Fcγ Receptor-mediated Phagocytosis in Macrophages Lacking the Src Family Tyrosine Kinases Hck, Fgr, and Lyn

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Abstract

M acrophage Fcγ receptors (FcγRs) mediate the uptake and destruction of antibody-coated viruses, bacteria, and parasites. W e examined FcγR signaling and phagocytic function in bone marrow–derived macrophages from mutant mice lacking the major Src family kinases expressed in these cells, Hck, Fgr, and Lyn. M any FcγR -induced functional responses and signaling events were diminished or delayed in these macrophages, including immunoglobulin (Ig)G-coated erythrocyte phagocytosis, respiratory burst, actin cup formation, and activation of Syk, phosphatidylinositol 3-kinase, and extracellular signal-regulated kinases 1 and 2. Significant reduction of IgG-dependent phagocytosis was not seen in hck−/−fgr−/− or lyn−/− cells, although the single mutant lyn−/− macrophages did manifest signaling defects. T hus, Src family kinases clearly have roles in two events leading to FcγR-mediated phagocytosis: one involving initiation of actin polymerization and the second involving activation of Syk and subsequent internalization. Since FcγR-mediated phagocytosis did occur at modest levels in a delayed fashion in triple mutant macrophages, these Src family kinases are not absolutely required for uptake of IgG-opsonized particles.

Key words: actin polymerization • Fcγ receptors • macrophage • phagocytosis • Src family kinases

Introduction

Clustering of Fcγ receptors (FcγRs) occurs upon binding of their ligand, the Fc portion of IgG, which is present in immune complexes or on antibody-coated cells. FcγR activation in macrophages results in the secretion of products involved in an inflammatory response, induction of antibody-dependent cellular cytotoxicity, and phagocytosis, which play key roles in our immune defense against infectious diseases (1, 2). T hrough these processes, FcγRs mediate the ingestion of viruses, bacteria, and parasites, as well as the antibody-dependent killing of cells expressing viral or tumor antigens (3, 4). In addition, internalization of antigens by phagocytosis leads to antigen processing and presentation to neighboring T cells.

Signaling through FcγRs has striking parallels to signaling via FcεRI and the T and B cell antigen receptors (5, 6). T hese receptors have been classified as members of the multichain immune recognition receptor family, and mediate signaling via homologous cytoplasmic sequences called the immunoreceptor tyrosine-based activation motif (ITAM)†. For class I and class III FcγRs, these sequences are present on the accessory γ chain (and the TCR-γ chain for FcγRIII), whereas for class II FcγRs they are present on the cytoplasmic portion of the ligand binding chain (7). ITAMs contain paired tyrosines and leucines or isoleucines in the consensus sequence YxxL/I/(x)7-12YxxL/I. T he ITAM is both necessary and sufficient for many FcγR-triggered signaling events and is involved in the recruitment, via Src homol-

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ology 2 (SH 2) domain–phosphotyrosine interactions, and activation of specific tyrosine kinases upon receptor clustering (1).

As has been analogously proposed for B cell and T cell receptors, this clustering of FcγRs by either the onset of phagocytosis or treatment with cross-linking antibodies rapidly stimulates the membrane-associated Src family tyrosine kinases, which in macrophages include Hck, Fgr, and Lyn (8–10). These kinases are believed to be responsible for phosphorylation of ITAM tyrosines of the FcγRs. The Src family tyrosine kinases are also capable of binding the FcγR after ITAM phosphorylation and may associate with it or with other receptor subunits at low affinity before receptor stimulation as well (11).

Both Src and Syk family kinases likely contribute to the phosphorylation of targets involved in downstream biochemical processes, such as phosphatidylinositol-4,5-bisphosphate (PIP2) breakdown, phosphatidylinositol 3-kinase (PI 3-kinase) activation, and stimulation of the mitogen-activated protein (MAP) kinase pathways. How these signaling events are connected to biological responses of the cell, including phagocytosis, is poorly understood. Syk has been shown to play a critical role in FcγR-mediated phagocytosis, both by the requirement for Syk to enhance formation of FcγR phagocytic function in transfected Cos cells (12) and by analysis of Syk-deficient macrophages, which are defective in internalization of antibody-coated RBCs (13, 14). Interestingly, syk−/− macrophages are able to bind IgG-coated particles normally and initiate the first steps in the signaling pathway leading to actin polymerization for phagocytic cup formation; however, they are completely unable to fuse the extended membranes around the phagocytic vesicle and hence fail to engulf the particle. Treatment of macrophages with inhibitors of PI 3-kinase produces a similar block in phagocytic vesicle closure (15).

We sought to examine the role of Src family tyrosine kinases in FcγR-mediated phagocytosis and signaling by using macrophages from genetically targeted mice lacking one or more of the Src family kinases expressed in these cells. Our results indicate that these Src family kinases are important for FcγR-induced formation of actin cups, γ chain phosphorylation, and activation of Syk. Interestingly, in the absence of these Src family kinases, most of these processes were delayed but not completely defective, suggesting that Src family kinases are not absolutely required for FcγR-mediated phagocytosis.

