Role of CrkII in Fcγ Receptor-mediated Phagocytosis*

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Phagocytosis of IgG-opsonized pathogens by Fcγ receptors requires extensive remodeling of the actin cytoskeleton, a process regulated by the small GTPase Rac. Rac was thought to be the guanine nucleotide exchange factor responsible for the activation of Rac, but recent evidence indicates that Fcγ receptor-mediated phagocytosis is unaffected in macrophages lacking all three isoforms of Rac. We therefore tested whether another GEF, DOCK180, participates in Fcγ receptor-initiated phagocytosis. DOCK180 associates with the adaptor protein Crk, which mediates recruitment of the GEF to sites of tyrosine phosphorylation. CrkII and DOCK180 were found to accumulate at the phagocytic cup. Knockdown of Crk or DOCK180 in murine macrophages using small interfering RNA inhibited phagocytosis of IgG-opsonized particles. Moreover, transfection of dominant negative CrkII prevented both recruitment of DOCK180 and the activation of Rac at the phagocytic cup. This is the first report of a role for either Crk or DOCK180 in Fcγ receptor-mediated phagocytosis. The Crk-DOCK180 complex is involved in the clearance of apoptotic cells, which unlike the ingestion of IgG-opsonized particles, is an anti-inflammatory process. The finding that CrkII-DOCK180 is also responsible, at least in part, for the effects of Fcγ receptors implies that additional, parallel pathways must account for the associated pro-inflammatory effect.

Phagocytosis is a key component of the innate immune response to infection. Phagocytic receptors recognize molecular features inherent to infectious microorganisms or bind host-derived opsonins, such as complement components or immunoglobulins that coat their surface. Fcγ receptors on the surface of phagocytes recognize pathogens opsonized by IgG. Upon engagement, the receptors become phosphorylated by Src family kinases and recruit and activate the kinase Syk (1). These early events culminate in extensive remodeling of the actin cytoskeleton, which is essential for engulfment of the pathogen.

Actin restructuring during Fc-mediated phagocytosis is regulated by the small GTPases Cdc42 and Rac. These GTPases act as molecular switches, recruiting effector proteins only when bound to GTP, a condition promoted by guanine nucleotide exchange factors (GEFs). The activation cycle is terminated by hydrolysis of GTP, which is facilitated by GTPase activating proteins. Although the relative contributions of Rac and Cdc42 to Fcγ-mediated phagocytosis are only partially understood (2), there is good evidence that Rac plays an essential role. Epitope-tagged Rac becomes enriched at sites of Fcγ-mediated phagocytosis (3), whereas expression of dominant negative Rac constructs drastically inhibits phagocytosis and the accumulation of actin underneath the IgG-opsonized particle (4). Indeed, recruitment of active Rac to the plasma membrane per se is sufficient to induce phagocytosis of latex beads (5).

Despite its importance, it remains unclear how stimulation of Fcγ receptors is linked to Rac activation. In particular, the identity of the GEF(s) responsible for activating Rac is controversial. Some evidence has implicated Vav isoforms, because microinjection of dominant negative Vav constructs was found to inhibit phagocytosis of IgG-opsonized red blood cells (6). On the other hand, it has recently been shown that macrophages deficient in all three isoforms of Vav display no defect in Fcγ-mediated phagocytosis (7).

It is known that all GEFs can be recruited to receptors at the plasma membrane by adaptor proteins (8). Such adaptors contain modular protein-protein interaction domains that allow the activated receptor to recruit downstream signaling molecules (9). One such adaptor, CrkII, contains an N-terminal SH2 domain that interacts with phosphorylated tyrosine residues followed by two SH3 domains (designated SH3-N and SH3-C) that bind proline-rich motifs (10). Interestingly, in Caenorhabditis elegans, CED-2 (the homologue of CrkII) has been shown to mediate phagocytic clearance of apoptotic cells (11) and interacts with a Rac GEF, DOCK180, via its SH3-N domain. Recently, CrkII was also found to be essential for the invasion of Listeria monocytogenes into non-phagocytic mammalian cells (12). Whether CrkII is also necessary for Fcγ-mediated phagocytosis is unknown.

