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The Src Family Kinases Hck and Fgr Regulate Neutrophil Responses to *N*-Formyl-Methionyl-Leucyl-Phenylalanine[1]

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Abstract

The chemotactic peptide formyl-methionyl-leucyl-phenilalanine (fMLP) triggers intracellular protein tyrosine phosphorylation leading to neutrophil activation. Deficiency of the Src family kinases Hck and Fgr have previously been found to regulate fMLP-induced degranulation. In this study, we further investigate fMLP signaling in $hck^{-/-}fgr^{-/-}$ neutrophils and find that they fail to activate a respiratory burst and display reduced F-actin polymerization in response to fMLP. Additionally, albeit migration of both $hck^{-/-}fgr^{-/-}$ mouse neutrophils and human neutrophils incubated with the Src family kinase inhibitor 4-amino-5-(4-chlorophenyl)-7-(*t*-butyl)pyrazolo[3,4-d]pyrimidine (PP2) through 3-μm pore size Transwells was normal, deficiency, or inhibition, of Src kinases resulted in a failure of neutrophils to migrate through 1-μm pore size Transwells. Among MAPKs, phosphorylation of ERK1/2 was not different, phosphorylation of p38 was only partially affected, and phosphorylation of JNK was markedly decreased in fMLP-stimulated $hck^{-/-}fgr^{-/-}$ neutrophils and in human neutrophils incubated with PP2. An increase in intracellular Ca^{2+} concentration and phosphorylation of Akt/PKB occurred normally in fMLP-stimulated $hck^{-/-}fgr^{-/-}$ neutrophils, indicating that activation of both phosphoinositide-specific phospholipase C and PI3K is independent of Hck and Fgr. In contrast, phosphorylation of the Rho/Rac guanine nucleotide exchange factor Vav1 and the Rac target p21-activated kinases were markedly reduced in both $hck^{-/-}fgr^{-/-}$ neutrophils and human neutrophils incubated with a PP2. Consistent with these findings, PP2 inhibited Rac2 activation in human neutrophils. We suggest that Hck and Fgr act within a signaling pathway triggered by fMLP receptors that involves Vav1 and p21-activated kinases, leading to respiratory burst and F-actin polymerization.

N-formylated peptides derived from bacterial or mitochondrial proteins are potent agonists of neutrophil (polymorphonuclear leukocyte; PMN[3]) responses implicated in host defense and inflammation. Mammalian granulocytes and macrophages express a whole spectrum of receptors that recognize these peptides, including those specific for the prototype ligand formyl-methionyl-leucyl-phenilalanine (fMLP) (reviewed in Ref. 1). Importantly, genetic

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deficiency of one of the mouse receptors for fMLP (mFPR), whose sequence is highly similar (76% identity) to the human receptor (huFPR), results in enhanced susceptibility to *Listeria monocytogenes* infection (2), highlighting the importance of cell stimulation with *N*-formylated peptides in innate immunity.

As classical Gαi-linked receptors, receptors for fMLP trigger a variety of intracellular signals deriving from activation of different phospholipases acting on membrane phospholipids (reviewed in Ref. 3). Activation of the phosphoinositide-specific phospholipase C (PLC) results in generation of inositol trisphosphate (IP_3), with the consequent mobilization of Ca^{2+} from intracellular stores, and diacylglycerol, with the consequent activation of various protein kinase C (PKC) isoforms. Ca^{2+}, PKC, as well as alternative mechanisms stimulate phospholipase D and phospholipase A_2, which can further feedback on activation of PKC (4–6). More recently, activation of the γ isoform of PI3K (PI3KIB) by the $\beta\gamma$ subunits of Gαi, with the consequent increase in phosphorylation of the 3 position in the inositol ring of phosphatidylinositol, has been demonstrated to play an essential role in stimulation of some PMNs responses by fMLP and other chemoattractants (7–9).

Besides activation of phospholipases and lipid kinases, fMLP receptors trigger a rapid tyrosine phosphorylation of several PMNs proteins (see Refs. 10–12 and references quoted therein). Among cytoplasmic tyrosine kinases, members of the Src (13–18) and the Tec families (19) in PMNs, and Pyk2 in a granulocytic cell line (20) have been implicated in signal transduction by fMLP. Additionally, signaling by receptors for either CXC and CC chemokines were reported to implicate Src family tyrosine kinases in granulocytes (21–23). Support for a role of tyrosine kinases in signal transduction by fMLP receptors derives from the evidence that inhibitory drugs, including some displaying significant specificity for Src or Tec family kinases, inhibit PMN responses to fMLP (16–18, 24, 25).

