Involvement of Iba1 in membrane ruffling and phagocytosis of macrophages/microglia

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SUMMARY

Ionized calcium binding adaptor molecule 1, Iba1, is an EF hand calcium binding protein whose expression is restricted to macrophages/microglia. In this study, Iba1 was shown to colocalize with F-actin in membrane ruffles induced by macrophage colony-stimulating factor and in phagocytic cups formed during zymosan phagocytosis. Expression of mutant Iba1 carrying either N- or C-terminal deletions or carrying a substitution in the calcium binding domain, suppressed the membrane ruffling and the phagocytosis. These results indicate that Iba1 is a key molecule in membrane ruffling and the phagocytosis of macrophages/microglia. Furthermore, Iba1 colocalized with a small GTPase Rac in the membrane ruffles and the phagocytic cups. The Iba1 mutants also suppressed membrane ruffling induced by dominant active Rac1V12, but do not affect microspikes by Cdc42V12 and stress fibers by RhoAV14. These observations suggest that Iba1 is involved in Rac and calcium signaling pathways.

Key words: Actin, Iba1, Membrane ruffling, Phagocytosis, Rac

INTRODUCTION

Macrophages are specialized phagocytes unique in their ability to locomote, change cell shape and incorporate miscellaneous foreign bodies (Downey, 1994; Rabinovitch, 1995). The macrophage lineage consists of freely living monocytes/macrophages and tissue-resident macrophages such as splenocytes, Kupffer cells, Langerhans cells, dendritic cells and microglia. In injured or diseased brain, resident microglia, which normally have small bodies with finely branched processes, are activated to proliferate and migrate to the site of the damage, and then are drastically transformed into expanded amoeboid shapes with the ability to clear invading pathogens or tissue debris (Nakajima and Kohsaka, 1993; Kreutzberg, 1996).

Morphogenesis, cell movement and phagocytosis are driven by dynamic reorganization of the actin cytoskeleton (Greenberg, 1995; Mitchison and Cramer, 1996). Recently, it has been recognized that organized actin remodeling is critically regulated by the Rho family of small GTPases, i.e. Rho, Rac and Cdc42 (Hall, 1998). In fibroblasts, Rho assemblies and maintains stress fibers and focal adhesions (Ridley and Hall, 1992), whereas in macrophages, which lack these structures, Rho induces cell contraction (Allen et al., 1997). Rac and Cdc42 promote the formation of lamellipodia and filopodia, respectively (Ridley et al., 1992; Kozma et al., 1995; Nobes and Hall, 1995; Allen et al., 1997). Rho GTPases can also regulate phagocytosis: Fc receptor-mediated phagocytosis requires Cdc42 and Rac, while complement receptor-mediated phagocytosis requires Rho (Cox et al., 1997; Hackam et al., 1997; Caron and Hall, 1998; Massol et al., 1998). These actin-based cellular events, however, are regulated not only by the GTPases but also by various signaling molecules. To understand the mechanisms underlying the advanced motility and phagocytosis of macrophages, macrophage-specific signaling should be intensively analyzed.

We have previously reported cDNA cloning of the rat iba1 (ionized calcium binding adaptor molecule 1) gene encoding 17 kDa of a small EF hand protein, expression of which is specifically restricted to the monocytic lineage, including microglia and macrophages (Imai et al., 1996). We further demonstrated enhanced Iba1 expression in activated microglia that were stimulated upon facial nerve axotomy (Graeber et al., 1998; Ito et al., 1998), astrocytoma (Tran et al., 1998) or Creutzfeldt-Jakob disease (von Eitzen et al., 1998). We therefore suggested that Iba1 should be a key molecule in functions of activated microglia and macrophages.

Meanwhile Utans et al. (1995) and Autieri et al. (1996) reported iba1-identical genes named AIF-1 (allograft inflammatory factor 1) and BART-1 (balloon angioplasty responsive transcript 1), respectively. AIF-1 and BART-1 were demonstrated to be upregulated in response to inflammation associated with rejection of a transplanted heart and with injury of the carotid artery due to balloon angioplasty, respectively. These groups also suggested a role of the gene in activated macrophages, but presented no physical evidence in support of their speculation.

Many groups including us have deposited sequences in the GenBank of iba1 counterparts cloned from various species, including human, mouse, rat, pig, fishes, Cyprinus carpio and...
**MATERIALS AND METHODS**

**cDNA clonings and construction of expression vectors**

Using primers based on the sequences of a mouse *iba1* genome clone (unpublished observation) and the human cosmids 1.11 sequence (Iris et al., 1993), mouse and human *iba1* cDNAs were amplified by PCR from J74A.1 and THP-1 cDNA pools, respectively. Mouse *cde42*, mouse *rac1*, human *rhoA* and rat *p21-activated kinase 1 (pak1)* cDNAs were also PCR-amplified. T7- and HA-tagging, deletions and point mutations were done by a PCR-based method described previously (Imai et al., 1991). For enhanced green fluorescent protein (EGFP)-tagging, cDNAs were inserted into pEGFP-C1 (Clontech). All of the manipulated cDNAs were ligated into pEF-BOS (Mizushima and Nagata, 1990) to obtain expression constructs.