In this work, we sought to determine the link between the early events in Fcγ receptor-mediated signaling and Rac activation in macrophages. We identified a critical role for CrkII in the phagocytosis of IgG-opsonized particles and provided evidence that this adaptor serves to recruit the Rac GEF DOCK180 to the site of ingestion. CrkII-DOCK180-mediated activation of

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4 The abbreviations used are: GEF, guanine nucleotide exchange factor; PBD, PAK-binding domain; YFP, yellow fluorescent protein; SH, Src homology; HA, hemagglutinin; siRNA, small interfering RNA; GST, glutathione S-transferase; PIP3, phosphatidylinositol 3,4,5-trisphosphate; GFP, green fluorescent protein; DIC, differential interference contrast.
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Rac therefore represents a conserved signaling pathway common to the phagocytosis of both IgG-opsonized particles and apoptotic cells.

EXPERIMENTAL PROCEDURES

Reagents and Antibodies—Fetal bovine serum, Dulbecco’s modified Eagle’s medium, Hepes-buffered solution/RPMI 1640 and phosphate-buffered saline (PBS) were from Wisent (St. Bruno, Quebec, Canada). Latex beads (3.1 mm in diameter) were from Bangs Beads (Fishers, IN). Mouse monoclonal antibody to Crk was from BD Biosciences. Goat antibody to DOCK180 and rabbit anti-CrkL were from Santa Cruz Biotechnology. Plasmids encoding enhanced green fluorescent protein-DOCK180 and FLAG-DOCK180 were generously provided by Dr. M. Matsuda (Osaka University, Japan). The plasmid encoding the fusion of the PBD-YFP was performed using an LSM510 laser scanning confocal microscope (Zeiss) with a 100× oil immersion objective. Quantitation of the fluorescence of PBD-YFP at the phagosomal cup was accomplished using Image J software (NIH) after subtracting for the background. Membrane-associated fluorescence was normalized to cytosolic fluorescence to eliminate the variable expression inherent to transient transfection. The phagocytic index of cells transfected with dominant negative mutants of CrkII was calculated by imaging random fields and counting the number of internalized beads in cells expressing suitably high levels of the transfected gene product. Phagocytic index of cells treated with siRNA was calculated by imaging random fields and counting the number of internalized beads divided by the number of cells in the field. In all cases, image acquisition settings were kept constant between conditions.

Immunoﬂuorescence of Crk in RAW cells was performed by fixing cells in 4% paraformaldehyde followed by permeabilization in 0.1% Triton, blocking in 5% milk, and then probing with mouse anti-Crk at 1:100 for 1 h. After washing with PBS, the samples were incubated with Cy3-conjugated anti-mouse antibody (1:1000) for 30 min and then washed and mounted using DAKO mounting medium (Dako Cytomation, Carpinteria, CA). An identical procedure was used for immunoﬂuorescence of DOCK180 in RAW cells, except that a goat anti-DOCK180 primary antibody (1:50) and the appropriate secondary antibody were used. For immunoﬂuorescence of HA- or FLAG-tagged proteins, we used mouse anti-HA at 1:1000 and mouse anti-FLAG at 1:1000 for 1 h.

Flow Cytometry—RAW cells were treated with siRNA to Crk or control siRNA 72 h before the experiment. Cells were probed with Fc Block antibody according to the manufacturer’s instructions (BD Biosciences) using a Cy2-conjugated secondary antibody at 1:1000. Cells were analyzed in a FACS-Calibur cytometer (BD Biosciences), and the results were analyzed using FlowJo software (Tree Star Flow Jo).

Cell Culture and Transfection—RAW264.7 cells were obtained from the American Type Culture Collection (Manassas, VA) and were grown in Dulbecco’s modified Eagle’s medium with 5% fetal bovine serum at 37 °C in 5% CO2 under a humidified atmosphere. Lipofection was carried out using the Amaxa system (Cologne, Germany), following the manufacturer’s guidelines, with 2 million cells and either 2 μg of cDNA or 1.5 μg of siRNA using program D32. Cells were analyzed 6–10 h after electroporation, unless otherwise stated.

Human macrophages were isolated by plating the lymphocyte/monocyte-rich layer of blood from healthy volunteers on coverslips bathed in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum, incubating them at 37 °C, followed by washing with sterile PBS and changing the medium daily for five days to remove platelets and lymphocytes.