To date, there have been relatively few studies of fMLP receptor signaling using PMNs isolated from mice with the genetic deficiency of Src family kinases (17, 18) or Syk (26). Importantly, whereas Src family kinase-deficient PMNs display a marked reduction in the degranulation response to fMLP (17, 18) or MIP-2 (23), $syk^{-/-}$ cells respond as wild-type (WT) cells to stimulation with fMLP and other chemoattractants (26). The defective degranulation response to fMLP and chemokines (17, 18, 21, 23) by Src family kinase-deficient PMNs might not necessarily reflect alterations in upstream Gαi-signaling, but simply the role played by these kinases in more distal signaling events regulating exocytosis, a possibility consistent with the physical association of Hck and Fgr with primary and secondary granule (13, 27). Indeed, very recent results showed that $hck^{-/-}fgr^{-/-}$ neutrophils and $fgr^{-/-}$ dendritic cells manifest a more robust signaling and functional responses to several murine-derived chemokines (28). To elucidate whether Src family kinases are implicated in signal transduction by fMLP receptors in PMNs, we investigated respiratory

[3]Abbreviations used in this paper: PMN, polymorphonuclear leukocyte; fMLP, formyl-methionyl-leucyl-phenilalanine; PLC, phospholipase C; IP_3, inositol trisphosphate; PKC, protein kinase C; WT, wild type; PP2, 4-amino-5-(4-chlorophenyl)-7-(*t*-butyl)pyrazolo[3,4-d]pyrimidine; PAK, p21-activated protein kinase; GEF, guanine nucleotide exchange factor; KO, knockout; T, temperature; CB, cytochalasin B; PAO, phenylarsine oxide; SB, sample buffer; SAPK, stress-activated protein kinase; Csk, C-terminal Src kinase.

burst activation and stimulation of a variety of signal transduction pathways in murine *hck*$^{-/-}$*fgr*$^{-/-}$ PMNs or human PMNs treated with the selective Src kinase inhibitor 4-amino-5-(4-chlorophenyl)-7-(*t*-bu-tyl)pyrazolo[3,4-d]pyrimidine (PP2) (29). In this study, we show that Fgr and Hck are essential for the fMLP-induced activation of a respiratory burst, as well as basal or fMLP-stimulated tyrosine phosphorylation of several Src family kinase substrates. Additionally, we demonstrate that these kinases act within a signaling pathway distinct from that leading to PLC and PI3K activation and regulate tyrosine phosphorylation of the guanine nucleotide exchange factor (GEF) Vav1 and activation of the Rac GTPase downstream targets p21-activated protein kinase (PAK) and JNK.

Materials and Methods

Cell preparation

Male and female C57BL/6J, 6- to 8-wk-old mice were used as WT controls. For generation of *hck*$^{-/-}$*fgr*$^{-/-}$ double knockout (KO) mice in this same background, see Ref. 30. Mouse bone marrow PMNs were isolated from femurs and tibias as described previously (30). Briefly, marrow cells were flushed from the bones using HBSS (137 mM NaCl, 0.53 mM KCl, 0.033 mM Na_2HPO_4, 0.4 mM $NaHCO_3$, 0.044 mM KH_2PO_4, and 2 mM HEPES (pH 7.4)) without Ca^{2+} and Mg^{2+}, and containing 0.1% BSA. Cells were centrifuged and, after hypotonic lysis of erythrocytes, resuspended in 3 ml of 45% Percoll (Amersham Biosciences) in Ca^{2+}/Mg^{2+}-free HBSS supplemented with 0.1% BSA. Bone marrow cells were then loaded on top of a Percoll discontinuous density gradient (31) and, after centrifugation at 1,600 × *g* for 30 min at room temperature (T), cells at the interface between 81% and 62% and 62% and 55% Percoll layer were harvested and diluted in Ca^{2+}/Mg^{2+}-free HBSS supplemented with 0.1%BSA. After an additional wash, PMNs were resuspended at 10 × 10^6/ml in modified HBSS with a total osmolarity of 308 mosmole/l (32) and HBSS supplemented with 0.5 mM $CaCl_2$ and 5 mM D-glucose (HGCa). Routinely, cell suspensions were left at room T for 1 h before assay. Human PMNs were prepared from buffy coats of healthy volunteers by centrifugation through Ficoll Paque Plus (Amersham Biosciences). Contaminating erythrocytes were removed by dextran-500 (Amersham Biosciences) sedimentation followed by hypotonic lysis. After isolation, cells were suspended in HGCa at 10 × 10^6/ml and left at room T for 1 h before assay. In some experiments, mouse or human cells were pretreated with 10 μM PP2 (Calbiochem) for 10 min at 37°C or 100 nM Wortmannin (Calbiochem) for 30 min at room T before assay.

Measurement of superoxide anion release

Superoxide generation was determined by reduction of ferricytochrome C (Sigma-Aldrich) (33). Cell suspensions were diluted to 2 × 10^6/ml for human and 5 × 10^6/ml for mouse PMNs with HGCa and added with 2 μg/ml cytochalasin B (CB) (Sigma-Aldrich). One hundred microliters of the cell suspension were then dispensed in 96-well microtiter plates, which had been previously precoated with 250 μg/ml human fibrinogen (Sigma-Aldrich). After 10 min of incubation at 37°C, 100 μl of HBSS supplemented with 0.5 mM $CaCl_2$ and 5 mM D-glucose (HGCa) containing 4 mM NaN_3 and 160 μM ferricytocrome C with or without the stimulus was added to each well. As stimuli, 10^{-6} M fMLP (Sigma-Aldrich) or 50 ng/ml PMA (Sigma-Aldrich) were used. Plates were incubated in an ELX 808 ultra

microplate reader (Bio-Tek Instruments), and the absorbance at 550 and 460 nm was recorded every minute. Differences in the absorbance at the two wavelengths were used to calculate nanomoles of O_2^- produced (33).