Materials and Methods

Reagents. The following antibodies and antisera were used in these experiments: supernatants from 2.4G2 (rat anti-Fc γ III) and MAR18.5 (mouse anti–rat IgG) (Jackson ImmunoResearch Laboratories); anti-Cbl, anti–extracellular signal–regulated kinase (Erk)1, and anti-c-Jun NH2-terminal kinase (JNK) (Santa Cruz Biotechnology); anti-JNK1 (Pharmingen); anti-Syk (16), anti-γ chain antisera (provided by J. P. Kinet, Harvard University, Boston, MA); anti-phosphotyrosine hybridoma 4G10 (17), fluorescein anti-rat IgG2b (Phar- mingen); and anti-SR BCs (Nordic Immunology). Protein A and protein G-Sepharose were from Amersham Pharmacia Biotech. Purified R eLP5 from Salmonella minnesota was obtained from List Biological Laboratories. Na232PO4 and γ-32P[γ-ATP] were from N EN Life Sciences.

Macrophages. Generation of the mouse strains with targeted deletions in hdx, fyn, or lyn genes has been described (18–20). All studies were carried out using bone marrow–derived macrophages cultured as described (20) except that RPMI 1640 medium was used in place of α-MEM. 20% L cell–conditioned medium (LCM) was used as a source of M-CSF. After 1–2 d in culture, nonadherent bone marrow cells, at concentrations of 0.5 × 107 well or 1 × 106 plate, were transferred to new 24-well or 100-mm plates, respectively. Confluent monolayers of adherent macrophages were used for phagocytosis or signaling experiments 5–7 d later. In some cases, nonadherent cells were frozen in liquid nitrogen and then thawed, counted, and plated as above for experiments 5–7 d later.

Phagocytosis A says. SR BCs (Accurate Chemical and Scientific Corp.) were washed in PBS and resuspended to a 5% solution in RPMI 1640. 1 ml of this solution was incubated with 400 μCi Na32PO4 for 2–3 h at 37°C. SR BCs were then washed twice in PBS and incubated at room temperature with a subagglutinating concentration (1:800) of anti-SR IgG for 30–45 min at room temperature. After two more washes in PBS, SR BCs were resuspended to the original volume (5% solution) but then further diluted in cold RPMI 1640 medium (1:300) for the phagocytosis assay. These 32Cr-labeled, antibody-coated erythrocytes will be referred to as 32Cr-EAs.

Confluent macrophages, in 24-well plates, were chilled on ice before addition of cold 32Cr-EAs (0.5 ml/well). Plates were centrifuged at 150 g for 5 min at 4°C to promote contact of 32Cr-EAs with the adherent macrophages. Phagocytosis was begun by aspiration of cold medium and addition of warm medium to the wells. Plates were immediately put at 37°C for the indicated time periods. Reactions were stopped by returning plates to the ice, followed by quick aspiration, washing, and hypotonic lysis of unengulfed 32Cr-EAs with 750 μl water for 1 min. After a subsequent wash in PBS, adherent macrophages with ingested 32Cr-EAs were lysed in 500 μl 1% SDS and transferred to tubes for quantitation of radioactivity in a γ-counter (CliniGamma; LKB-Wallac). Percent phagocytosis was calculated based on the total cpm of replicate wells treated as described above but without hypotonic lysis of unengulfed 32Cr-EAs. Each time point represents the average of four replicate wells. Internalization of EAs was blocked by known inhibitors of phagocytosis such as cytochalasin D (2 μM), which inhibits actin polymerization, and wortmannin (100 μM), which inhibits the enzyme PI 3-kinase.

Phagocytosis of C3-opsonized FITC-labeled zymosan particles (Molecular Probes) was carried out as described (21). In brief, zymosan particles (5 × 107 beads) were incubated in 100 μl of mouse serum for 30 min at 37°C to fix C3bi onto the surface of the yeast particle. Immunofluorescent staining with anti–C3 polyclonal antisera (Sigma Chemical Co.) confirmed uniform opsonization of the zymosan particles with C3 (not shown). Washed C3-opsonized particles (5 × 107) were added to confluent monolayers of macrophages cultured in 96-well black-sided tissue culture plates, and the plates were centrifuged at 150 g for
30 s and incubated on ice for 30 min. Unbound particles were removed by washing with PBS, and phagocytosis was initiated by addition of medium at 37°C. At the indicated times, plates were removed from the 37°C incubator, the designated wells were washed, and phagocytosis was stopped by addition of 2% paraformaldehyde. The total fluorescence was measured in a fluorescent plate reader (CytoFluor II; PerSeptive Biosystems), after which fluorescence from bound but not internalized zymosan particles was quenched by addition of 2 mg/ml trypan blue in 0.15 M NaCl/20 mM sodium citrate buffer, pH 4.4. The percent phagocytosis was calculated by dividing the value of the trypan blue–quenched signal by the total fluorescence for each well. Each time point was performed in six-well replicates. Attachment of C3-opsinized yeast particles to macrophages was blocked by addition of anti–Mac-1 (CR3; β2 integrin, CD11b/CD18) mAb (not shown).