**Calcium blotting**

Glutathione-S-transferase (GST), GST-iba1(12-84) and GST-iba1(84-147) were expressed in *E. coli* using pGEX vectors (Pharmacia), and purified on glutathione-Sepharose columns (Pharmacia). The proteins, resolved by 12% SDS-PAGE, were electroblottered onto a BA83 nitrocellulose membrane (Schleicher & Schell) under the conditions described by Maruyama et al. (1984). After three washings in a buffer containing 10 mM imidazole-HCl, pH 6.8, 60 mM KCl and 5 mM MgCl₂, the membrane was incubated in the same buffer containing 0.1 μCi/ml [³⁵S]CaCl₂ (Amersham) for 5 minutes, washed twice in 50% ethanol for 2 minutes each, and autoradiographed. The amounts of GST-iba1(12-84), GST-iba1(84-147), GST and sheep testis calmodulin (UCB-Bioproducts) examined were 120, 120, 120 and 30 pmol per lane, respectively, because calmodulin contains four calcium binding sites.

**Cell culture**

MG5 microglia and A1 astroglia cell lines were established from a p53-deficient mouse in our laboratory (Ohsawa et al., 1997). The A1 conditioned medium, which contained M-CSF, was prepared from an overnight culture of A1 cells in DMEM (Gibco BRL) containing 10% fetal calf serum (FCS). MG5 cells were maintained in a 7:3 (v:v) cocktail of the A1 conditioned medium and DMEM containing 10% FCS (growth medium). The cells were plated on glass coverslips and incubated for 48 hours in the growth medium before the examination. NIH 3T3 cells from Japanese Collection of Research Bioresources (JCRB) were grown in DMEM supplemented with 10% FCS.

**Immunocytochemistry**

Cells were fixed with 3.7% formaldehyde for 10 minutes at room temperature, washed with PBS and permeabilized with PBS containing 0.1% Triton X-100 for 5 minutes. The cells were blocked with PBS containing 3% normal goat serum and 3% BSA (blocking buffer) for 15 minutes, and incubated with 2 μg/ml anti-iba1 polyclonal antibody (Imai et al., 1996) and/or 1 μg/ml anti-Rac monoclonal antibody clone 23A8 (Upstate Biotechnology) in the blocking buffer for 2 hours at room temperature. After washing with PBS, the cells were incubated for 1 hour with secondary antibodies, Texas Red-conjugated anti-rabbit IgG (1:50, Amersham), FITC-conjugated anti-mouse IgG (1:200, Tago), and/or FITC-conjugated anti-rabbit IgG (1:200, Tago). To visualize F-actin, 2 units/ml Texas Red-phalloidin (Molecular Probes) were incubated together with the secondary antibodies. After washing, the cells were mounted in PermaFluor (Immunon), and examined under a fluorescence microscope AX70 (Olympus) or a confocal laser scanning microscope CLSM2010 (Molecular Dynamics). Tag-staining with an anti-T7 (0.2 μg/ml, Novagen) or anti-hemagglutinin (HA) monoclonal antibody (0.5 μg/ml, clone 3F10, Roche diagnostics) was performed under essentially the same conditions except that the solutions used in the steps following permeabilization contained 0.05% Triton X-100 and that, for 3F10, the secondary antibody was FITC-conjugated anti-rat IgG (1:200, Tago) or Texas Red-conjugated anti-rat IgG (1 μg/ml, Southern Biotechnology Associates).

**Microinjection**

MG5 cells were cultured in DMEM containing 10% FCS for 16 hours before microinjection. Microinjection was performed with a Leica Mechanical Micromanipulator fitted with an Eppendorf Femtotip (0.5 μm) diameter that was pneumatically driven by a Narishige IM300 microinjector. A plasmid mixture dissolved in PBS (0.6 μg/μl) was microinjected into the nucleus while under observation with a Leica DMI8RB inverted microscope. After a 4 hour incubation, the microinjected cells were identified by EGFP fluorescence or by staining using anti-T7 or anti-HA antibody. Proteins were microinjected into the cytoplasm together with 2 μg/μl FITC-conjugated lysine fixable dextran (Molecular Probes) to mark the injected cells. NIH 3T3 cells were plated onto poly-L-lysine-coated coverslips, and cultured in DMEM containing 10% calf serum for 24 hours. The cells were placed in serum-free DMEM for 16 hours before microinjection.

**Real-time recording of EGFP-Iba1 translocation in living cells**

Microinjected cells on a glass coverslip were incubated with 0.1 mg/ml Texas Red-conjugated zymosans (Molecular Probes). Confocal sections were obtained with a CLSM2010 equipped with a 100x plan fluor objective (Nikon), and 488 nm and 568 nm argon/krypton lasers. The emission signals of EGFP-Iba1 and Texas Red-zymosans were obtained separately using a detector filter of 530 nm and 590 nm, respectively. The images from the two channels were superimposed using ImageSpace™ software (Molecular Dynamics).

