Dissociation of Recruitment and Activation of the Small G-protein Rac during Fcγ Receptor-mediated Phagocytosis*

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Rho-family proteins play a central role in most actin-dependent processes, including the control and maintenance of cell shape, adhesion, motility, and phagocytosis. Activation of these GTP-binding proteins is tightly regulated spatially and temporally; however, very little is known of the mechanisms involved in their recruitment and activation in vivo. Because of its inducible, restricted signaling, phagocytosis offers an ideal physiological system to delineate the pathways linking surface receptors to actin remodeling via Rho GTPases. In this study, we investigated the involvement of early regulators of Fcγ receptor signaling in Rac recruitment and activation. Using a combination of receptor mutagenesis, cellular, molecular, and pharmacological approaches, we show that Src family and Syk kinases control Rac and Vav function during phagocytosis. Importantly, both the immunoreceptor tyrosine-based activation motif within Fcγ receptor cytoplasmic domain and Src kinase control the recruitment of Vav and Rac. However, Syk activity is dispensable for Vav and Rac recruitment. Moreover, we show that Rac and Cdc42 activities coordinate F-actin accumulation at nascent phagosomes. Our results provide new insights in the understanding of the spatiotemporal regulation of Rho-family GTpase function, and of Rac in particular, during phagocytosis. We believe they will contribute to a better understanding of more complex cellular processes, such as cell adhesion and migration.

Reorganization of the actin cytoskeleton drives cell shape changes during processes such as cell adhesion, migration and chemotaxis, bacterial invasion, immune synapse formation, and phagocytosis (1, 2). Generally induced locally, these morphological changes originate from the activation of a given set of cell surface receptors and the induction of signaling pathways that activate Rho family GTP-binding proteins, which control localized actin polymerization. Understanding how Rho-GTpase signaling is locally activated is therefore crucial to many essential aspects of molecular cell biology.

Rho proteins cycle between inactive GDP-bound and active GTP-bound conformations. Only the latter can bind downstream effectors. Conversion to the GTP-bound form is catalyzed by guanine nucleotide exchange factors (GEFs), whereas inactivation back to the GDP-bound form is mediated by GTPase-activating proteins. Inducible activation of Rho GTPases requires their targeting to membranes, where they will interact with both GEFs and downstream targets (3). Although Rho proteins can bind to membranes directly, by virtue of their posttranslationally added prenylation, their subcellular localization is also regulated, as Rho GTP-binding proteins are held soluble and inactive in the cytosol through an interaction between their prenyl moiety and the effector region of GDP dissociation inhibitor proteins (4). The mechanisms by which Rho proteins are recruited and activated in particular regions of the cells to control actin polymerization are still poorly understood. Furthermore, the deciphering of these mechanisms is complicated by several factors, for example by the fact that different Rho-family members can be involved in a given cellular process, leading to synergistic or antagonistic cross-talk, as shown during cell migration or adherens junction formation (5, 6). Because of its spatiotemporally restricted and inducible signaling, the phagocytic uptake of model particles offers an ideal biological system to delineate the pathways linking the localized ligation of surface receptors to actin remodeling via Rho proteins (1).