Respiratory Burst. FcγR-mediated internalization of immune complexes leading to oxidative burst in the phagocytic vacuole was monitored using the FcOxyBURST® reagent from Molecular Probes. The reagent consists of insoluble BSA–anti-BSA immune complexes in which the BSA is covalently linked with dichlororhodaminefluorescein (H₂DCF). Oxidation of the H₂DCF to DCF in the phagocytic vesicle produces green fluorescence that can be monitored by flow cytometry (22). For this assay, macrophages (either resting or primed by 12-h incubation with 10 ng/ml LPS plus 20 U/ml IFN-γ) were removed from culture dishes and placed in suspension in HBSS at 2 × 10⁶/ml. Suspended cells (500 μl aliquots) were maintained at 37°C for 15 min, then collected by flow cytometry to determine background fluorescence. Flow cytometry showed that 530 nm at a rate of ~200 cells/s. Cells were then stimulated with the FcOxyBURST® reagent at a final concentration of 120 μg/ml, and development of fluorescence was monitored (the reaction tube was maintained at 37°C). Changes in mean fluorescence of the population were plotted at 0.67-s intervals. In some experiments, cells were preincubated with cytchalasin D (3 μM) to inhibit phagocytosis.

Endocytosis of Immune Complexes. FcγR-mediated endocytosis of small soluble immune complexes was determined using flow cytometry to measure the uptake of PE-labeled heat-aggregated IgG. Biotinylated rabbit IgG (250 μl of a 10 mg/ml solution in PBS; Pierce Chemical Co.) was heated to 63°C for 30 min to partially denature and aggregate the immunoglobulins. These complexes were labeled by addition of 5 μg of PE-conjugated streptavidin. Cultured macrophages were placed in suspension in HBSS plus 1% BSA medium at either 4°C or 37°C, then incubated with immune complexes at a final concentration of 250 μg/ml for varying periods of time. At the end of the incubation period, cells were washed in HBSS once and surface-bound immune complexes were removed by treatment with PBS, pH 2.5, for 1 min at 4°C. After an additional HBSS wash, cells were fixed in 1% paraformaldehyde in PBS. Cells were analyzed by flow cytometry to measure endocytosis of IgG. In some experiments, cells were preincubated with cytchalasin D (3 μM) before incubation with immune complexes.

Photomicroscopy. Macrophages grown on coverslips were synchronized on ice for EA phagocytosis as described above. At 5 and 15 min after addition of 37°C medium, the cells were washed twice in PBS then fixed in 3% paraformaldehyde for 15 min on ice. After two more PBS washes, cells were stained with rhodamine-conjugated phalloidin (Molecular Probes) for 30 min at room temperature. Subsequently, cells were washed twice in PBS, then incubated with FITC-conjugated goat anti-rabbit IgG (1:50 dilution) in PBS/5% BSA for 45 min at room temperature. Cells were washed twice in PBS, fixed again in 1% paraformaldehyde for 10 min, then viewed by fluorescence microscopy. Digital images were captured in two fluorescent channels on a DeltaVision fluorescence microscope (Applied Precision).

Cellular Activation, Immunoprecipitation, and Immunoblotting. Before stimulation, macrophages were placed in growth medium lacking LCM for 3–4 h. Plates were cooled on ice and then incubated for 30–45 min with chilled supernatant from the rat anti-FcγR 2.4G2 hybridoma. After washing with PBS, a secondary cross-linking anti-rat antibody (MAR 18.5 or F(ab’)₂ fragment mouse anti-rat IgG) was added at 10 μg/ml in PBS and cells were immediately placed at 37°C to begin stimulation. For LPS or CSF-1 stimulation, cells were starved as above and LPS (1 μg/ml) or LCM was then added directly to culture plates at 37°C. At the indicated times, the stimulated cell monolayers were washed with cold PBS containing 1 mM Na₂VO₄ and then lysed in 0.7 ml lysis buffer (20 mM Tris-HCl, pH 8, 137 mM NaCl, 10% glycerol, 1% Triton X-100, 2 mM EDTA, 2 mM Na₂VO₄, 10 mM sodium pyrophosphate, 80 mM disodium β-glycerophosphate, 1 μg/ml aprotinin, 10 μg/ml leupeptin, and 100 μg/ml Pefabloc® SC [Boehringer Mannheim Biochemicals]). Plates were rocked at 4°C for 15 min to extract cellular proteins, and lysates were then centrifuged at 17,000 g for 15 min to remove insoluble material. Lysates were precleared with 30 μl of protein G-Sepharose, and protein concentration in the precleared supernatant was determined by the Coomassie blue dye binding assay (Bio-Rad ad).

For immunoblotting, 30–100 μg of total lysate protein was separated on 8 or 10% SDS-polyacrylamide gels under reducing conditions. For Erk kinase mobility shift assays, lysates were separated on SDS-PAGE gels having an acrylamide/bisacrylamide ratio of 12:1. For immunoprecipitation experiments, 1 μg of anti-Cbl or 5–10 μl of anti-Syk or anti-γ-chain antibody was added to equivalent amounts of lysate protein for 1 h at 4°C, after which 30–40 μl of protein A-Sepharose was added for an additional 2–4 h with constant rocking. For immunoprecipitation with 4G10, hybridoma supernatant was first bound to protein G-Sepharose, and protein concentration in the precleared supernatant was determined by the Coomassie blue dye binding assay (Bio-Rad ad).