Phagocytosis Assays—Latex beads were opsonized with 3.75 mg/ml human IgG for 1 h at 37 °C. Cells grown on glass coverslips were changed to prewarmed serum-free Hepes-buffered solution/RPMI 1640 and were overlaid with 25 μl of opsonized latex beads and incubated at 37 °C to initiate phagocytosis. In most experiments, phagocytosis was synchronized by centrifugation of the beads onto the cells at 1500 revolutions/min for 10 s. Unless stated otherwise, RAW cells were incubated with beads for 10 min at 37 °C followed by labeling of external beads using Cy5-conjugated donkey anti-human antibody and then fixed with 4% paraformaldehyde. In some experiments, RAW cells were pretreated with 100 μM LY294002 for 15 min prior to phagocytosis.

Microscopy, Immunofluorescence, and Image Analysis—Analysis of the distribution and density of Crk, DOCK180, and PBD-YFP was performed using an LSM510 laser scanning confocal microscope (Zeiss) with a 100× oil immersion objective. Quantitation of the fluorescence of PBD-YFP at the phagosomal cup was accomplished using Image J software (NIH) after subtracting for the background. Membrane-associated fluorescence was normalized to cytosolic fluorescence to eliminate the variable expression inherent to transient transfection.

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Immunoblotting—Lysates were analyzed by SDS-PAGE using 7–10% polyacrylamide gels. Proteins were transferred to nitrocellulose membranes, blocked for 1 h in 5% milk in PBS.
with 0.05% Tween 20 (PBS/Tween), and then probed overnight with the primary antibody at 4 °C. We used 1:2500 for mouse anti-Crk, 1:100 for anti-DOCK180, 1:50 for anti-CrkL, 1:2000 for anti-GAPDH, and 1:10000 for anti-phosphotyrosine antibodies. After washing in PBS/Tween, the blots were incubated with horseradish peroxidase-conjugated secondary antibodies (1:2000–1:5000) for 30 min, washed vigorously, and then visualized by enhanced chemiluminescence (Amersham Biosciences).

Aggregated IgG was prepared by heating a 12.5 mg/ml solution of IgG in PBS to 56 °C for 30 min and then removing high-molecular-weight aggregates by centrifugation. The supernatant was applied to the cells for 7 min at 37 °C. For immunoblotting for phosphotyrosine, membranes were blocked with 0.5% bovine serum albumin in Tris-buffered saline (TBS), and washes were in TBS with 0.1% Tween 20. Quantitation of protein bands was performed using Image J (NIH) on scanned blots and included correction for the background.

**Protein Expression, Purification, and Pulldown Assay**—Plasmid constructs containing glutathione S-transferase (GST) alone or fused to full-length wild-type CrkII or CrkII W170K were expressed in *Escherichia coli* and purified as described previously (12). Sedimentation of proteins from solubilized lysates of RAW cells that interact with GST fusion proteins was performed essentially as previously described (12). Approximately 2 × 10⁶ RAW cells were seeded in 10-cm plates and grown for 24 h. The cells were rinsed with ice-cold PBS and solubilized by the addition of 1 ml of ice-cold lysis buffer (1% Triton X-100, 50 mm Tris-HCl, pH 7.5, 150 mm NaCl, 3 mm sodium-orthovanadate HCl-activated, 20 mm NaF, 1 mm phenylmethylsulfonyl fluoride, and 10 μg/ml each of aprotinin and leupeptin). The RAW cell lysate was centrifuged at 12,000 × g for 10 min at 4 °C, and the supernatant was recovered and used for the pulldown assay. Equal quantities of GST fusion proteins and RAW cell lysates were used for each precipitation. The total protein in the precipitates was separated on 8% SDS-polyacrylamide gels and analyzed by immunoblotting for DOCK180.

**Statistics**—Unless otherwise stated, all experiments were performed at least in triplicate. For comparisons of means, Student’s *t* tests were used. A probability <0.05 was deemed significant.

**RESULTS**

**CrkII Is Recruited to the Phagocytic Cup during Fcγ-mediated Phagocytosis**—To confirm that Crk is expressed in macrophages, we analyzed whole cell lysates of human blood monocyte-derived macrophages and murine RAW264.7 cells (hereafter called RAW cells) by immunoblotting using a monoclonal antibody that recognizes the two known splice variants of Crk (10). As expected, we detected two bands, corresponding to CrkII (~40 kDa) and CrkI (~28 kDa). As shown in Fig. 1A, CrkII is expressed at higher levels than CrkI in both human and murine macrophages. Next, we determined whether Crk is recruited to the phagosome during Fcγ-mediated phagocytosis. Human macrophages were allowed to ingest IgG-opsonized latex beads and were subsequently immunostained for endogenous Crk. A distinct recruitment of Crk to the nascent phagosome was noted (Fig. 1B). A similar recruitment was also observed in RAW macrophages (Fig. 1C), indicating that Crk is present and mobilizes to phagosomes in both human and murine phagocytes. Of note, the association of endogenous Crk was clearly observable at the earliest stages of phagocytic cup formation but was virtually absent from formed (sealed) phagosomes (Fig. 1D). The accumulation of Crk at the phagocytic...
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A schematic of the domain structure of CrkII and of the mutants used in this study. The recruitment of CrkII to the phagocytic index. RAW macrophages were transfected with either wild-type (WT) or dominant negative alleles of HA-tagged CrkII before incubation with IgG-opsonized latex beads. The phagocytic index was calculated relative to cells transfected with wild-type CrkII. Data are means ± S.E. of at least 70 individual determinations.