Phagocytosis is the mechanism by which cells take up large particles (>=0.5 μm) into an intracellular compartment, the phagosome (7). Phagocytic uptake is initiated by the direct or opsonin-mediated recognition of ligands exposed on the particle surface by specific receptors present at the surface of phagocytic cells (e.g. macrophages, neutrophils, and receptor-transfected cells). Receptor clustering triggers intracellular signaling pathways that lead to the reorganization of the actin cytoskeleton, which is essential for particle uptake. The receptor for the Fc portion of immunoglobulins (FcγR) is the best studied phagocytic receptor (8–10). Binding of IgG-opsonized particles to FcγR induces the activation of Src kinases, which phosphorylate two tyrosines in the receptor immunoreceptor tyrosine-based activation motif (ITAM) domain (11). ITAM phosphorylation is crucial for the formation of actin-rich cups and thus for particle uptake, as shown in experiments using hck/lyn/fgr-deficient mice, FcγR mutants, or Src kinase inhibitors (12–15). Phosphorylated ITAM motifs act as docking sites for a non-receptor tyrosine kinase, Syk, which undergoes auto-phosphorylation and whose activation is necessary for phagocytosis (16, 17). Moreover, surface expression of a FcγR/Syk chimera is sufficient to stimulate particle uptake (18). If Syk activation clearly lies downstream of Src kinase activity as demonstrated in hck/lyn/fgr-deficient macrophages (12, 17), its role in actin polymerization downstream of FcγR is still controversial. On one hand, actin polymerized normally at nascent phagosomes in macrophages derived from Syk-deficient mice (16). On the other hand, actin polymerization was impaired in FcγR-transfected DT40 lymphocytes engineered to lack Syk (19), whereas piceatannol, a specific inhibitor of Syk (20), blocked actin accumulation at nascent phagosomes in murine macrophages (15). Nevertheless, taken together, these data show that Src kinase activity and ITAM-dependent signaling are essential for actin polymerization and particle uptake during FcγR-mediated phagocytosis.

This work was supported in part by grants from the Wellcome Trust (068556/Z/02/Z) and Biotechnology and Biological Sciences Research Council (28/C18637). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 Supported by a fellowship from the French foundation “La Fondation pour la Recherche Médicale” (SPE200302637107).
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3 The abbreviations used are: GEF, guanine nucleotide exchange factor; FcγR, Fcγ receptor; ITAM, immunoreceptor tyrosine-based activation motif; SRBC, sheep red blood cells; PBS, phosphate-buffered saline; GFP, green fluorescent protein; WT, wild-type.

8756 JOURNAL OF BIOLOGICAL CHEMISTRY VOLUME 281 • NUMBER 13 • MARCH 31, 2006
Rho GTP-binding proteins play a critical role during FcγR-mediated uptake, as inhibition of either Rac or Cdc42 blocks phagocytosis (19, 21, 22). Rac and Cdc42 are activated and recruited to forming phagosomes in response to IgG-particle challenge (21, 23, 24). Elegant fluorescence resonance energy transfer studies have recently confirmed that active forms of Rac and Cdc42 accumulate rapidly, coincidentally with F-actin, underneath IgG-opsonized particles (25). It is thought that each of these two Rho proteins controls the local recruitment of the Arp2/3 complex, thereby actin polymerization at phagosomes (26). Nevertheless, the role of Rac and Cdc42 in actin polymerization at forming phagosomes is still controversial, as two independent studies have reported that expression of dominant negative Rac or Cdc42 did not completely block F-actin accumulation at nascent phagosomes, although each individually inhibited FcγR-mediated phagocytosis (19, 22). Another unresolved issue is whether Rac and Cdc42 are recruited in their active form or locally activated. Our published data support the latter, as the inactive (N17) form of Rac is recruited to nascent phagosomes (21). Furthermore, the recruitment of Rac (either N17 or wild-type) to nascent phagosomes occurs in the absence of Vav-exchange activity, despite the fact that Vav activity is necessary for Rac activation and phagocytosis (23).

Intriguingly, whether and how the tyrosine kinase and Rho signaling pathways are connected to control actin polymerization and phagocytosis downstream of FcγR has not yet been elucidated. Because activation of Src and Syk kinases corresponds to the first signaling events detected after binding of IgG-opsonized particles, they are good candidates to control small GTPase function during phagocytosis. Src and Syk kinases have been described to phosphorlyate Vav and enhance Vav exchange factor activity in vitro as well as during T- and B-cell receptor signaling (27, 28). In line with this, we have shown that Syk is still recruited to nascent phagosomes in macrophages expressing dominant negative Vav, suggesting that Vav acts downstream of initial FcγR signaling (23).

Herein, we show that early FcγR signaling controls recruitment and Vav-dependent activation of Rac and actin polymerization. Remarkably, the kinase activity of Src, but not Syk, controls Rac and Vav recruitment to nascent phagosomes, whereas Syk activity is essential for Rac activation, and therefore, for particle uptake. We also show that Rac activation is dispensable for actin polymerization at nascent phagosomes although necessary to coordinate with Cdc42-actin polymerization and engulfment during FcγR-mediated phagocytosis.