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For detection of γ chain phosphorylation, macrophages were incubated in phosphate-free RPMI 1640 (Irvine Scientific) containing 1% FCS and 32PO₄ (3.1 mCi/100-mm plate) for 5 h at 37°C. Cells were then stimulated as described above, except that incubation with MAR 18.5 was performed on ice. Lysates were sequentially immunoprecipitated, first with control anti-IgG antisera and then with anti-γ chain antisera. Washed immunoprecipitates were then separated on a 15% reducing SDS-PAGE gel, and 32PO₄-labeled proteins were detected by autoradiography.

JNK and PI 3-Kinase Assays. Anti-JNK immunoprecipitates were assayed for kinase activity using glutathione S-transferase (GST)-cJun as a substrate in kinase assay buffer (25 mM Hepes, pH 7.6, 20 mM MgCl₂, 20 mM disodium β-glycerophosphate, 1 mM Na₂VO₄, and 2 mM dithiothreitol) as described (23). PI 3-kinase
assays were performed using either antiphosphotyrosine (4G10) or anti-Cbl immunoprecipitates as described previously (24). $^{32}$P incorporation into phosphatidylinositol 3-phosphate was quantified by PhosphorImager® analysis (Molecular Dynamics).

Results

Impaired FcγR-mediated Phagocytosis and Respiratory Burst in hck$^{-/-}$fgr$^{-/-}$lyn$^{-/-}$ Macrophages. To examine the role of Src family tyrosine kinases in FcγR-mediated phagocytosis, we studied this process in primary cultures of bone marrow-derived macrophages isolated from mice deficient in the Src family members prevalent in these cells, Hck, Fgr, and Lyn. In initial studies using a nonsynchronous assay in which macrophages were incubated with EAs at 37°C for 20–30 min, we observed no great difference in the phagocytic ability of wild-type versus hck$^{-/-}$fgr$^{-/-}$lyn$^{-/-}$ cells, as determined by microscopic counting of internalized phase-bright EAs (13; and data not shown) or uptake of $^{51}$Cr-labeled EAs (see below). However, when initiation of phagocytosis was synchronized by first binding the EAs to macrophages on ice and then placing them at 37°C to start phagocytosis, we noted that the wild-type macrophages internalized bound EA particles quite rapidly (within a few minutes) while the process was delayed in hck$^{-/-}$fgr$^{-/-}$lyn$^{-/-}$ macrophages. As shown in Fig. 1 A, after 5 min at 37°C, wild-type cells had ingested large numbers of phase-bright EAs, but hck$^{-/-}$fgr$^{-/-}$lyn$^{-/-}$ cells had ingested very few.

To measure the rate of phagocytosis accurately, we used an assay for phagocytosis based on the ingestion of $^{51}$Cr-labeled EAs (see Materials and Methods). Fig. 1 B shows a representative time course experiment in which the delayed onset of phagocytosis in hck$^{-/-}$fgr$^{-/-}$lyn$^{-/-}$ macrophages is evident. At 2–10 min, the defect in phagocytosis in mutant macrophages was greatest. At these early time points, while wild-type macrophages had already internalized nearly all of
mediated activation of respiratory burst in LPS/IFN-γ-primed cells were completely inactive and LPS/IFN-γ-primed cells mounted a considerably blunted response. The FcγR-dependent oxidative burst in this assay was blocked by preincubation of cells with cytochalasin D (not shown), indicating that signal transduction leading to formation of F-actin is required for assembly of the NADPH oxidase within the phagocytic vesicle, as reported previously (22, 25, 26).

In contrast to phagocytosis and induction of respiratory burst, wild-type and triple mutant macrophages displayed equivalent endocytosis of soluble immune complexes (Fig. 2 B). Using a flow cytometric assay for internalization of fluorescently labeled heat-aggregated IgG, we observed equal labeling of wild-type and mutant cells at varying times after exposure to the complexes. Unlike phagocytosis or induction of respiratory burst, internalization of immune complexes was not blocked by preincubation of cells with cytochalasin D (not shown). This is consistent with the observation that phagocytosis of immune complexes requires γ chain signaling to F-actin formation while endocytosis does not (27).

Figure 2. Normal immune complex endocytosis but impaired FcγR-mediated activation of respiratory burst in hck−/−fgr−/−lyn−/− macrophages. (A) Suspension macrophages (resting or primed by 12-h incubation in LPS/IFN-γ) were incubated with 120 μg/ml of Fc Oxy8R STβ light chains and the development of fluorescence resulting from oxidative burst was monitored by flow cytometry. (B) Endocytosis of heat-aggregated rabbit IgG was determined by exposing cells to PE-conjugated immune complexes for varying periods of time, then removing the surface-bound complexes by acid treatment. The amount of remaining fluorescence representing internalized immune complexes was determined by flow cytometry. The data shown are for a 20-min incubation of cells with immune complexes at 37°C (solid lines) or 4°C (broken lines). Equivalent uptake of complexes was seen also in wild-type and mutant cells at earlier and later time points (not shown).