**Phagocytic cup formation assay**

To synchronize ingestion, MG5 cells on glass coverslips were arrested by 10 minutes of incubation in the growth medium at 4°C, and the medium was replaced with medium containing 1 mg/ml zymosan particles (Sigma). The cells were incubated at 4°C for another 10 minutes to allow the particles to attach onto the cell surfaces, and washed to remove unbound particles. The cells were then warmed to 37°C by a medium change to initiate particle engulfment. After 5 minutes of incubation at 37°C, the cells were fixed, and stained with Texas Red-conjugated phalloidin. Cells to which zymosan(s) attached were examined, and those forming phagocytic cup(s) were counted; the activity of phagocytic cup formation was expressed as the ratio of numbers of the cup-forming cells.

**Measurement of activation of Rac and Cdc42**

Activation of Rac and Cdc42 were measured by an affinity pull-down assay using the immobilized Cdc42/Rac binding domain of PAK1 (Manser et al., 1998). The PAK1(70-106) peptide was
expressed in *E. coli* as GST fusion protein, and immobilized by adsorption to glutathione-Sepharose beads. After stimulation, cells were lysed in a buffer containing 25 mM Hepes, pH 7.3, 0.15 M NaCl, 5 mM MgCl₂, 0.5 mM EGTA, 20 mM 2-glycerophosphate, 10 mM NaF, 2 mM sodium vanadate, 0.5% Triton X-100, 4% glycerol, 5 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 5 µg/ml leupeptin and 5 µg/ml pepstatin. The lysate was clarified by centrifugation, immediately mixed with the immobilized GST-PAK1(70-106), and gently rotated at 4°C for 30 minutes. The beads were collected by centrifugation, and washed three times with the same buffer. The bound proteins were eluted by heating in the SDS-sample buffer, separated by 12.5% SDS-PAGE, and then transferred onto an Immobilon P membrane (Millipore). Pulled-down GTP-bound forms of Rac and Cdc42 were detected by western blot analysis using 0.4 µg/ml anti-Rac and 0.5 µg/ml anti-Cdc42 antibodies (Santa Cruz), respectively, and visualized with an ECL system (Amersham).

**Intracellular calcium monitoring**

Intracellular calcium concentrations ([Ca²⁺]ᵢ) were monitored by a fura-2 method as described by Grynkiewicz et al. (1985) with minor modifications (Inoue et al., 1998), using a highly sensitive intensifier target video camera C2400 and an Argas 50 image processor (Hamamatsu Photonics). MG5 cells were plated at 2×10⁴ cells/cm² on a poly-L-lysine-coated glass coverslip set underneath a silicon rubber wall Flexiperm (Heraeus Biotecnology), and cultured in the growth medium for 24 hours and then in DMEM containing 10% FCS for 16 hours. The cells were washed with a balanced salt solution (BSS) containing 20 mM Hepes, pH 7.4, 150 mM NaCl, 5 mM KCl, 1.2 mM CaCl₂, 1.2 mM MgCl₂ and 10 mM glucose, and incubated with 10 µM fura-2 acetoxyethyl ester (fura-2/AM) at 37°C in BSS. After 30 minutes of incubation, the cells were washed with BSS, and then stimulated with 300 ng/ml M-CSF for some minutes. Finally the cells were stimulated by 100 µM ATP to check their ability to respond (Inoue et al., 1998). Raw data were recorded as 500 nm emission of fura-2 excited alternatively at 340 nm and 380 nm, and [Ca²⁺]ᵢ was expressed as the ratio of the fluorescence intensities at 340 nm and 380 nm.

**RESULTS**

**Structure and calcium binding activity of Iba1**

We have cloned mouse and human *iba1* cDNAs (DDBJ/EMBL/GenBank accession numbers D86382 and D86438, respectively) by PCR, and aligned the deduced amino acid sequences with the rat Iba1 sequence reported previously (Imai et al., 1996) (Fig. 1A). All three proteins consist of 147 amino acids, which are highly conserved among them throughout their entire sequences. Iba1 consists of two EF hand motifs, putative calcium-binding domains, and unique N- and C-terminal regions whose peptide sequences display no homology to any other reported sequences.

On comparison with the consensus of EF hand motifs (Heizmann and Hunziker, 1991), the first EF hand was considered to be a good match while the second was not (Fig. 1B). Therefore, we constructed GST fusion proteins with Iba1 proteins that had been cleaved so as to contain only one EF hand (amino acids 12-84 and 84-147), and the resulting proteins, GST-Iba1(12-84) and GST-Iba1(84-147), were subjected to calcium blot analysis (Fig. 1C,D). Ionized calcium was found to bind to calmodulin (lane 4) and GST-Iba1(12-84)
(lane 1), carrying the first EF hand, but not to GST (lane 3) or to GST-Iba1(84-147) (lane 2), carrying the second EF hand. These observations suggest that the first EF hand does indeed possess preferential calcium-binding affinity, which is greater than that of the second EF hand.

