules are unable to bind to the PDGFR F5 mutant, in which all of the five tyrosines were replaced by phenyalanines (15). PAE cells, which did not express Iba1, were co-transfected with the Iba1-inducible construct and PDGFR mutant-expression vectors. Similar expression levels were seen for all the PDGFRs in the stable transfectants, as measured by fluorescence-activate cell sorting analysis (data not shown). Without stimulation with PDGF, the morphology of the cells was identical in the presence and absence of Iba1 expression (data not shown). When the Iba1-nonexpressing cells were stimulated with PDGF, obvious membrane ruffles were formed in the cells co-transfected with WT PDGFR receptor or Y740/51 mutant, which is capable of PI3K activation, in agreement with a previous report (25). In contrast, cells expressing F5 or Tyr-1021 receptor did not respond to PDGF (Fig. 4). These observations indicate the necessity of PI3K signaling for PDGF-induced membrane ruffling in the absence of Iba1. On the other hand, after the induction of Iba1, apparent membrane ruffles were formed in the cells transfected with Tyr-1021 mutant, capable of activation of PLC-γ. Membrane ruffling was also detected in the Iba1-expressing cells with the WT or Y740/51 mutant, as in the Iba1-nonexpressing cells (Fig. 4). Cells expressing Tyr-771, Tyr-1009, or kinase inactive receptor (Arg-635) did not show PDGF-induced membrane ruffling regardless of Iba1 expression (data not shown). These observations strongly suggest that PLC-γ is the key signaling molecule in Iba1-dependent and wortmannin-resistant membrane ruffling.
Requirement of Iba1 in PLC-γ-dependent Rac Activation—

Iba1 was recently demonstrated to function together with Rac in the membrane ruffling of microglia, and Rac was shown to be activated during their membrane ruffling (12). To investigate whether Rac is also activated in Iba1- and PLC-γ/H9253-dependent membrane ruffling, the activation of Rac was monitored by pull-down assay with the Cdc42/Rac interactive binding domain of PAK (19) using PAE transfectants. In the absence of Iba1, PDGF stimulation efficiently converted Rac into the GTP-bound form in the cells expressing WT and the Y740/51 mutant but not in the cells expressing the Tyr-1021 (Fig. 5A), Tyr-771, or Tyr-1009 (data not shown) mutant. All lysates contained equal amounts of total Rac. These results indicate that Rac was activated through the PI3K-dependent pathway in the absence of Iba1. However, in the presence of Iba1, in addition to WT- or Y740/51-expressing cells, Tyr-1021-expressing cells also showed Rac activation in response to PDGF (Fig. 5B). These observations indicate the existence of an Iba1- and PLC-γ-dependent Rac-activating pathway that triggers the formation of membrane ruffles.

Inhibitory Effects of PLC-γ Mutants on Iba1-dependent Membrane Ruffling and Rac Activation—To confirm the involvement of PLC-γ in Iba1-dependent and PI3K-independent Rac activation, we investigated the effects of PLC-γ mutants that act as dominant negative forms against endogenous PLC-γ. PLC-γ1-Y771F/Y783F had phenylalanines substituted for ty-
A protein and FITC-dextran (Injected cells were located by fluorescence of epidermal growth factor-induced membrane ruffling was inhibited in PLC-/H9253 FLAG-tagged PLC-Y783F-expressing cells (Fig. 6 lower panel, upper panel, the PI3K N-terminal SH2 domain but does not suppress PLC-γ signaling (17). Into the PAE cells expressing both PDGFR Tyr-1021 and Iba1, we microinjected GST-PLC-γ1-2SH2 or GST-P13K SH2(N) fusion protein together with FITC-conjugated dextran to mark the cells that were injected. The cells injected with GST-PLC-γ1-2SH2 fusion protein were stimulated with PDGF and stained with phalloidin. PDGF-induced membrane ruffling was inhibited in the GST-PLC-γ1-2SH2-injected cells, whereas the cells injected with GST-P13K SH2 (N) were not inhibited.

Next we investigated whether PLC-γ1-Y771F/Y783F mutant blocked Iba1-dependent Rac activation. We induced the transient expression of WT PDGFR, and WT or mutant Iba1 and PLC-γ1 in CHO cells, which lacked PDGFR expression (26), and then examined Rac activity by GST-PAK pull-down assay. Under the conditions we used, in CHO cells expressing PDGFR only, Rac was not activated by PDGF stimulation (Fig. 6B, lanes 1 and 2), and membrane ruffles were not formed (data not shown). In the cells expressing both Iba1 and PDGFR, Rac was activated (lanes 3 and 4) and membrane ruffles were formed (data not shown) in response to PDGF, indicating that CHO cells also contain the pathway exerting Iba1-dependent Rac activation. In cells expressing Iba1 and PDGFR, expression of the PLC-γ1-Y771F/Y783F mutant completely suppressed Rac activation in response to PDGF (lanes 13 and 14). These results indicate the specific involvement of PLC-γ in Iba1-dependent Rac activation.