In the bound EAs, phagocytosis by hck−/−fgr−/−lyn−/− macrophages was minimal. At later time points, however, the hck−/−fgr−/−lyn−/− macrophages internalized 35S-labeled EAs moderately well. By 30 min, wild-type macrophages had internalized 88 ± 3% (n = 26 experiments; ±SEM) of the bound EAs, whereas triple mutant cells phagocytosed a lower fraction of the bound EAs, varying from 13 to over 79% in different experiments (mean of 41 ± 6%, n = 22 experiments; ±SEM). Variation in the triple mutant phagocytosis was seen with different lots of anti-SR B Cs used to opsonize RBCs and with different serum samples used for macrophage culture. Poorer phagocytosis by the mutant macrophages was not due to defective recognition of EAs, since both mutant and wild-type macrophages bound EAs similarly on ice (Fig. 1 A, 0 time) and displayed equivalent cell-surface levels of FcγRII and III as determined by flow cytometry (Fig. 1 D). In contrast to FcγR-mediated phagocytosis, internalization of complement C3b-bound zymosan particles was equivalent in wild-type and hck−/−fgr−/−lyn−/− bone marrow-derived macrophages (Fig. 1 C). Likewise, triple mutant macrophages had no impairment in cytokine or nitrite production after stimulation with LPS, bacterial DNA, or inflammatory cytokines (20; and data not shown).

The induction of respiratory burst after binding of insoluble immune complexes to FcγR was also severely impaired in the triple mutant cells (Fig. 2 A). Although resting or LPS/IFN-γ-primed wild-type cells displayed robust oxidative burst after ingestion of H2DCF-labeled immune complexes, resting hck−/−fgr−/−lyn−/− cells were completely inactive and LPS/IFN-γ-primed cells mounted a considerably blunted response. The FcγR-dependent oxidative burst in this assay was blocked by preincubation of cells with cytochalasin D (not shown), indicating that signal transduction leading to formation of F-actin is required for assembly of the NADPH oxidase within the phagocytic vesicle, as reported previously (22, 25, 26).

Delayed Actin Cup Formation in hck−/−fgr−/−lyn−/− Macrophages. An early event in the phagocytic process is the polymerization of actin underlying the membrane with engaged phagocytic receptors (2). Therefore, we examined phagocytic cup formation in wild-type and mutant macrophages by staining cells for polymerized actin with rhodamine-phalloidin. Fluorescein-conjugated anti–rabbit IgG was used to visualize the bound EA particles. In agreement with the delayed phagocytosis seen in hck−/−fgr−/−lyn−/− macrophages, there were very few actin cups formed by mutant cells at 5 min after binding of EA particles, whereas a high fraction of EA particles bound by wild-type macrophages at 5 min had already elicited actin cup formation (Fig. 3). By 15 min, some actin cup formation was clearly evident in the mutant macrophages. Thus, in the absence of Hck, Fgr, and Lyn, actin polymerization was slowed but not blocked, correlating closely with what was seen by examining internalization of labeled EA particles.

Impaired FcγR Signal Initiation in hck−/−fgr−/−lyn−/− Macrophages. As protein phosphorylation on tyrosine residues is essential for FcγR-mediated phagocytosis to occur (28) and Src family members are believed to be crucial elements in propagation of the tyrosine kinase signal, we examined several important signaling reactions downstream of FcγR activation in wild-type and hck−/−fgr−/−lyn−/− macrophages. After antibody-mediated FcγR cross-linking, hck−/−fgr−/−lyn−/− macrophages showed dramatically reduced levels of overall tyrosine phosphorylation (Fig. 4). Even at later time points (>20 min), when phagocytosis was clearly proceeding in hck−/−fgr−/−lyn−/− macrophages.
Src Family Kinases and Fcγ Receptor Phagocytosis

(Fig. 1, A and B), very few changes in tyrosine phosphorylation were seen. Since initiation of FcγR I and FcγR III signaling and phagocytosis is dependent on tyrosine phosphorylation of the accessory γ chain, we examined this event in 32PO₄-labeled macrophages stimulated with FcγR cross-linking antibodies. As the γ chain is phosphorylated on threonine and serine residues under resting conditions (29, 30), immunoprecipitation of the γ chain from 32P-labeled wild-type or hck⁻/⁻, fgr⁻/⁻, lyn⁻/⁻ macrophage lysates revealed the presence of a faster migrating phosphoband present at all time points examined (Fig. 5 A). This band was not detected in anti-IgG immunoprecipitates from the same lysates (data not shown), suggesting that this protein is indeed the constitutively phosphorylated γ chain. However, after FcγR cross-linking for 15 min (Fig. 5 A; and as early as 2 min, data not shown), a slower migrating protein appeared in the γ chain immunoprecipitates from wild-type cells only. Since the induced phosphorylation of the γ chain after FceR I or FcγR cross-linking is predominantly on tyrosine residues (29, 30), this species most likely represents newly tyrosine-phosphorylated γ chain. In contrast to wild-type cells, no detectable

Figure 3. Reduced actin cup formation in hck⁻/⁻, fgr⁻/⁻, lyn⁻/⁻ macrophages. Phagocytosis of EAs in wild-type (top panels, Wt) and triple mutant (middle and bottom panels) macrophages was initiated as described in the legend to Fig. 1. After 5 min (top and middle panels) or 15 min (bottom panels), unbound EAs were removed by washing and macrophages were fixed in 3% paraformaldehyde. Cells were stained with rhodamine-conjugated phalloidin to detect F-actin at sites of phagocytic cup formation (left), and with FITC-conjugated anti-rabbit IgG to detect the bound SRBCs (middle). An overlay of the F-actin and anti-rabbit IgG staining demonstrates colocalization of bound EAs with phagocytic cups (right). Cells were examined in all focal planes for the presence of actin cups. The middle panels show a focal plane that includes the nucleus of the triple mutant cells to localize cellular structures, since these cells had very low F-actin formation at all focal planes. Arrows indicate representative phagocytic cups colocalized with SRBCs. Bar, 10 μm.
induction of γ chain phosphorylation was observed in stimulated hck−/−fgr−/−lyn−/− macrophages in four independent experiments.