Dominant Negative CrkII Inhibits Fcγ-mediated Phagocytosis—To determine the functional role of CrkII in phagocytosis, we expressed dominant negative mutant forms of the adaptor in RAW macrophages. These mutants contain amino acid substitutions in either the SH2 (R38K), SH3-N (W170K), or SH3-C (W276K) domains of the protein (Fig. 2A). The R38K mutation eliminates an arginine residue found in all SH2 domains that is known to be essential for the recognition of phosphotyrosine groups (13). Similarly, the W170K and W276K mutations alter conserved tryptophans required for binding of SH3 domains to proline-rich motifs (14). Mutants of CrkII at Arg-38 and Trp-170 have been previously shown to disrupt the interaction of CrkII with its binding partners (15). Transfection of the dominant negative mutants of CrkII significantly inhibited phagocytosis, with the greatest inhibition occurring with the R38K and W170K mutants (Fig. 2B; p < 0.01 for all mutants versus control). In particular, expression of the R38K mutant decreased phagocytosis by almost 75% relative to cells transfected with wild-type CrkII. This mutant also decreased the ability of the cells to spread on the substratum (not illustrated), consistent with an effect on actin remodeling. Transfection of wild-type CrkII had no significant effect on internalization compared with untransfected cells (data not shown).

Knockdown of Crk by siRNA Inhibits Fcγ-mediated Phagocytosis—Because overexpression of inhibitory mutants can have undesirable effects on related yet distinct pathways, we sought to confirm the role of Crk by alternative, more specific means. To this end, we introduced siRNA to Crk into RAW cells by electroporation and determined the levels of the protein 48–72 h later. As a control for our siRNA studies, we used a non-targeting siRNA (Dharmacon, Lafayette, CO) designed not to interact with any gene products in mouse cells. Although Crk expression was unaffected in cells transfected with control siRNA, the expression of both isoforms was markedly depressed in cells treated with Crk siRNA. CrkI and CrkII protein levels dropped by >75% and 50%, respectively (p < 0.05 versus control for both) (Fig. 3A and B). Importantly, this effect was specific to the Crk gene. Not only was the expression of unrelated housekeeping proteins such as GAPDH unaffected,
but the levels of the related protein CrkL were similarly unaltered (Fig. 3A).

In parallel experiments, we exposed siRNA-transfected cells to IgG-opsonized beads and determined the phagocytic index (Fig. 3, C–F). Cells pretreated with Crk siRNA had a lower ability to ingest particles compared with non-targeting siRNA controls (Fig. 3G). The inhibition was never complete, but it was highly significant ($p < 0.01$) and of the same order as the reduction in protein expression (see above). This likely reflects heterogeneity in the delivery of siRNA by electroporation, because immunostaining revealed the presence of two populations of cells: one with near-normal expression and another with negligible levels (not shown). Together with the data from the dominant negative constructs, these results indicate that Crk has an essential function in Fcγ-mediated phagocytosis.

**Knockdown of Crk Does Not Affect Fcγ Receptor Levels or Function**—Silencing the Crk gene was expected to alter the adaptor function of this protein and its downstream effectors without impairing receptor activation. To validate this notion, we determined the surface density and activation state of Fcγ receptors in cells treated with control or Crk-directed siRNA. Flow cytometric determinations revealed that the surface expression of Fcγ receptors was indistinguishable in control and Crk-depleted cells (Fig. 4A). The ability of these receptors to become activated was evaluated by quantifying the induction of tyrosine phosphorylation triggered by cross-linking. As shown in Fig. 4B, a robust increase in phosphotyrosine content was induced by aggregated IgG in both control and Crk-depleted cells. These results provide evidence that Crk knockdown does not affect Fcγ receptor expression, targeting, or activation and imply that Crk plays a specific role in signaling subsequent transduction events.