**Colocalization of Iba1 with F-actin in membrane ruffles**

Iba1 is highly expressed in a microglial cell line, MG5, which was established in our laboratory from a p53-deficient mouse (Ohsawa et al., 1997). MG5 was shown to retain a number of specialized properties of microglia/macrophages, for instance high motility and strong phagocytic activity, and to proliferate in the presence of M-CSF. To elucidate the functions of Iba1 in microglial/macrophages, we first examined the intracellular localization of endogenous Iba1 in growing MG5 cells by immunocytochemistry. The anti-Iba1

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**Fig. 2.** Colocalization of Iba1 with F-actin in membrane ruffles. (A) Intracellular localization of Iba1 in MG5 cells. MG5 cells cultured in the growth medium were fixed, stained with anti-Iba1 antibody, and observed by fluorescence microscopy. Scale bars, 20 μm. (B) Colocalization of Iba1 with F-actin in membrane ruffles. MG5 cells were maintained for 48 hours on a glass coverslip in the growth medium and further cultured for 16 hours in DMEM containing 10% FCS to starve the cells for M-CSF. Ruffling was then induced by 30 ng/ml M-CSF (R&D Systems Inc.). After 5 minutes, the cells were fixed, doubly stained with the anti-Iba1 antibody and Texas Red-conjugated phalloidin, and observed by confocal microscopy. Scale bars, 10 μm.

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**Fig. 3.** Effects of EGFP-Iba1 mutants on membrane ruffling induced by M-CSF. MG5 cells were cultured in DMEM containing 10% FCS for 16 hours and then expression plasmids encoding EGFP-Iba1 mutants were microinjected into the nuclei. After a 4 hour incubation, the cells were stimulated with 30 ng/ml M-CSF for 5 minutes, fixed, stained and observed by confocal microscopy. The first column depicts the structures of the EGFP-Iba1 mutants. The second column shows the Texas Red-conjugated phalloidin staining for F-actin, and the third column shows EGFP fluorescence of the EGFP-Iba1 mutants. The fourth column shows a merge of the images of the EGFP-Iba1 mutants (green) and F-actin (red). Colocalization appears in yellow. (A) full-length Iba1 fused with EGFP, EGFP-Iba1; (B) EGFP-Iba1(1-115); (C) EGFP-Iba1(114-147); (D) EGFP-Iba1(1-29); (E) EGFP-Iba1(30-147); (F) EGFP-Iba1(30-120); (G) EGFP-Iba1(EF2EF2). Scale bars, 10 μm.
antibody clearly visualized Iba1 not only in the cytoplasm and nucleus but also in lamellipodia and membrane ruffles, in which the leading margin of the cell rose up and made wave-like structures (Fig. 2A).

In order to confirm the localization of Iba1 in membrane ruffles, growth factor-starved MG5 cells were stimulated with M-CSF, doubly stained with the anti-Iba1 antibody and Texas Red-conjugated phalloidin to visualize F-actin, and observed with a confocal laser scanning microscope. The quiescent MG5 cells cultured in the absence of M-CSF exhibited contracted and rounded up cell bodies carrying phalloidin-stained retraction fibers in the cell periphery, while Iba1 showed no colocalization with F-actin (Fig. 2B). Within 5 minutes, M-CSF stimulation rapidly induced actin reorganization to form many marked membrane ruffles and lamellipodia, and to make the cell periphery detach from the substratum and extend. Iba1 vigorously accumulated in membrane ruffles and closely colocalized with F-actin (Fig. 2B). These observations suggest that Iba1 contributes to the formation and regulation of membrane ruffles by cooperating with actin.

**Involvement of Iba1 in the formation of membrane ruffles induced by M-CSF**

To determine whether Iba1 really participated in the formation of membrane ruffles, we constructed expression plasmids encoding various mouse Iba1 mutants that were fused with EGFP, and microinjected these plasmids into nuclei of MG5 cells. After a 4 hour incubation, the cells were stimulated with M-CSF for 5 minutes, and then transiently expressed EGFP-Iba1 mutants were observed by confocal microscopy.

**Fig. 4. Iba1 in zymosan phagocytosis.**

(A) Translocation of EGFP-Iba1 in living MG5 cells that were phagocytosing zymosan particles. Cells expressing EGFP-Iba1 were incubated with 0.1 mg/ml Texas Red-conjugated zymosans, and a series of confocal images were captured at the times indicated. EGFP-Iba1 and the zymosan particles are shown in green and red, respectively. Arrowheads indicate the particles undergoing phagocytosis. Scale bars, 10 μm.