In the widely held model of immune receptor activation, it is proposed that the initial phosphorylation of ITAMs by Src family kinases allows for the binding and subsequent activation by phosphorylation of Syk. Therefore, we assessed the phosphotyrosine content of Syk in cells stimulated through the FcγR (Fig. 5 B). In wild-type macrophages, there was a rapid and sustained induction of Syk phosphorylation after FcγR cross-linking, which began to decline only after 30 min of stimulation. The picture was quite different in hck−/−fgr−/−lyn−/− macrophages, as Syk phosphorylation was not detectable until cells had been stimulated for at least 20 min and the level of Syk phosphorylation achieved was much lower. The enzymatic activity of Syk was similarly compromised in FcγR-stimulated hck−/−fgr−/−lyn−/− macrophages (13).

A divagation of the PI 3-kinase pathway is seen impaired in hck−/−fgr−/−lyn−/− Macrophages. To correlate downstream signaling events with the delayed phagocytosis seen in mutant macrophages, we next assayed components of the PI 3-kinase pathway, known to have a role not only in phagocytosis (15) but also in membrane trafficking (31) and other cytoskeletal-induced events (32). Although it is not known precisely how FcγRs activate PI 3-kinase, this may involve Cbl, a multidomain adaptor protein that is one of the major tyrosine kinase substrates in signaling complexes formed after engagement of FcγRs on macrophages (33).

Cbl binds to and recruits the p85 regulatory subunit of PI 3-kinase via a phosphotyrosine–SH2 domain interaction (34). In bone marrow–derived macrophages from wild-type mice, the phosphorylation of Cbl was detected very early after receptor cross-linking and the peak in this reaction strikingly paralleled that of Syk phosphorylation (Fig. 5 C). In hck−/−fgr−/−lyn−/− macrophages, we observed both a delayed onset and diminished peak in Cbl phosphorylation, again parallel to that seen for Syk.

We next examined the formation of Cbl–PI 3-kinase complexes in FcγR-stimulated macrophages by assessing the amount of lipid kinase activity found in Cbl immunoprecipitates. In wild-type macrophages stimulated through the FcγR, we observed an eightfold increase in Cbl-associated PI 3-kinase activity, which peaked 5 min after receptor cross-linking (Fig. 6 A). The activity detected in response to FcγR cross-linking of hck−/−fgr−/−lyn−/− cells was greatly reduced, reaching only a twofold induction, and did not occur until 20 min after receptor cross-linking. In contrast, the amount of Cbl-associated PI 3-kinase activity in wild-type and hck−/−fgr−/−lyn−/− macrophages stimulated through the CSF-1 receptor was comparable (Fig. 6 B), indicating that the mutant cells did not harbor any intrinsic defects in PI 3-kinase but rather that the reduced activation observed after FcγR stimulation was due to the paucity of tyrosine-phosphorylated Cbl with which p85 could associate (see Fig. 5 C).

In addition to Cbl, other tyrosine-phosphorylated proteins may also interact with the p85 regulatory subunit of PI 3-kinase, affecting its subcellular location and activation (35, 36). Therefore, we also assayed PI 3-kinase activity in...
total antiphosphotyrosine immunoprecipitates from stimulated macrophages. The induction of phosphotyrosine-associated PI 3-kinase activity in FcγR-stimulated cells was less than that seen associated with Cbl, but the PI 3-kinase activity in antiphosphotyrosine immunoprecipitates was similarly delayed and reduced in hck−/−fgr−/−lyn−/− macrophages relative to wild-type cells (Fig. 6 C). However, in cells stimulated with CSF-1 via its receptor tyrosine kinase, the fold induction of phosphotyrosine-associated PI 3-kinase activity was high in both mutant and wild-type cells (Fig. 6 D).

Activation of the Erk but Not JNK MAP Kinase Pathway Is Impaired in hck−/−fgr−/−lyn−/− Macrophages. Activation of the MAP kinases Erk1 (p44) and Erk2 (p42) also occurs after FcγR cross-linking in murine macrophages or human monocytes, although a specific role for this family of serine/threonine kinases in phagocytosis has not been determined (37, 38). We used a gel mobility shift assay to assess levels of phosphorylated, activated Erk1/2 in FcγR-stimulated macrophages. After FcγR cross-linking, Erk1/2 activation was observed within 5–10 min in wild-type cells, but only weakly after 20 min in hck−/−fgr−/−lyn−/− macrophages (Fig. 7 A). These findings were also confirmed by in vitro kinase assays of Erk1/2 activity using the fusion protein GST-Elk1 as substrate (data not shown). In contrast, Erk1/2 activation occurred similarly in both wild-type and mutant cells after LPS stimulation (20; Fig. 7 B), indicating that there was not a general block in activation of this pathway in hck−/−fgr−/−lyn−/− cells.