### EXPERIMENTAL PROCEDURES

**DNA Constructs**—Eucaryotic pRK5 expression vectors encoding human FcγRIIA (FcγR), myc-tagged N17Rac, N17Cdc42, Wasp (amino acids 201–310), Vav wild-type (Vav-WT), VavC (comprising just the carboxy-terminal SH3-SH2-SH3 domains), VavΔ342-348 (containing a 6-amino-acid deletion in the catalytic DH domain, VavΔDH), eGFP-p59HckWT and eGFP-RacWT have been previously described (21, 23, 29). Hemagglutinin-tagged pCMV-VavΔ342-348 was a kind gift from Charles Abrams (University of Pennsylvania, Philadelphia, PA).

Plasmids expressing truncated versions of the FcγR were generated from the pRK5-FcγRIIA template by inverse PCR using the Expand Long Template PCR System (Roche Applied Science) using the following primers: 5′-tagatcttgccttcatgttcaag-3′ and 5′-gaagatcttcatgttcaag-3′ for FcγRΔ240; 5′-tagatcttgccttcatgttcaag-3′ and 5′-gaagatcttcatgttcaag-3′ for FcγRΔ275. The resulting PCR products, which contained terminal BgIII restriction sites were digested and religated overnight at 16 °C. To generate the FcγR(Y/F)2 mutant, tyrosines 282 and 298 were sequentially substituted with phenylalanines on pRK5-FcγRIIA by reverse PCR, using the QuickChange site-directed mutagenesis kit (Stratagene). We used the following combinations of primers (mutation underlined): 5′-gctgagcgggctcatgacctgaaccc-3′ and 5′-gggttcagagctgacctgacgac-3′ to introduce the Y282F substitution; 5′-gagatcttaataaggctcatgacctc-3′ and 5′-gagatcttcatgttcaagatgttttattcagc-3′ for the Y298F mutation. Mutagenesis products were transformed into One Shot TOP10 chemically competent Escherichia coli (Invitrogen), according to the manufacturer’s instructions. All constructions were checked by DNA sequencing (MWG), amplified, and prepared for transfection using the Qiagen maxi-prep kit.

**Antibodies and Drugs**—Mouse monoclonal anti-Rac (clone23A8) and anti-phosphotyrosine (clone 4G10) antibodies were purchased from Upstate Biotechnology, the anti-myc antibody (clone 9E10) was from Roche Applied Science. All conjugated secondary antibodies (donkey) were purchased from Jackson ImmunoResearch Laboratories. PP2 and piceatannol were purchased from Calbiochem-Biosciences and dissolved in dimethyl sulfoxide (Me2SO). The maximum final concentration of Me2SO never exceeded 0.1% (v/v) in vehicle- or drug-treated cells.

**Cell Culture and Transfection**—Cells from the murine macrophage J774.A1 and simian kidney fibroblast COS-7 cell lines were maintained in complete medium, Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% heat-inactivated fetal calf serum (PAA laboratories), and penicillin/streptomycin (100 units/ml and 100 µg/ml, respectively, Invitrogen). COS-7 cells were seeded on coverslips in 6-cm dishes (100 cells/dish) and transfected with the calcium/phosphate protocol as described previously (29). Briefly, DNA/calcium phosphate precipitates (10 µg of DNA/400 µl of calcium phosphate/6-cm dishes containing 3.6 ml of fresh complete medium) were added onto the cells for 16–18 h, washed, and incubated in fresh complete medium for an additional 6 h.

**Drug Treatments and Phagocytosis Assay**—Transfected COS-7 were transferred to 10 mm Hepes-buffered serum-free Dulbecco’s modified Eagle’s medium (SFM) for a period of 18 h and then incubated with drugs for 30 min. Drugs were maintained in the medium during a challenge with IgG-opsonized sheep red blood cells (SRBC, Cappel) prepared as previously described (21, 23). Briefly, 0.4 µl of SRBC were opsonized with rabbit anti-SRBC IgG during 30 min in 1 ml of gelatin veronal buffer (Sigma) and washed once with gelatin veronal buffer. IgG-SRBC were allowed to adhere for 15 min at 4 °C and synchronized phagocytosis was induced for 15 min at 37 °C.