(B) Colocalization of Iba1 with F-actin in phagocytic cups. MG5 cells arrested by cooling at 4°C were incubated with 1 mg/ml zymosans for 10 minutes to allow the particles to fall and attach to the cells, and then warmed to 37°C to start synchronized phagocytosis. The cells were fixed at the times indicated, doubly stained with the anti-Iba1 antibody and Texas Red-conjugated phalloidin, and observed by fluorescence microscopy. Scale bars, 20 μm. (C) Inhibition of the formation of phagocytic cups by the anti-Iba1 antibody. Anti-Iba1 antibody (10 μg/μl) or normal rabbit IgG (10 μg/μl) was microinjected into the cytoplasm of MG5 cells together with FITC-conjugated dextran to mark the injected cells. The cells were incubated with 1 mg/ml zymosans for 10 minutes at 4°C, and warmed to 37°C to start synchronized phagocytosis. After 5 minutes, the cells were fixed and stained with Texas Red-conjugated phalloidin. Among FITC-fluorescent cells to which zymosan(s) attached, cells forming phagocytic cup(s) were counted, and the activity of phagocytic cup formation was expressed as the ratio of numbers of both cells. At least 85 cells were observed for each column. Data shown are the mean of three independent experiments. (D) Inhibition of the formation of phagocytic cups by the Iba1 mutants. Expression plasmids encoding EGFP-Iba1 mutants were microinjected into the nuclei of MG5 cells. After a 4 hour incubation, EGFP-fluorescent cells were evaluated for phagocytic cup formation by the same method as described in C. The number of cells examined was as follows: Iba1, 51; Iba1(1-115), 50; Iba1(114-147), 49; Iba1(1-29), 57; Iba1(30-147), 36; Iba1(30-120), 32; Iba1(EF2EF2), 57.
Expression of EGFP or EGFP-iba1 did not alter the morphology of the cells, and the addition of an EGFP-tag to either the N or C terminus of Iba1 did not affect its intracellular localization in either the absence or presence of M-CSF stimulation (data not shown). These results indicate the EGFP-tagging to be neutral at least with respect to the translocation of Iba1.

We then examined the effects of a series of EGFP-Iba1 mutants bearing the structures illustrated in Fig. 3. In the absence of M-CSF, none of the mutants affected cell morphology, whereas some of them did in the presence of M-CSF. In contrast to the cell expressing the full-length Iba1 fusion (Fig. 3A) or noninjected cells seen as neighbors, the cells injected with Iba1 mutants carrying a deletion of the C-terminal region (EGFP-Iba1(1-115), Fig. 3B) or the N-terminal region (EGFP-Iba1(30-147), Fig. 3E) exhibited no membrane ruffles or extension of the cell periphery. However, both of these mutants colocalized with F-actin in the cytoplasm and beneath the plasma membrane. The Iba1 mutant carrying deletions of both of the N- and C-terminal regions [EGFP-Iba1(30-120)] displayed a diffuse distribution in the cytoplasm and no colocalization with F-actin, and no longer inhibited the ruffling formation (Fig. 3F). Massive deletions of N- or C-terminal sequences, EGFP-Iba1(114-147) and EGFP-Iba1(1-29), had no effect on the ruffling formation (Fig. 3C,D), while EGFP-Iba1(114-147) colocalized with F-actin beneath the membrane but EGFP-Iba1(1-29) did not. Even after an additional 4 hour incubation, these three mutants, EGFP-Iba1(30-120), -Iba1(114-147) and -Iba1(1-29), could not inhibit the ruffling formation (data not shown). Thus Iba1 plays an important role in the formation of membrane ruffles induced by M-CSF, and the results suggest that both the N- and C-terminal regions are essential for the function of Iba1.

To eliminate the calcium binding ability of Iba1, we constructed another Iba1 mutant, EGFP-Iba1(EF2EF2), in which the functional first EF hand loop (amino acids 58-69) was replaced by the nonfunctional second one (amino acids 94-105). As shown in Fig. 3G, expression of EGFP-Iba1(EF2EF2) inhibited the formation of membrane ruffles in a manner similar to that of EGFP-Iba1(1-115) or EGFP-Iba1(30-147), suggesting that the calcium-binding ability is also necessary for the function of Iba1.

**Involvement of Iba1 in the formation of phagocytic cups produced during zymosan ingestion**

Phagocytosis is one of the most important physiological functions of macrophages/microglia (Rabinovitch, 1995; Kreutzberg, 1996) and is driven by actin-based mechanisms (Greenberg, 1995), similar to the process of membrane ruffling. MG5 cells expressing EGFP-Iba1 were therefore exposed to Texas Red-conjugated zymosans to stimulate phagocytosis, and dynamic movement of EGFP-Iba1 in the living cells was observed by time-lapse confocal microscopy (Fig. 4A). Until a zymosan particle attached to the cell surface, EGFP-Iba1 was diffusely distributed in the cytoplasm (0 minutes). By 1 minute after attachment, EGFP-Iba1 had actively gathered into a crown-shaped extension of the cell cortex surrounding the particle, and subsequently concentrated to pack around the particle (3 minutes). After 5 minutes, the EGFP-Iba1 that had accumulated around the particle gradually dispersed back into the cytoplasm.

During a type of phagocytosis, including that of a zymosan, a crown-like structure rich in actin, termed the phagocytic cup, forms within the initial few minutes, and disappears during the next few minutes (Greenberg et al., 1991; Allen and Aderem, 1995). To confirm that Iba1 is translocated into the phagocytic cups, MG5 cells were incubated with zymosans for 5 or 10 minutes, and then stained with the anti-Iba1 antibody and phalloidin. At 5 minutes, circular bundles of F-actin collaring the particles were seen to form phagocytic cups, in which Iba1 colocalized with F-actin (Fig. 4B). At 10 minutes, the cells had fully internalized most of the zymosans while both Iba1 and F-actin had faded away from the surroundings of the particles, and the cups had disappeared (Fig. 4B). These results indicate that Iba1 is a significant component of the phagocytic cups, and Iba1 participates in the early steps of phagocytosis.