Recent reports have demonstrated a requirement for Rac1 and Cdc42 in FcγR-mediated phagocytosis (39) and have implicated these GTPases in activation of the JNK pathway (40). Using a solid-phase in vitro kinase assay, we assayed for JNK activity in wild-type and hck−/−fgr−/−lyn−/− macrophages and observed activation of JNK beginning 20 min after FcγR cross-linking (Fig. 7 C). In sharp contrast to all other signaling reactions we studied, this response was similar in both wild-type and triple mutant macrophages. Probing of stimulated cell lysates with an antibody that specifically recognizes the phosphorylated, active form of JNK1 revealed a single band of the appropriate molecular weight in both wild-type and mutant cells (Fig. 7 C).
allowed for earlier detection of activated JNK (2–5 min), yet again no difference in the response of the two cell types to FcγR cross-linking was noted (data not shown).

The Kinases Hck, Fgr, and Lyn Are Redundant for Phagocytosis but Not for Specific Signaling Events. Although Hck, Fgr, and Lyn have all been shown to be receptor associated and activated upon FcγR stimulation, the roles of these kinases may not be redundant (19, 41). To address this issue, we examined the rate of phagocytosis in bone marrow–derived macrophages from hck<sup>−/−</sup> fgr<sup>−/−</sup> and lyn<sup>−/−</sup> single mutant mice. All mutant macrophages displayed similar cell surface expression of FcγRII and III as determined by flow cytometry (not shown). Likewise, cells from all three mutant mouse strains phagocytosed <sup>51</sup>Cr-EAs with approximately the same kinetics as wild-type macrophages (Fig. 8, A and B; and data not shown).

Although clearly not a dramatic defect, it should be noted that in some experiments we did observe a slight delay in phagocytosis in lyn<sup>−/−</sup> macrophages (Fig. 8 B).

Interestingly, when we assayed biochemical signaling reactions in single and double mutant macrophages, diverse roles for Hck, Fgr, and Lyn became apparent. In antiphosphotyrosine immunoblots of total cell lysates (Fig. 9 A) or Syk immunoprecipitates (Fig. 9 B), we detected a rapid and robust response in FcγR–cross-linked wild-type and hck<sup>−/−</sup> fgr<sup>−/−</sup> macrophages. Thus, neither Hck nor Fgr is crucial for the initial phosphotyrosine response after FcγR cross-linking. However, in macrophages lacking Lyn, there was a marked decline in the global phosphotyrosine response and a decrease in Syk phosphorylation, although not as complete as in cells lacking all three kinases. The reduced tyrosine phosphorylation observed in lyn<sup>−/−</sup> macrophages compared with hck<sup>−/−</sup> fgr<sup>−/−</sup> cells suggests that, of the three principal Src family kinases expressed in macrophages, Lyn plays the dominant role in FcγR signal transduction, including initiation of Syk phosphorylation. However, lack of this kinase alone was not sufficient to physiologically impair FcγR–mediated phagocytosis, at least under the conditions examined.

**Discussion**

To examine the role of Src family kinases in FcγR–mediated signaling and phagocytosis, we have analyzed these functions in macrophages derived from mice lacking the three predominant family members expressed in these cells, Hck, Fgr, and Lyn. Indicative of an important role for Src family kinases in FcγR signaling, macrophages deficient in these kinases exhibited poor proximal (γ chain and Syk tyrosine phosphorylation) and downstream (PI 3-kinase and MAP kinase activity) signaling reactions. FcγR–mediated phagocytosis was also defective in triple mutant macrophages at early times after binding of antibody-coated erythrocytes, but by 20–30 min, substantial phagocytosis did occur. Likewise, FcγR–mediated induction of respiratory burst was also dramatically reduced in triple mutant cells.

Src family kinases are thought to initiate signaling after
clustering of multisubunit immune recognition receptors by phosphorylating tyrosines in the ITAM sequence of receptor cytoplasmic domains, thereby allowing for recruitment of Syk (or ZAP-70 in T cells) to the phosphorylated ITAMs (5, 6, 42). Consistent with this view, we did not detect any inducible FcRγ chain phosphorylation in hck<sup>-/-</sup>frg<sup>-/-</sup>lyn<sup>-/-</sup> macrophages. Moreover, both the phosphorylation and enzymatic activity of Syk after FcγR engagement were markedly reduced in the triple mutant macrophages. Eventually a small degree of Syk tyrosine phosphorylation may be important for the observed phagocytosis in these cells. The low amount of induced phosphorylation correlated well with the initiation of significant phosphorylation did occur, and the onset of this phosphorylation and enzymatic activity of Syk after FcγR engagement were markedly reduced in the triple mutant macrophages. Eventually a small degree of Syk tyrosine phosphorylation did occur, and the onset of this phosphorylation correlated well with the initiation of significant phosphorylation following the receptor complex and activation by Src family kinases. As noted above, Src family kinases are thought to be responsible for phosphorylation of ITAM sequences in lymphocyte immune recognition receptors and can phosphorylate the ITAM in FcγRIIa in vitro (49). Although we failed to observed γ chain phosphorylation in hck<sup>-/-</sup>frg<sup>-/-</sup>lyn<sup>-/-</sup> cells, γ chain phosphorylation also does not occur normally in syk<sup>-/-</sup> macrophages after FcγR ligation (14). The lack of γ chain phosphorylation in syk<sup>-/-</sup> cells may be due to the fact that Syk is able to protect the phosphorylated ITAMs from phosphatases by binding to them. Alternatively, Syk may directly amplify ITAM phosphorylation after recruitment to the receptor complex and activation by Src family kinases.