**Immunofluorescence**—Cells were washed once with phosphate-buffered saline (PBS) and fixed in cold 4% (w/v) paraformaldehyde for 15 min at 4 °C. Free aldehyde groups were neutralized in 13.3 mg/ml of NH4Cl/PBS for 10 min. Cells were then permeabilized with 0.1% Triton X-100 in PBS for 5 min and blocked with 1% bovine serum albumin/PBS for 15 min. For immunostaining, cells were incubated for 30 min with antibodies diluted in 1% bovine serum albumin/PBS supplemented with excess human IgG (Sigma) to prevent nonspecific binding of the antibodies to Fcγ receptors. FITC-, rhodamine- and Cy5-conjugated donkey anti-rabbit IgG were used to detect opsonized SRBC. Myc-tagged constructs were visualized using mouse monoclonal anti-myc followed by fluorescein isothiocyanate-conjugated anti-mouse IgG. F-actin was stained using Alexa546-conjugated phalloidin (Molecular Probes). Coverslips were mounted in Mowiol mountant (Calbiochem), and images were captured using a Zeiss LSM 510 confocal microscope.

**Determination of Rac Activity Levels**—The Cdc42/Rac interactive-binding domain of Pak1 (PAK-CRIB) fused to glutathione S-transferase (GST) was prepared for GTPase pull-down assays as previously
Rac Regulation during FcγR Phagocytosis

Src and Syk kinase activities are necessary for FcγR-mediated phagocytosis (12, 15, 16). Moreover, the first detectable signaling events after particle binding are Src-mediated tyrosine phosphorylation of the FcγR ITAM domain and the accumulation to nascent phagosomes of several phosphorylated proteins, including Src kinases themselves and the Src substrate Syk (31). We used a pharmacological approach to investigate the role of Src and Syk kinases in the activation of Rac signaling. PP2, a selective inhibitor of Src-family kinases (14), and piceatannol, a selective Syk inhibitor (20), were titrated for inhibition of FcγR-mediated phagocytosis in J774.A1 macrophages (data not shown). Compared with control, MeSO-treated cells, macrophages treated with PP2 (10 μM), and piceatannol (75 μM) exhibited a marked reduction in their FcγR-dependent ability to phagocytose but were as competent in binding particles, confirming that the drugs specifically act on phagocytic signaling (Fig. 1A). MeSO treatment did not affect the binding and phagocytic properties of any of the cell types we used (data not shown).

We next examined the effect of these drugs on FcγR-induced Rac activation. Endogenous GTP-loaded Rac was precipitated from lysates of control, drug-treated and SRBC-challenged J774.A1 using the Rac/Pak-CRIB pull-down method (30). Briefly, lysates were incubated for 45 min at 4°C with 10 μg of a 50% slurry of Pak-CRIB-GST coupled to glutathione-agarose beads to precipitate GTP-loaded GTases. Beads were subsequently washed three times in cold, modified PD buffer (without deoxycholate or SDS) and resuspended in 20 μl of 2× sample buffer. Equal amounts of beads and total cell lysates were analyzed by SDS-PAGE and immunoblotting, using a monoclonal anti-Rac antibody. Fold activation of Rac was assessed, relative to the level of Rac in the total cell lysate, by quantification of autoradiographic exposures using ImageJ software.

Analysis of Vav Phosphorylation Level—J774.A1 macrophages seeded at 2 × 10⁶ cells/10-cm dish were starved in 10 ml of HEPES-containing SFM during 1.5 h, treated with the drugs for an additional 30 min, then challenged with 15 μl of IgG-SRBC in 3 ml of cold HEPES-buffered SFM for 15 min at 4°C. Cells were washed once to remove unbound particles, reincubated at 37°C for 10 min, washed again on ice with 10 ml of ice-cold PBS, and scraped in 200 μl of lysis buffer (50 mM Tris-HCl, pH 7.4, 1% Nonidet P-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, protease inhibitor mixture 1 μg/ml, 1 mM Na3VO4, 1 mM NaF). Whole cell lysates were analyzed by SDS-PAGE and immunoblotting, using a monoclonal anti-phosphotyrosine antibody. Total Vav levels were assessed after membrane stripping and immunoblotting. Fold phosphorylation of Vav was related to the level of Vav in the total cell lysate, by quantification of autoradiographic exposures using ImageJ software.