To determine whether Iba1 is required for the formation of phagocytic cups, we microinjected the anti-Iba1 antibody into cytoplasm of MG5 cells. After a 5 minute incubation with zymosans at 37°C, the cells were stained with phalloidin, and then microinjected cells forming phagocytic cup(s) were counted. As shown in Fig. 4C, microinjection of the anti-Iba1 antibody decreased the phagocytic cup-forming cells (44%) but...
microinjection of normal rabbit IgG did not (89%). When the EGFP-iba1 mutants shown in Fig. 3 were expressed in MG5 cells, EGFP-iba1(1-115), EGFP-iba1(30-147) and EGFP-iba1(EF2EF2) effectively inhibited the formation of the phagocytic cups (24%, 47% and 47%, respectively) in contrast to the normal formation seen in most of the cells expressing the full-length fusion product, EGFP-iba1 (96%) (Fig. 4D). Expression of EGFP-iba1(114-147), EGFP-iba1(1-29) or EGFP-iba1(30-120) did not significantly inhibit cup formation (84%, 88% and 78%, respectively). In both cases, the numbers of zymosans attached to the cells were not affected by the antibody or Iba1 mutant (data not shown); however, the inhibited cells could not form membrane protrusions surrounding the particles, resulting in failure of phagocytosis.

These observations indicate that Iba1 plays an important role in phagocytosis at the step of the formation of phagocytic cups. Iba1 is also suggested to be involved in the common mechanisms underlying membrane ruffling and phagocytosis because the mutants that did not exhibit membrane ruffles and extensions in Fig. 3 were the same as those that were inhibited in phagocytic cup formation in Fig. 4D.

**Involvement of Rac GTPase in the formation of membrane ruffles and phagocytic cups**

Membrane ruffling induced by growth factors and some types of phagocytosis were recently reported to be regulated by a member of the Rho GTPase family, Rac (Ridley et al., 1992; Allen et al., 1997; Cox et al., 1997; Caron and Hall, 1998;
To investigate whether Iba1 participates in a Rac signaling pathway leading to actin reorganization, we examined the localization of Iba1 and Rac in MG5 cells under M-CSF stimulation or in zymosan phagocytosis. By immunocytochemistry with the anti-Iba1 and anti-Rac antibodies, Rac was demonstrated to be translocated and closely colocalized with Iba1 in membrane ruffles (Fig. 5A) and phagocytic cups (Fig. 5B).

To further examine the contribution of Rac and other Rho family GTPases, Cdc42 and Rho, toward the formation of membrane ruffles, dominant negative forms of the GTPases, Rac1N17, Cdc42N17 and RhoAN19, were expressed in MG5 cells that were subsequently stimulated with M-CSF. Rac1N17 inhibited membrane ruffling and the extension of the cell periphery (Fig. 6A) in a manner similar to the Iba1 mutants shown in Fig. 3B,E,G, and furthermore destroyed the colocalization of endogenous Iba1 with F-actin (data not shown). By contrast, neither Cdc42N17 nor RhoAN19 affected membrane ruffling (Fig. 6A) or Iba1 translocation. Microinjection of Clostridium botulinum exoenzyme C3 transferase (C3 toxin), which selectively and tightly inactivates Rho signals by ADP-ribosylation (Aktories et al., 1989; Sekine et al., 1989), did not inhibit membrane ruffling and Iba1 translocation (data not shown).

Intracellular activation of Rac was assayed using the Rac/Cdc42-binding domain of PAK (Burbelo et al., 1995) as a pull-down ligand, by which GTP-bound forms of Rac and Cdc42 were separated from GDP-bound forms (de Rooij and Bos, 1997; Manser et al., 1998). In response to M-CSF, endogenous GDP-Rac was significantly converted to the activated GTP-bound form within 1 minute and the elevated activity was sustained for 20 minutes, while activation of Cdc42 was not clearly observed (Fig. 6B). These results indicate that not Cdc42 but Rac was specifically activated in M-CSF-stimulated MG5 cells.

Similarly, we examined an involvement of Rho GTPases in zymosan phagocytosis by microinjecting the dominant negative forms of the GTPases and assessing phagocytic activities, as shown in Fig. 4. Cells expressing Rac1N17 and Cdc42N17 reduced the phagocytic cup formation markedly to 24% and partially to 67%, respectively, while RhoAN19 or C3 toxin did not suppress the formation (Fig. 6C). By the PAK pull-down assay, activation of Rac was apparently observed at 5 minutes and enhanced at 15 minutes, while that of Cdc42 was slight even after 15 minutes (Fig. 6D). These results indicate that Rac activation was also necessary for the promotion of zymosan phagocytosis.

**Participation of Iba1 in a Rac signaling pathway leading to membrane ruffling**

Next we examined the effects of the three inhibitory Iba1 mutants, EGFP-Iba1(1-115), EGFP-Iba1(30-147) and EGFP-Iba1(EF2EF2), against a continuous strong signal generated by dominant active forms Rac1V12. As shown in Figs 7 and 8A, MG5 cells overexpressing Rac1V12 and EGFP-Iba1 fully extended the cell body and formed marked membrane ruffles, in which EGFP-Iba1 was translocated. In contrast, EGFP-Iba1(1-115) dramatically inhibited cell extension, and partially suppressed membrane ruffling, whereas EGFP-Iba1(30-147) and EGFP-Iba1(EF2EF2) predominantly suppressed membrane ruffles on a dorsal cell surface, and hardly inhibited cell extension. These observations suggest the involvement of different mechanisms in the ruffling and the extension caused by RacV12, and of Iba1 in both the mechanisms.