**DOCK180 Is Recruited to the Phagocytic Cup in a CrkII-dependent Manner**—One of the best-characterized Crk-binding proteins is DOCK180, which was described to display GEF activity (16, 17). It therefore seemed conceivable that CrkII functions at the nascent phagocytic cup by recruiting DOCK180. To test this hypothesis, we immunostained endogenous DOCK180 in RAW macrophages during the course of phagocytosis of IgG-opsonized beads (Fig. 5A). We also transfected RAW macrophages with either FLAG- or green fluorescent protein (GFP)-tagged DOCK180 and visualized the cells during the course of particle ingestion. An obvious accumulation of DOCK180 at the cup was observed very early during phagocytosis, whether by immunostaining of the endogenous protein or of epitope-tagged constructs (Fig. 5, B and C), resembling the pattern noted earlier with CrkII.

DOCK180 binds to Crk through a C-terminal proline-rich motif that is recognized by an SH3 domain of the adaptor (16). In addition, DOCK180 possesses a DHR-1 domain that binds phosphatidylinositol 3,4,5-trisphosphate (PIP3) (18). To analyze the contribution of the DHR-1 domain to DOCK180 recruitment to the phagosome, we pretreated RAW cells with LY294002, a potent and specific inhibitor of phosphatidylinositols 3-kinase, the enzyme that generates PIP3. Pretreatment for 15 min with the inhibitor at a concentration of 100 μM was sufficient to eliminate the formation of PIP3 at the phagocytic cup, as monitored using the pleckstrin homology (PH) domain

![FIGURE 4. Knockdown of Crk does not affect Fcγ receptor expression or function](image)

A RAW cells treated with either control (Ctrl) siRNA or siRNA to Crk were probed 72 h after electroporation with an antibody to murine FcγRII/III followed by a Cy2-labeled secondary antibody and then analyzed by flow cytometry. Black tracing denotes cells treated with siRNA to Crk; gray tracing denotes control siRNA-treated cells. Histogram is representative of three separate experiments.

![FIGURE 5. DOCK180 is recruited to the cup in a CrkII-dependent manner](image)

A RAW cells were incubated with IgG-opsonized beads for 1 min at 37 °C and then probed for endogenous DOCK180. For all panels, arrowheads denote the phagocytic cup, whereas asterisks denote beads. Insets are corresponding DIC images. B RAW cells were transfected with FLAG-tagged DOCK180 and incubated with IgG-opsonized beads for 1 min at 37 °C and then probed for the FLAG epitope. C RAW cells were transfected with GFP-tagged DOCK180 and incubated with IgG-opsonized beads for 1 min and then visualized by confocal microscopy. D RAW cells were transfected with GFP-tagged DOCK180 and treated with 100 μM LY294002 for 15 min prior to incubation with IgG-opsonized beads for 1 min. RAW cells were co-transfected with GFP-tagged DOCK180 (E) and HA-tagged CrkII and incubated with IgG-opsonized beads for 1 min followed by immunostaining for HA (F). RAW cells were co-transfected with GFP-tagged DOCK180 (G) and HA-tagged CrkII(R38K) and then incubated with IgG-opsonized beads for 1 min followed by immunostaining for HA (H). Images are representative of three separate experiments.
of Akt linked to GFP (Akt-PH-GFP) (19, 20) (data not shown). Recruitment of DOCK180 to the cup persisted under these conditions (Fig. 5D), implying that the interaction between PIP₃ and the DHR-1 domain is dispensable and suggesting that association through CrkII may be more important.

Accordingly, when RAW cells were co-transfected with GFP-DOCK180 and HA-tagged CrkII, we observed a high degree of colocalization of these proteins at the phagocytic cup using confocal microscopy (Fig. 5, E and F). To determine whether the recruitment of DOCK180 was in fact mediated by CrkII, RAW cells were co-transfected with the HA-tagged CrkII(R38K). This mutation disrupts the ability of the SH2 domain in CrkII to interact with phosphotyrosines at sites of receptor activation and, as a result, CrkII(R38K) was not recruited to the phagocytic cup (Fig. 5H). Importantly, expression of the dominant negative CrkII blocked recruitment of DOCK180 to sites of phagocytosis (Fig. 5, compare G with E).