Scoring—Binding and phagocytosis indices were determined by counting the number of particles respectively associated to and internalized by 100 cells, whether J774.A1 or FcγR-expressing COS-7 cells. Protein recruitment to nascent phagosomes was scored blindly by confocal microscopy and is expressed as the percent positive phagosomes out of a population of >100 phagosomes, corresponding to an average of 20 cells/coverslip.

Statistics—A paired t test was used to determine the statistical significance of the results. Data sets were considered different for p values < 0.05.

RESULTS

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neither had any significant effect on SRBC binding (Fig. 3A). Importantly, PP2, but not piceatannol, abrogated F-actin accumulation at nascent phagosomes, indicating that Src-family, but not Syk kinases, control F-actin accumulation at nascent phagosomes (Fig. 3B). It is well documented that expression of Src-like kinases and Syk kinases is not restricted to hematopoietic cells (35, 36) and both kinase activities appear to be expressed in COS cells (33). The differential effect of piceatannol on Rac activation and F-actin cup formation conflicts with some (26) but not all published (19, 22) studies, which prompted us to re-examine the respective roles of Rac and Cdc42 in FcγR-induced actin polymerization. We used different constructs known to interfere directly with activation of, or downstream signaling from, these Rho family members. Each construct blocked phagocytosis in COS-7 cells by at least 70% (data not shown). Dominant negative (N17) myc-tagged Rac was first co-expressed with FcγR in COS-7 cells. After challenge with IgG-SRBC, the percent of F-actin-positive nascent phagosomes was scored. Fig. 4 shows that specific inhibition of Rac activation had no effect on F-actin accumulation at nascent phagosomes. Neither N17Rac, thought to titrate endogenous Rac-specific GEFs, nor dominant negative Vav mutants (known to block Rac but not Cdc42 activation during FcγR-mediated phagocytosis (23)), impaired F-actin accumulation at nascent phagosomes (Fig. 4). Because Rac and Cdc42 activities control FcγR-mediated phagocytosis independently of each other, we next examined whether Cdc42 plays the major role in actin polymerization. Fig. 4 shows that N17Cdc42 overexpression did not abolish F-actin accumulation underneath bound SRBC. Independent confirmation was obtained in cells co-transfected with the Cdc42-binding fragment (amino acids 201–310) of the effector WASP, which also failed to block F-actin accumulation at nascent phagosomes (Fig. 4). Interestingly, we noticed a difference in actin cup morphology, dependent on whether Rac or Cdc42 activity was blocked (Fig. 4, insets). In cells expressing N17Rac, the cups were well organized actin rings, whereas in N17Cdc42- or WASP-expressing cells, F-actin accumulated in a discontinuous structure (Fig. 4 and data not shown). These results demonstrate that neither Rac nor Cdc42 activities are essential per se for actin polymerization at nascent phagosomes and suggest that these small GTP-binding proteins could have complementary functions and coordinate actin polymerization during FcγR-mediated phagocytosis. To test this hypothesis, we inhibited the activity of both Rac and Cdc42 by expressing either the PAK-CRIB fragment (which prevents endogenously activated Rac or Cdc42 from interacting with downstream effectors) or the combination of N17Cdc42 and VavΔDH to block their activation. In both cases, simultaneous inhibition of endogenous Rac and Cdc42 abrogated F-actin accumulation at sites of particle binding (Fig. 4). Together, these results strongly suggest that Rac and Cdc42 are the main Rho proteins that coordinate actin polymerization at nascent phagosomes, explaining why they control FcγR-mediated uptake.