To determine whether Iba1 specifically participates in Rac signaling, we examined the effects of these Iba1 mutants on cells activated by dominant active forms of other Rho GTPases, Cdc42V12 and RhoAV14. As reported previously for macrophages (Allen et al., 1997), expression of Cdc42V12 induced MG5 cells to form microspikes (Fig. 8B). Coexpression of EGFP, EGFP-Iba1 or EGFP-Iba1(1-115) had no clear effect on the microspike formation. RhoAV14 has been reported to induce stress fibers in fibroblasts (Ridley and Hall, 1992); however, expression of RhoAV14 made MG5 cells shrink up and detach from the substratum. Thus, we examined the effect of Iba1(1-115) upon RhoAV14 in NIH 3T3 cells.
RhoA V14 certainly induced the formation of stress fibers and the contraction of the cell bodies. EGFP-Iba1 neither colocalized with the stress fibers nor affected stress fiber formation and cell contraction (Fig. 8C, middle). Although a portion of EGFP-Iba1(1-115) colocalized with the stress fibers, it did not inhibit the formation and the contraction (Fig. 8C, bottom). The other inhibitory Iba1 mutants, EGFP-Iba1(30-147) and EGFP-Iba1(EF2EF2), also had no effect on the processes induced by Cdc42V12 and RhoA V14 (data not shown). These results suggest that Iba1 participates specifically in Rac signaling but not in that of Cdc42 or Rho.

M-CSF-induced calcium oscillation in MG5 cells
The EGFP-Iba1 mutant that was expected to lose calcium-binding ability, EGFP-Iba1(EF2EF2), effectively inhibited membrane ruffling and phagocytosis (Figs 3G, 4D, 7), suggesting that calcium signaling is crucial for Iba1 to operate. We therefore examined changes in \([\text{Ca}^{2+}]_i\) in MG5 cells stimulated with M-CSF using a fura-2/AM system. The cells rapidly responded to M-CSF with repeated \([\text{Ca}^{2+}]_i\) transients but did not respond to stimulation by BSA, although all the cells kept the potential to respond following ATP stimulation (Fig. 9). These observations indicate that M-CSF could efficiently mobilize calcium, which could in part be responsible for the M-CSF-stimulated events by modifying the behavior of Iba1.

DISCUSSION
Macrophages and microglia exhibit advanced locomotive and phagocytic activities, both of which are essential for their function of migration to the site of inflammation and scavenging pathogens and cell debris (Downey, 1994; Rabinovitch, 1995; Kreutzberg, 1996). We have previously reported cDNA cloning of the rat \(iba1\) gene, which was highly and specifically expressed in a monocytic lineage including microglia and macrophages (Imai et al., 1996), and upregulated markedly in these cells in their activated state (Ito et al., 1998). In this study, we have examined mouse and human Iba1 amino acid sequences, which appear to be more than 90% identical to the rat sequence (Fig. 1A). These facts suggest that Iba1 has an essential role in the functions of the lineage.

To analyze the role of Iba1 in microglia/macrophages, we first examined its intracellular localization in MG5 cells. In the absence of M-CSF, Iba1 existed diffusively in the cytoplasm; however, in response to M-CSF, Iba1 was translocated to membrane ruffles and colocalized with F-actin (Fig. 2B). During phagocytosis of zymosans, Iba1 accumulated in phagocytic cups, together with F-actin (Fig. 4A,B); furthermore, microinjection of anti-Iba1 antibody blocked the phagocytic cup formation (Fig. 4C).

Iba1 can be structurally divided into three parts: the N- and C-terminal regions, and the EF hand insert region (Fig. 1A). The N- and C-terminal regions have unique amino acid sequences that show no homology with any other peptides. We therefore prepared Iba1 mutants, EGFP-Iba1(30-147) and EGFP-Iba1(1-115), carrying truncations of the N- and C-terminal regions, respectively. In quiescent MG5 cells, these mutants did not affect the cell morphology, by contrast, in the presence of M-CSF and zymosans, the mutants effectively
inhibited the formation of membrane ruffles and phagocytic cups, respectively (Figs 3B, E, 4D). In addition, EGFP-Iba1(30-120), missing both the N- and C-terminal regions, simply distributed diffusively in the cytoplasm, and had no effect on membrane ruffling or phagocytosis (Figs 3F, 4D). These results indicate that Iba1 is significant in membrane ruffling and phagocytosis, and suggest that the N- and C-terminal regions relay cellular signals from molecule to molecule. In fact, under a continuous strong signal of Rac1V12, the N- and C-terminal deletion mutants showed different effects upon ruffling (Fig. 7).