We further assessed the association of CrkII and DOCK180 by carrying out GST pulldown assays on protein extracts obtained from RAW cells. Pulldown experiments showed that GST-CrkII, a fusion protein constructed using the full-length wild-type form of CrkII, interacts directly with DOCK180, as previously reported (16). In contrast, GST-CrkII(W170K), a fusion of GST with a form of CrkII bearing a point mutation in the SH3 domain that binds DOCK180, failed to pull down DOCK180 as did GST alone (Fig. 6). Together, these data suggest that DOCK180 is recruited to the phagocytic cup by CrkII.

Knockdown of DOCK180 by siRNA Inhibits Fcγ Receptor-mediated Phagocytosis—To assess the importance of CrkII-DOCK180 signaling to phagocytosis, we treated RAW cells with siRNA to DOCK180. Immunoblotting performed 72 h after transfection showed that the expression of DOCK180 had decreased by almost 50% compared with cells exposed to control siRNA (p < 0.001) (Fig. 7, A and B). No further decrease in the content of DOCK180 was achieved with longer treatments with siRNA, probably reflecting the incomplete nature of the transfection protocol. Nonetheless, in parallel experiments where the phagocytic index of siRNA-treated cells was measured, depletion of DOCK180 caused a significant impairment of Fcγ-mediated phagocytosis relative to controls (p < 0.05) (Fig. 7C).

CrkII and DOCK180 Are Required for the Recruitment of Active Rac to the Phagocytic Cup—Our findings suggested that CrkII-DOCK180 signaling is required for Fcγ-mediated phagocytosis. DOCK180 is the founding member of a newly recog-
nized family of GEFs that do not possess a Dbl domain (8). Instead, DOCK180 has a DHR-2 or DOCKER domain capable of loading Rac (but not Rho or Cdc42) with GTP (17, 21). We therefore tested whether DOCK180 recruitment via CrkII contributes to the activation of Rac in forming phagosomes. RAW cells were co-transfected with CrkII(R38K) and YFP-tagged PBD. The latter construct consists of the p21 Rac-GTP-binding domain of PAK fused to YFP and is a sensitive indicator of Rac activity (22). In cells co-transfected with wild-type CrkII and PBD-YFP, the Rac-GTP indicator was recruited to phagocytic cups, as expected (Fig. 8, A and B). In contrast, co-transfection with the dominant negative CrkII(R38K) completely ablated the accumulation of PBD-YFP at the cup (Fig. 8, C–E). These data indicate that functional CrkII is required for Rac activation at sites of Fcγ receptor-initiated phagocytosis.

Additionally, we tested whether DOCK180 was directly involved in Rac activation. RAW cells were treated with siRNA to DOCK180 and 48 h later were transfected by lipofection with the Rac-GTP indicator, YFP-tagged PBD. Co-transfection with siRNA and the PBD-YFP was not feasible due to the progressive loss of expression of the PBD-YFP over periods >24 h after transfection. The PBD-YFP accumulation at the phagocytic cup was assessed after 72 h of treatment with siRNA. The depletion of DOCK180 prevented the accumulation of the Rac-GTP indicator at the phagocytic cup, which was readily observed in cells treated with control (non-targeting) siRNA (Fig. 9). These data suggest that DOCK180 is necessary for Rac activation at sites of Fcγ receptor-mediated phagocytosis.

**DISCUSSION**

This report describes a role for CrkII and DOCK180 during Fcγ receptor-mediated phagocytosis. Both proteins were found to accumulate at the phagocytic cup, and inhibition of their function by transfection of dominant negative constructs or by gene silencing using siRNA impaired the ability of macrophages to engulf IgG-coated targets.
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Two splice variants of Crk have been identified. As in most cells, CrkII is the predominant isoform in macrophages, whereas CrkI, which lacks the C-terminal SH3 domain and a linker region, is expressed at lower levels. It is therefore likely that the more abundant and versatile isoform, CrkII, is mainly responsible for the biological effects reported here. CrkI, a related protein that exhibits 60% amino acid homology to CrkII, is the product of a distinct gene (10). Like other adaptor proteins, CrkI and II consist of SH2 and SH3 domains and are therefore capable of bridging tyrosine-phosphorylated proteins with partners bearing proline-rich motifs (9). Crk isoforms are likely to be recruited to the activated Fcγ receptor complex by phosphotyrosine moieties generated upon stimulation of Src family kinases or of Syk. Not only are the tyrosines of the receptor ITAM motif phosphorylated, but multiple other phosphotyrosines are generated on ancillary proteins recruited to the receptor complex. These include the ubiquitin ligase Cbl (23) and the scaffolding protein Gab2 (24). Of note, the SH2 domain of Crk was reported to bind preferentially to phosphotyrosines in the context of a pY-X-X-P motif, which is found in both Cbl and Gab isoforms. Moreover, Gab1 was recently found to associate stably with Crk (10, 12).