Vav activity controls Rac activation during FcγR-mediated phagocytosis (23). As activation of Vav downstream of T- and B-cell receptor ligation is known to correlate with its tyrosine phosphorylation (28), we investigated whether the activation of Vav, just like Rac, was Src- and Syk-dependent. The stimulation of J774.A1 macrophages with IgG-SRBC led to a 3-fold increase in the amount of tyrosine-phosphorylated Vav (Fig. 5), consistent with what was described for FcγR cross-linking in RAW 264.7 murine macrophages (37). By contrast, in cells treated with PP2 or piceatannol, Vav phosphorylation levels were no longer influenced by a challenge with IgG-SRBC (1.1- and 1.2-fold difference,
respectively, Fig. 5). We conclude that Src and Syk kinase activities control Vav phosphorylation during FcγR-mediated phagocytosis, which provides a likely mechanism for the observed abolition of Rac activation by PP2 and piceatannol.

Catalytically inactive mutants of Vav are still recruited to nascent phagosomes (23). To better understand the pathways involved in localizing Vav at sites of particle binding, we first investigated whether FcγR signaling controlled Vav enrichment at nascent phagosomes. Two truncation mutants of the FcγR were engineered, deleting half (FcγRΔH9253R275, missing the ITAM domain) or all (FcγRΔH9253R240) of the 73 amino acids of its cytoplasmic tail, as well as a point mutant in which tyrosine residues at positions 282 and 298 were substituted phenylalanines (FcγR(Y/F)2, Fig. 6)

FIGURE 4. Rac and Cdc42 activities coordinate actin polymerization during FcγR-mediated phagocytosis. COS-7 cells were co-transfected with FcγRIIA and GFP, myc-tagged (N17Rac, VavC, VavΔDH, N17Cdc42, WASP (amino acids 201–310), Pak-CRIB), or hemagglutinin-tagged (VavΔDH) constructs as indicated, challenged with IgG-SRBC, and stained for F-actin, tag (Myc or HA), and SRBC as shown. Representative images are shown in A, with arrowheads indicating typical cup morphologies, better seen in the insets. The percent F-actin-positive phagosomes (compared with control values, arbitrarily set at 100) is given in B. Results are expressed as the mean ± S.D. of at least three independent experiments.

FIGURE 5. Tyrosine phosphorylation of Vav is dependent on Src and Syk kinase activities. J774-A1 macrophages were left unstimulated (−, white bars) or challenged (+, black bars) with IgG-SRBC after pretreatment with Me2SO (DMSO), PP2 or piceatannol (Pic) as indicated. Vav phosphorylation levels were first assessed by immunoblotting whole cell lysates with a murine monoclonal anti-phosphotyrosine antibody (4G10). Membranes were then stripped and analyzed for total Vav content using a rabbit polyclonal anti-Vav antibody. Top, representative example. Bottom, quantification. For each treatment, the unchallenged condition was arbitrarily set at 1. Results are expressed as the mean ± S.D. of at least three independent experiments.
SRBC, Fig. 6B, black bars) confirming the crucial importance of the ITAM-containing region and the tyrosine residues it harbors for particle internalization. We then co-transfected COS-7 cells with FcγR constructs and either myc-tagged wild-type Vav (VavWT) or GFP, as negative control. VavWT was recruited to nascent phagosomes in FcγRWT-expressing cells (Fig. 6C, bottom, compare recruitment of Vav, black bars, with GFP, white bars) as previously described in macrophages (23, 40). However, Vav recruitment was impaired (Fig. 6C) in cells expressing truncated FcγRs, suggesting that the ITAM-containing region of the FcγR-cytoplasmic tail controls local recruitment of Vav. Therefore, we next analyzed whether Src and Syk kinase activities are necessary to recruit Vav underneath bound particles. COS-7 cells co-expressing FcγRWT and either myc-tagged VavWT or GFP were treated with Me2SO, PP2, or piceatannol before a challenge with IgG-SRBC. As shown in Fig. 6C, PP2 pretreatment blocked Vav recruitment to nascent phagosomes, whereas piceatannol had no effect. These results strongly suggest that Src, but not Syk, activity, through phosphorylation of the FcγR ITAM domain controls recruitment of the RacGEF Vav.