The remaining insert region contains two EF hand motifs, of which the first was demonstrated to match the consensus sequence well and have calcium binding activity, whereas the second was not (Fig. 1B-D). Accordingly, calcium binding ability was considered to be lost in another Iba1 mutant, EGFP-Iba1(EF2EF2), in which the first EF hand was replaced by the second. Indeed, EGFP-Iba1(EF2EF2) effectively inhibited membrane ruffling (Fig. 3G) and phagocytic cup formation (Fig. 4D). These results suggest that calcium binding ability is necessary for Iba1 to work, and that the state of Iba1 should be under the control of calcium signals.

Calcium is believed to be a major signal transducer modulating the actin cytoskeleton, mainly because calcium is biochemically known to regulate many of actin binding proteins, and to activate some of protein kinases and phosphatases involved in actin remodeling (Hartwig and Yin, 1988; Stossel, 1993; Janmey, 1994). Some reports demonstrated that cell movement and phagocytosis were accompanied by coordinated mobilization of intracellular calcium (Marks and Maxfield, 1990; Hishikawa et al., 1991; Meagher et al, 1991). In fact, we show M-CSF-evoked calcium transients for the first time (Fig. 9). In our preliminary study with MG5 cells, chelation of cytoplasmic free calcium by BAPTA-AM suppressed M-CSF-induced membrane ruffling (data not shown). These observations suggest the biological importance of calcium in cytoskeleton control; however, to understand how calcium orchestrates these regulatory molecules, including Iba1, further studies are required.

Both immunostaining and EGFP-tagging showed nuclear localization of wild-type Iba1 (Figs 2, 3A). In contrast, all of the inhibitory mutants were excluded from the nucleus (Fig. 3B, E, G). In the cells expressing the mutant, however, endogenous Iba1 visualized by immunostaining was precisely localized in the nucleus (data not shown). In addition, anti-Iba1 antibody microinjected into cytoplasm inhibited phagocytic-
cup formation (Fig. 4C). These observations indicate that the loss of nuclear localization is not responsible for the inhibitory effects of the mutant, and that the cytoplasm is the main place from where Iba1 exerts its effects upon the ruffling and phagocytosis. The biological roles of nuclear Iba1 remain to be clarified.

Cells contain a variety of integrated actin structures, among which Iba1 was localized selectively in membrane ruffles and phagocytic cups (Figs 2B, 4B) but not in filopodia or stress fibers. This observation suggests that Iba1 is not a simple F-actin associate but a molecule functioning in specific signaling pathways. In the absence of any appropriate stimulus, however, overexpressed Iba1 by itself did not actively induce any kind of actin reorganization in MG5 cells expressing endogenous Iba1, and even in NIH 3T3 cells, which do not express endogenous Iba1. We therefore hypothesize that Iba1 acts as an adaptor, linking signaling molecules activated by M-CSF or zymosans to molecules regulating the actin cytoskeleton.

Rac is known to be a multifunctional molecular switch, turning on signals to the nucleus, the generation of radicals and the actin cytoskeleton, and is therefore recognized to be a key player in the respiratory burst of phagocytes (Bokoch, 1995). In this study using MG5 cells, dominant negative Rac1N17 inhibited M-CSF-induced membrane ruffling, while neither Cdc42N17, RhoAN19 (Fig. 6A) nor C3 toxin did (data not shown). Dominant active Rac1V12 induced marked cell extension and membrane ruffling, and Iba1 accumulated in these membrane ruffles (Figs 7, 8A). By pull-down assays, we showed that M-CSF induced activation of endogenous Rac but not that of Cdc42 (Fig. 6B). Furthermore, the three inhibitory Iba1 mutants suppressed Rac1V12-induced cell extension or membrane ruffling (Fig. 7), whereas the mutants had no effect on filopodia and stress fibers induced by Cdc42V12 and RhoAV14, respectively (Fig. 8B,C). These results apparently indicate that Rac activation is required for the formation of membrane ruffles in MG5 cells and suggest that Iba1 participates specifically in Rac signaling, but not in that of Cdc42 or Rho.

Fc receptor-mediated phagocytosis has been reported to be regulated by Rac and Cdc42 (Cox et al., 1997; Caron and Hall, 1998; Massol et al., 1998), and to be accompanied by pseudopod extensions and phagocytic cups (Greenberg, 1995), whose morphology is almost indistinguishable from that shown in zymosan phagocytosis of MG5 cells. As expected, Rac1N17 completely inhibited phagocytic cup formation, and Cdc42N17 partially did (Fig. 6C). On examination with the pull-down assay, however, zymosan stimulation was shown to induce only Rac activation within 5 minutes (Fig. 6D). These results indicate that Rac activation is the initial and main signal for zymosan phagocytosis, at least in MG5 cells.

Membrane ruffles and phagocytic cups are huge multimolecular complexes consisting of Rac and various proteins associated directly or indirectly with Rac and actin, and, in response to cellular signals, these molecules work cooperatively to organize remodeling of the actin structures. Consequently, the relative positions of Iba1 and Rac could not be easily defined. We have not yet obtained data demonstrating direct binding of Iba1 to Rac. To understand the precise topology of Rac and Iba1, we are presently trying to analyze biochemical properties of Iba1 and to identify Iba1-associating molecules.

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