The middle SH3 domain of CrkII is known to associate with a proline-rich motif in DOCK180 (16). This interaction most likely accounts for the recruitment of DOCK180 to sites of phagocytosis. By contrast, little is known about the function of the C-terminal SH3 domain of CrkII. Although no binding partners for this domain have yet been identified, the domain is therefore capable of bridging tyrosine-phosphorylated proteins, CrkI and II consist of SH2 and SH3 domains and are responsible for the biological effects reported here. CrkL, a related protein that exhibits 60% amino acid homology to CrkII, is expressed at lower levels. It is therefore likely that the more abundant and versatile isoform, CrkII, is mainly responsible for the internalization of primary macrophages from mice deficient in all three isoforms of Vav. In these triple knock-out cells phagocytosis of IgG-opsonized particles proceeded normally (7). It is therefore likely that the microinjected dominant negative alleles may have had effects other than the inhibition of Vav isoforms. Together, these observations argue that one or more GEFs other than Vav are primarily responsible for loading GTP onto Rac during Fcγ-mediated phagocytosis.

Our data suggest that the CrkII-DOCK180 complex contributes to Rac activation at the phagocytic cup during internalization of IgG-opsonized particles. Not only are these proteins recruited to the cup with a spatial and temporal pattern that is compatible with the initiation of Rac activation, but interference with their function impaired the stimulation of Rac. This was ascertained using PBD-YFP to probe for active Rac/Cdc42 (Figs. 8 and 9) (22). A role for CrkII-DOCK180 in particle engulfment is not unprecedented. These proteins are also engaged in the disposal of apoptotic cells mediated by the phosphatidylserine receptor in C. elegans (11, 32) and by integrins in mammalian cells (33). This commonality of function is intriguing given the fundamental differences between the phagocytosis of apoptotic and IgG-coated particles (34). The former is an ongoing process that is characterized by active suppression of inflammation (34, 35). In contrast, Fcγ receptor-mediated phagocytosis is typically associated with induction of the oxidative burst (36) and with the release of pro-inflammatory cytokines (35). This important distinction had been attributed to the engagement of different adaptor and GEF systems during the engulfment of apoptotic and IgG-opsonized particles (11, 37). Our finding that CrkII-DOCK180 is also responsible, at least in part, for the effects of Fcγ receptors implies that additional, parallel pathways must be differentially engaged in both cases. The source of the pro-inflammatory signals generated by Fcγ receptors, which are seemingly unrelated to Vav and CrkII-DOCK180, must be sought.