We have shown that Rac is recruited to nascent phagosomes independently of Vav exchange activity, suggesting that Rac is locally activated by Vav (23). How Rac is recruited to nascent phagosomes is still poorly understood. As we did for Vav, we studied the recruitment of a GFP-tagged version of wild-type Rac (Rac-GFP) in COS-7 cells co-transfected with the different FcγR constructs. As described previously (21, 23), overexpressed Rac is specifically recruited underneath bound particles during FcγR-mediated phagocytosis (39 ± 8 Rac-GFP positive phagosomes per 100 analyzed phagosomes, Fig. 7, compare Rac-GFP to GFP alone). However, recruitment of Rac-GFP was abrogated in cells expressing the FcγRΔ240, Δ275, and (Y/F)2 mutants, suggesting that the ITAM-containing region, already described to control phagocytosis and Vav recruitment, also regulates the signaling pathway leading to Rac recruitment at nascent phagosomes. To examine the role of Src and Syk kinases in Rac recruitment to nascent phagosomes, FcγR-expressing COS cells were treated with Me2SO, PP2, or piceatannol, challenged with IgG-SRBC, and scored for specific recruitment of Rac-GFP underneath bound particles. Remarkably, pretreatment with PP2, but not with piceatannol, abolished Rac-GFP recruitment (Fig. 7, compare black, Rac-GFP, with white, GFP bars). To exclude the possibility of artifacts in the scoring of recruitment, we scored the recruitment of a GFP-tagged version of the hematopoietic, Src-family kinase, p59Hck. Ectopic expression of wild-type kinase had no significant effect on SRBC binding or phagocytosis and did not alter the adverse consequence of FcγR mutation or PP2 treatment on phagocytosis. For example, PP2 reduced by 78% the number of SRBC.
internalized by COS cells expressing FcγRIIA and p59Hck. Interestingly, as shown in Fig. 8, p59Hck was recruited to nascent phagosomes in an ITAM-dependent but PP2-independent manner, suggesting that potential particle-induced membrane convolution does not bias our scoring method. We therefore conclude that Rac accumulation at nascent phagosomes is controlled by the activity of Src kinases, but is independent of Syk activity.

**DISCUSSION**

Phagocytosis is an actin-driven process driven by Rho-family GTPases (10, 41). Activation of Rac and Cdc42 is required for FcγR-mediated phagocytosis (10, 41); Rac and Cdc42 are also recruited to nascent phagosomes (21, 23, 25). However, the exact role of these Rho proteins and the mechanisms regulating their function during uptake are still poorly understood. Using macrophages and FcγR-transfected...
COS cells (21, 23, 32, 33), we show that inhibition of Rac/Cdc42 activities block F-actin accumulation at nascent phagosomes (Fig. 9). These data extend previous findings showing that toxin B, an inhibitor of most Rho proteins (42), blocked both FcyR-induced accumulation of F-actin and phagocytosis (21, 22). Interestingly, full Fc-actin rings formed underneath SRBC in N17Rac-expressing cells, whereas only discontinuous structures were observed in cells expressing N17Cdc42, confirming published data (22) and supporting the idea that Rac and Cdc42 orchestrate remodeling of the actin cytoskeleton during FcyR uptake (Fig. 9 (25, 43, 44)).

We also show that pharmacological inhibition of Src- and Syk-family kinases abolished not only uptake but also FcyR-induced activation of Rac and tyrosine phosphorylation of Vav, which controls Rac activation during phagocytosis (23). Vav GEF activity is controlled by phosphorylation (28, 45–47). Because Vav is likely to be phosphorylated by Syk (16), itself activated downstream of Src substrate (17), we propose that a linear cascade leads sequentially from ligated FcyR to Src-mediated ITAM phosphorylation, SH2-dependent recruitment and activation of Syk, Vav activation, and Vav-mediated activation of Rac (Fig. 9). This would account for our observation that inhibition of Vav does not affect the recruitment of tyrosine-phosphorylated proteins or Syk to nascent phagosomes (23). The early steps of this pathway (FcyR → Src → Syk → Vav) closely resemble signaling induced by other ITAM-containing immunoreceptors (T-cell and B-cell receptors), where Syk/ZAP70 directly interacts with the SH2 domain of Vav (28). We postulate that it also applies to other ITAM-containing phagocytic receptors acting in a Src- and Rac-dependent manner, such as Dectin-1 (48) and CEACAM3 (49, 50).