When the cells expressing PDGFR and PLC-γ1 were stimulated with PDGF, Rac activation was induced (lanes 7 and 8), whereas this Rac activation was inhibited by additional expression of mutant Iba1-(1-115), which effectively suppresses the membrane ruffling of MG5 cells (12) (lanes 11 and 12). PDGFR did not cause Rac activation in the cells expressing PDGFR and mutant PLC-γ1-Y771F/Y783F or mutant Iba1-(1-115) (lanes 5, 6, 9, and 10). These results suggest a functional link between PLC-γ and Iba1 in membrane ruffling and Rac activation.

**Ligand-induced Tyrosine Phosphorylation of PLC-γ**—During the ligand-induced activation process, PLC-γ is known to be phosphorylated by receptor tyrosine kinases (27); thus we next analyzed the tyrosine phosphorylation of PLC-γ in response to growth factors stimulating membrane ruffling. In mammals, two types of PLC-γ are known, PLC-γ1 and PLC-γ2. As shown in Fig. 7, PAE cells selectively express PLC-γ1, whereas the microglial cell line MG5 predominantly expresses PLC-γ2. The PAE cells expressing Iba1 and WT PDGFR were stimulated with PDGF, immunoprecipitated with an anti-PLC-γ1 antibody, and immunoblotted with an anti-phosphotyrosine antibody. 4G10. Phosphorylation of PLC-γ1 was detected 30 s after PDGF stimulation, and after 2 min, the signal was greatly intensified (Fig. 7A). When MG5 cells were stimulated with M-CSF, phosphorylation of PLC-γ1 was, by contrast, undetectable even after 2 min. On the other hand, phosphorylation of PLC-γ2 was clearly detected in MG5 cells after M-CSF stimulation (Fig. 7B). These observations indicate that, in response to peptide growth factors, PLC-γ1 and -γ2 are phosphorylated in these Iba1-expressing cells, including macrophages/microglia, and exert their activity during membrane ruffling.

**DISCUSSION**

In our previous report (12), Iba1 was revealed to be a macrophage/microglia-specific EF hand protein responsible for M-CSF- and Rac-induced membrane ruffling. In this study, to analyze the functions of Iba1 in more detail, we introduced an Iba1 expression construct into Swiss 3T3, PAE, and CHO cells, stimulated the cells with PDGF, and examined signaling profiles leading to Rac activation and subsequent membrane ruffling. As a result, we elucidated a novel signaling pathway...
where, in the presence of Iba1, Rac is activated in a manner dependent on PLC-γ.

In response to PDGF, Iba1-expressing Swiss 3T3 cells exhibited enhanced membrane ruffling and increased chemotactic activity in comparison with Iba1-nonexpressing Swiss 3T3 cells (Figs. 1B and 2). Recent experiments have suggested that PI3K plays a role in receptor tyrosine kinase-mediated membrane ruffling and chemotaxis in many types of cells (24, 25, 28–30). Among them, in Swiss 3T3 and PAE cells, PI3K is reported to be exclusively responsible for Rac activation (24, 25, 31, 32). Indeed, treatment with wortmannin or LY294002 effectively suppresses the PDGF-induced membrane ruffling of Iba1-nonexpressing Swiss 3T3 cells (Fig. 3). In addition, in the absence of Iba1, PDGF could not induce Rac activation and membrane ruffling in PAE cells expressing PDGFR mutants incapable of activating PI3K (Fig. 4 and 5A). By contrast, membrane ruffling of Iba1-expressing Swiss 3T3 cells was not significantly inhibited by wortmannin or LY294002 (Fig. 3). Furthermore, in the presence of Iba1, PDGF stimulation actually induced Rac activation and membrane ruffling in PAE cells expressing the PDGFR mutant that is capable of activating PLC-γ but not PI3K (Figs. 4 and 5). These findings indicate the existence of an Iba1-dependent, PI3K-independent pathway leading to Rac activation and membrane ruffling, and suggest the involvement of PLC-γ in this pathway. In fact, dominant negative forms of PLC-γ clearly inhibited Iba1-dependent membrane ruffling (Fig. 6, A and B) and Rac activation (Fig. 6C). PLC-γ was further shown to be phosphorylated during membrane ruffling (Fig. 7). These observations indicate that PLC-γ is specifically involved in Iba1-dependent Rac activation and membrane ruffling and that Iba1 is the molecule responsible for connecting the signaling pathways of Rac and PLC-γ.