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REFERENCES

1. Greenberg, S., and Grinstein, S. (2002) Curr. Opin. Immunol. 14, 136–145
2. Hoppe, A. D., and Swanson, J. A. (2004) Mol. Biol. Cell 15, 3509–3519
3. Caron, E., and Hall, A. (1998) Science 282, 1717–1721
4. Cox, D., Chang, P., Zhang, Q., Reddy, P. G., Bokoch, G. M., and Greenberg, S. (1997) J. Exp. Med. 186, 1487–1494
5. Castellano, F., Montcourrier, P., and Chavrier, P. (2000) J. Cell Sci. 113, 2955–2961
6. Patel, I. C., Hall, A., and Caron, E. (2002) Mol. Biol. Cell 13, 1215–1226
7. Hall, A. B., Gakidis, M. A., Glogauer, M., Wilsbacher, J. L., Gao, S., Swat, W., and Brugge, J. S. (2006) Immunity 24, 305–316
8. Schmidt, A., and Hall, A. (2002) Genes Dev. 16, 1587–1609
9. Buday, L. (1999) Biochim. Biophys. Acta 1422, 187–204
10. Feller, S. M. (2001) Oncogene 20, 6348–6371
11. Wang, X., Wu, Y. C., Fadok, V. A., Lee, M. C., Gengyo-Ando, K., Cheng, L. C., Ledwich, D., Hsu, P. K., Chen, J. Y., Chou, B. K., Henson, P., Mitani, S. Grinstein, unpublished data.
S., and Xue, D. (2003) Science 302, 1563–1566
12. Sun, H., Shen, Y., Dokainish, H., Holgado-Madruga, M., Wong, A., and Ireton, K. (2005) Cell Microbiol. 7, 443–457
13. Tanaka, M., Gupta, R., and Mayer, B. J. (1995) Mol. Cell. Biol. 15, 6829–6837
14. Dalgarno, D. C., Botfield, M. C., and Rickles, R. J. (1997) Biopolymers 43, 383–400
15. Kiyokawa, E., Hashimoto, Y., Kurata, T., Sugimura, H., and Matsuda, M. (1998) J. Biol. Chem. 273, 24479–24484
16. Hasegawa, H., Kiyokawa, E., Tanaka, S., Nagashima, K., Gotoh, N., Shibuya, M., Kurata, T., and Matsuda, M. (1996) Mol. Cell. Biol. 16, 1770–1776
17. Brugnera, E., Haney, L., Grimsley, C., Lu, M., Walk, S. F., Tosello-Trampont, A. C., Macara, I. G., Madhani, H., Fink, G. R., and Ravichandran, K. S. (2002) Nat. Cell Biol. 4, 574–582
18. Cote, J. F., Motoyama, A. B., Bush, J. A., and Vuori, K. (2005) Nat. Cell Biol. 7, 797–807
19. Haugh, J. M., Codazzi, F., Teruel, M., and Meyer, T. (2000) J. Cell Biol. 151, 1269–1280
20. Marshall, J. G., Booth, J. W., Stambolic, V., Mak, T., Balla, T., Schreiber, A. D., Meyer, T., and Grinstein, S. (2001) J. Cell Biol. 153, 1369–1380
21. Kiyokawa, E., Hashimoto, Y., Kobayashi, S., Sugimura, H., Kurata, T., and Matsuda, M. (1998) Genes Dev. 12, 3331–3336
22. Scott, C. C., Dobson, W., Botelho, R. J., Coady-Osberg, N., Chavrier, P., Knecht, D. A., Heath, C., Stahl, P., and Grinstein, S. (2005) J. Cell Biol. 169, 139–149
23. Tsygankov, A. Y., Teckchandani, A. M., Feshchenko, E. A., and Swam-nathan, G. (2001) Oncogene 20, 6382–6402
24. Gu, H., Botelho, R. J., Yu, M., Grinstein, S., and Neel, B. G. (2003) J. Cell Biol. 161, 1151–1161
25. Kizaka-Kondoh, S., Matsuda, M., and Okayama, H. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 12177–12182
26. Akakura, S., Kar, B., Singh, S., Cho, L., Tibrewal, N., Sanokawa-Akakura, R., Reichman, C., Ravichandran, K. S., and Birge, R. B. (2005) J. Cell. Physiol. 204, 344–351
27. Massol, P., Montcourrier, P., Guillemot, J. C., and Chavrier, P. (1998) EMBO J. 17, 6219–6229
28. Garcia-Garcia, E., and Rosales, C. (2002) J. Leukocyte Biol. 72, 1092–1108
29. Han, J., Luby-Phelps, K., Das, B., Shu, X., Xia, Y., Mosteller, R. D., Krishna, U. M., Falck, J. R., White, M. A., and Broek, D. (1998) Science 279, 558–560
30. Das, B., Shu, X., Day, G. J., Han, J., Krishna, U. M., Falck, J. R., and Broek, D. (2000) J. Biol. Chem. 275, 15074–15081
31. Cox, D., Tseng, C. C., Bjekic, G., and Greenberg, S. (1999) J. Biol. Chem. 274, 1240–1247
32. Reddien, P. W., and Horvitz, H. R. (2000) Nat. Cell Biol. 2, 131–136
33. Albert, M. L., Kim, I. L., and Birge, R. B. (2000) Nat. Cell Biol. 2, 899–905
34. Maderna, P., and Godson, C. (2003) Biochim. Biophys. Acta 1639, 141–151
35. Fadok, V. A., Bratton, D. L., Konowal, A., Freed, P. W., Westcott, J. Y., and Henson, P. M. (1998) J. Clin. Invest. 101, 890–898
36. Yamamoto, K., and Johnston, R. B., Jr. (1984) J. Exp. Med. 159, 405–416
37. Henson, P. M., Bratton, D. L., and Fadok, V. A. (2001) Nat. Rev. Mol. Cell Biol. 2, 627–633