We show for the first time that the FcyR ITAM-containing region and Src activity control Rac and Vav recruitment to nascent phagosomes (Fig. 9). Piceatannol blocks Rac and Vav activation but not recruitment strengthening our hypothesis that Rac is first recruited and then locally activated by Vav (23). One explanation could be that Vav and Rac are recruited together. However, apart from the DH domain (which is dispensable for Rac recruitment to nascent phagosomes (23)) no potential Rac binding site has been described on Vav. A complex of inactive Rac and GDP dissociation inhibitor could interact with Vav, as shown in T-cells (51, 52). However, we have shown that a Vav mutant, lacking both the amino-terminal and the DH regions of Vav, can still be recruited together with Rac at nascent FcγR phagosomes (23). We thus favor the hypothesis of an independent recruitment of Vav and Rac (Fig. 9). Rac recruitment could involve GDP dissociation inhibitor and an as yet unidentified GDP dissociation inhibitor displacement factor. GDP dissociation inhibitor displacement factor activities have been characterized for Rab proteins (53) and described for Ran (54) but not for Rho proteins, although several molecules have been proposed to act as GDP dissociation inhibitor displacement factor-like factors, including Ca2+-, protein kinase Cε, ezrin-radixin-moesin (ERM) proteins (57), and cytoplasmic receptor chains (58). However, it is hard to imagine that calcium or ERM play a significant role in our system, as they are either acting downstream of Rac signaling or dispensable during FcγR-mediated phagocytosis (59, 60). There are also several candidate regulators of Vav recruitment to nascent phagosomes. The Tec kinase, Itk (inducible T-cell kinase) is activated downstream of Src-like and phosphoinositol 3-kinases (61) and controls Vav recruitment to the immunological synapse after T-cell receptor activation (62). However, during FcγR-mediated phagocytosis, phosphoinositol 3-kinase activation is controlled by Syk (17), whose activity is dispensable for Vav recruitment (this work). Several adaptor proteins, e.g. SLP76 and BLNK, both of which are expressed in macrophages (63), have been involved in immunoreceptor signaling. In particular, BLNK has been implicated in B-cell receptor-induced localization of Vav to lipid rafts and Rac activation (64). However, in macrophages deficient for SLP-76 and BLNK, FcγR-mediated phagocytosis proceeds normally (63) suggesting that these proteins are dispensable for Vav recruitment during this process. Finally, our data are compatible with a role for Syk (but not Syk activity) in the independent recruitment of Rac and/or Vav, for example through the phosphorylatable linker region tyrosines (45). The molecular mechanisms involved in Rac and Vav recruitment to nascent phagosomes are currently under investigation in the laboratory.

In conclusion, we have established that Rac and Cdc42 coordinate local actin polymerization during FcγR-mediated phagocytosis. We have also demonstrated that Rac recruitment and activation are differentially controlled downstream of FcγR ligation; Src-family kinases trigger two signaling pathways that control on one hand, the Syk-independent recruitment of Rac and Vav to nascent phagosomes, and, on the other hand, the Syk-dependent activation of Vav and Rac. Similar approaches to the ones described here can be used to investigate the spatiotemporal regulation of Cdc42, Arf6, and Rab11, other small GTPases necessary for FcγR-mediated phagocytosis. Our results confirm that signaling molecules (e.g. Vav, Syk) are conserved, at least functionally, between hematopoietic and non-hematopoietic cells such as fibroblasts, explaining why transfection of phagocytic receptors into COS-7 or HeLa cells (65) is sufficient to induce phagocytic signaling. We believe this model will continue to provide useful information for the understanding of more complex cellular processes, for example cell migration. In this respect, it is worth noting that Rac controls at least in part, Src-induced actin-driven morphological changes in fibroblasts (66, 67).

Acknowledgments—We thank S. Berhane and J. C. Patel for technical assistance and reagents.

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Rac Regulation during FcγR Phagocytosis

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