Recent studies have provided increased evidence for PI3K-independent Rac activation. Wortmannin-treated macrophages still induced ruffling at the dorsal surface after M-CSF stimulation (33). N-Formyl-Met-Leu-Phe induced wortmannin- and LY294002-resistant Rac activation in neutrophils (34–36). In our studies, pretreatment with wortmannin did not block M-CSF-induced membrane ruffling of microglial cell line MG5 and primarily cultured microglia (data not shown). These observations strongly suggest the existence of a PI3K-independent pathway leading to Rac activation and membrane ruffling. Kundra et al. (37) showed that the mutant PDGFR, which lacks the binding site for PLC-γ, could not transduce chemotactic signals when expressed in TRMP cells. Expression of another PDGFR mutant that induces increased PLC-γ activation by PDGF showed increased chemotactic activity in PAE cells (31).

Among them, in Swiss 3T3 and PAE cells, PI3K is reported to be closely related. It is likely that Iba1 may modulate the parallel results shown with mutant PLC-γ and WT Iba1. The functions of Iba1 and PLC-γ in Rac activation are considered to be closely related. It is likely that Iba1 may modulate the PLC-γ-dependent signaling pathway. During membrane ruffling, complicated machineries are constructed by Iba1, PLC-γ, and other signaling molecules, including Rac, which co-operate in interactions between each them. However, unfortunately, we have no clear evidence to demonstrate direct binding among Iba1, PLC-γ, and Rac. We are now investigating conditions that would support their association.

Activated PLC-γ translocates to the inside surface of the cell membrane and catalyzes the hydrolysis of phosphatidylinositol 4,5-bisphosphate to form diacylglycerol and inositol 1,4,5-trisphosphate, which are capable of activating protein kinase C (PKC) and mobilizing intracellular calcium ([Ca^{2+}]_i), respectively. In our preliminary experiments, membrane ruffling of Iba1-expressing Swiss 3T3 cells was not suppressed by pretreatment with phorbol ester to down-regulate PKC (data not shown). On the other hand, M-CSF-induced membrane ruffling of MG5 cells was accompanied by [Ca^{2+}]_i spikes and was completely inhibited by chelation of cytoplasmic free calcium with O,O'-Bis(2-aminophenyl)ethylene glycol-N,N,N',N'-tetraacetic acid, tetraacetoxyethyl ester (12). Iba1 actually possesses calcium-binding activity; furthermore, an Iba1 mutant without calcium-binding activity suppressed M-CSF-induced membrane ruffling (12). Several pieces of evidence indicate the significance of calcium signaling in actin remodeling (3, 39). These observations point to the importance of [Ca^{2+}]_i in Iba1-related membrane ruffling and suggest that [Ca^{2+}]_i mobilization is a strong candidate for linking PLC-γ and Iba1 molecules.

Iba1 also translocates to the cell membrane during mem-
brane ruffling (12). In our preliminary studies, Iba1 was demonstrated to bind to phosphatidylserine in the presence of calcium (data not shown), suggesting that Iba1 translocates to the phosphatidylserine-rich inner surface of the cell membrane in a calcium-dependent manner. Iba1 was further shown to bind to phosphatidylinositol 4,5-bisphosphate, the significant substrate of PLC-γ. Iba1 may, directly or indirectly, support translocation of PLC-γ to the cell membrane and its substrate, and may induce Rac activation by potentiating the activity of PLC-γ. Indeed, overexpression of PLC-γ in CHO cells enhanced activation of Rac (Fig. 6C). Although it seems rather inconsistent with data presented here, mutant Iba1 suppressed membrane ruffling induced by activated RacV12 in our previous paper (12). Preliminarily, mutant Iba1 exhibited loss of ability to translocate, localized constitutively to the cell membrane, and disrupted surrounding actin architecture (data not shown). RacV12 showed a tendency to be excluded from the site where mutant Iba1 accumulated (data not shown). In our hypothesis, Iba1 may also function in translocation of Rac, and mutant Iba1 may suppress membrane ruffling by inhibiting appropriate translocation of RacV12.

Macrophages are extremely motile. To express this phenotype, macrophages have to contain highly integrated mechanisms that regulate dynamic reorganization of the actin cytoskeleton. In addition to the well-known PI3K-organized Rac mechanisms that regulate dynamic reorganization of the actin cytoskeleton, PLC-γ may induce Rac activation by potentiating the activity of PLC-γ. Herein, we have shown direct evidence that PLC-γ activates Rac and causes membrane ruffling in the presence of Iba1. This finding indicates that macrophages/microglia have at least dual control pathways to determine the precise molecular mechanisms underlying Iba1- and PLC-γ-dependent activation of Rac and membrane ruffling.

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