Figure 10. Model for the role of Src family kinases in FcγR-mediated phagocytosis. Src family kinases are responsible for phosphorylation of the FcγRγ chain, recruitment of Syk to the receptor, and activation of Syk, which then mediates signaling leading to internalization of the bound particle. The results presented here, however, demonstrate an additional function of Src family kinases to promote actin polymerization and phagocytic cup formation, as evidenced by the significant delay in these events in hck<sup>-/-</sup>frg<sup>-/-</sup>lyn<sup>-/-</sup> macrophages and their normal kinetics in Syk-deficient macrophages. Other signaling events possibly involving Syk (indicated by the broken arrow) include ITAM phosphorylation, stimulation of slow actin cup formation, and recruitment and/or autoactivation. These additional signaling reactions may account for residual phagocytosis in the absence of the Src family kinases.
kinases. Thus, comparison of these two knockout cell types does not definitively determine which kinases are responsible for ITAM phosphorylation.

We cannot rule out the possibility that the slow activation of signaling and phagocytosis in hck-/-fgr-/-lyn-/- macrophages is accomplished by another Src-related kinase. However, current evidence does not favor this possibility. No compensatory increase in expression of other known Src family kinases has been detected in these cells (20), although very low levels of Src appear to be expressed in the cultured macrophages used in these experiments. By breeding the triple mutant mice to Src mutant mice, we have recently generated quadruple mutant src-/-hck-/-fgr-/-lyn-/- mice; macrophages from these mice exhibit phagocytosis at rates and levels similar to those seen in the triple mutant macrophages (data not shown). Moreover, treatment of cells with the tyrosine kinase inhibitor PP1, which inhibits Src family but not Lyn kinases (50), also did not completely block EA phagocytosis (data not shown). Together, these observations suggest that FcγR phagocytosis is not completely dependent on Src family kinases.

The possibility of functional redundancy between the Src family kinases in FcγR signaling and phagocytosis was examined using macrophages isolated from single or double mutant mice. Interestingly, macrophages lacking Hck and Fgr exhibited normal FcγR signaling and phagocytosis, suggesting that neither of these two kinases is crucial for the initial response to FcγR cross-linking. In contrast, macrophages lacking Lyn showed a significant defect in signaling, while phagocytosis in these mutant macrophages occurred at a normal, or near normal rate. Apparently phagocytosis requires only a low level of FcγR signaling. Thus, FcγR signaling reactions were largely dependent on Lyn, whereas some residual function could be supplied by Hck and/or Fgr. For FcγR-mediated phagocytosis, however, there was functional redundancy between the different Src family members.

The signaling events downstream of Syk activation that are important for FcγR-mediated phagocytosis are still not well understood, but a leading candidate is PI 3-kinase activation. Recruitment of PI 3-kinase to the membrane may be mediated by association with Cbl, which becomes tyrosine phosphorylated after FcγR cross-linking and is translocated to the membrane where the phosphoinositide substrates of PI 3-kinase are located (33). Since FcγR-induced Cbl phosphorylation and phosphotyrosine- and Cbl-associated PI 3-kinase activities were diminished in hck-/-fgr-/-lyn-/- macrophages, it is likely that decreased amounts of D3 phosphoinositides were formed in the membrane, which may contribute to the reduced phagocytosis in these cells. In contrast, it appears that activation of Erk1 and Erk2 is not required for FcγR phagocytosis, since we were unable to detect significant activation of these kinases in hck-/-fgr-/-lyn-/- macrophages even at time points when these cells showed some phagocytosis. Similarly, Karimi and Lennartz (37) concluded that although MAP kinase activation occurs during IgG-mediated phagocytosis in human monocytes, it is not required, since treatment of cells with a selective inhibitor of this pathway blocked Erk2 activation without affecting phagocytosis. Taken together, these results suggest that MAP kinase activity does not play a central role in signaling to cytoskeletal rearrangements necessary for IgG-mediated phagocytosis, but rather may be involved in signaling to other FcγR-mediated events in leukocytes, such as regulation of transcription.

In summary, this report describes a genetic approach to dissect the signaling pathways emanating from the FcγR. Our findings indicate that Src family tyrosine kinases play an important role in directing polymerization of actin adjacent to signaling receptors. Src family tyrosine kinases also greatly promote the activation of the Syk tyrosine kinase, which is essential for completion of phagocytosis. In macrophages lacking detectable Src family kinases, phagocytosis, respiratory burst, actin cup formation, Syk activation, and activation of downstream signaling events were all delayed and decreased to some extent, but not abolished entirely, demonstrating an important although nonessential role of these kinases in FcγR-mediated functional responses.

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