Measurement of actin polymerization

Polymerization of actin was measured using a phalloidin binding assay. Purified PMNs (2×10^6) in 200 μl of RPMI 1640 were stimulated with fMLP at room T. Cells were fixed by addition of 200 μl of 2% paraformaldehyde, then incubated for 20 min at 4°C. Cells were then washed and resuspended in 200 μl of 0.2% Triton X-100 in PBS, and stained with 0.2 μM rhodamine-phalloidin (Molecular Probes) for 30 min on ice. Following staining, cells were washed twice with 4°C PBS and resuspended in 10% buffered formalin (Sigma-Aldrich), and the fluorescence bound to cells was determined by flow cytometry using a BD Biosciences FACScan. The mean fluorescence intensity of the cell population was determined.

Transwell migration asssays

PMN migration was assessed using Transwell filters of 3- or 1-μm pores (BD Biosciences), precoated with 20% FCS in PBS and inserted in 24-well plates. The bottom chamber was filled with 0.7 ml of RPMI 1640 containing different concentrations of fMLP (see *Results*), and the top chamber was filled with 0.1×10^6 cells in 0.2 ml of RPMI 1640. Plates were incubated at 37°C/5% CO_2 for 60 or 90 min in assays performed with 3-or 1-μm pores, respectively. At the end of the incubation, the inserts were removed, plates were centrifuged at 1,000 rpm for 10 min, and the supernatants were aspirated. Cells were kept at −20°C overnight and then lyzed with 200 μl of hexadecyltrimethylammonium bromide diluted in 50 mM potassium phosphate buffer (pH 6.5) to achieve a final concentration of 0.5%. Myeloperoxidase activity was assayed by adding 15 μl of a 1.25 mg/ml *o*-dianisidine solution and 15 μl of 0.05% hydrogen peroxide. The enzymatic reaction was stopped after 15 min by addition of 20 μl of 1% sodium azide, and adsorbance was read at 450 nm. To calculate the number of migrated cells, different numbers of PMNs were plated in 24-well plates and, after centrifugation, lyzed as described above. Myeloperoxidase activity of different numbers of PMN was used to obtain a reference standard curve for each set of assayed cells, i.e., WT vs $hck^{-/-}fgr^{-/-}$ mouse PMNs, and control vs PP2-treated human PMNs.

Measurement of cytosolic-free Ca^{2+}

PMNs, suspended in HGCa, were loaded with 2 μM fura 2-AM (Calbiochem) for 20 min at 37°C and, after a 5-fold dilution with HGCa, incubated for an additional 30 min. The cells were then washed twice and resuspended in HGCa at 20×10^6/ml. Fluorescence changes of 5×10^6 PMNs kept at 37°C under stirring, were monitored with a PerkinElmer LS-5B luminescence spectrometer using 335 and 380 nm excitation and 505 nm emission wavelengths. Calcium concentrations were calculated as described in Ref. 34.

Immunoprecipitation and immunoblotting

Mouse or human PMNs (10×10^6/ml) in HGCa were pretreated with 2 μg/ml CB (Sigma-Aldrich) for 10 min at 37°C and then stimulated with 10^{-6} M FMLP or 50 ng/ml PMA for the time indicated in the results. At the appropriate time, cell activation was stopped by addition of one-half volume of ice-cold HBSS (without Ca^{2+} and glucose) containing a 3-fold concentration of protease inhibitors mixture tablet (Roche Molecular Biochemicals) supplemented with 3 mM Na_3VO_4, 30 μM phenylarsine oxide (PAO) (Sigma-Aldrich), 75 μg/ml pepstatin (Sigma-Aldrich), and 3 mM diisopropyl-fluorophosphate (Sigma-Aldrich). Samples were kept in ice for 10 min before lysis with 4× sample buffer (SB; 100 mM Tris (pH 6.8), 200 mM 2-ME, 4% SDS, 20% glycerol, and 0.4% bromophenol blue). For Vav1 immunoprecipitation, cells were lyzed in SB without 2-ME, and bromophenol blue and lysates (200 μg of total proteins) were diluted 10 times with 1% Triton buffer (1% Triton, 100 mM NaCl, and 50 mM HEPES (pH 7.4)) and then incubated for 2 h with anti-Vav1 Ab (Santa Cruz Biotechnology) preadsorbed with protein A immobilized to Trysacril (Pierce). Immunocomplexes were collected by centrifugation, washed three times with TBS supplemented with 0.2% Triton, 1 mM Na_3VO_4, and 10 μM PAO, and then boiled in SB. Samples were separated on SDS-PAGE gels and transferred to nitrocellulose Hybond C (Amersham Biosciences). After quenching with 3% BSA in TBS for 1 h, blots were incubated overnight at 4°C with primary Abs, followed by HRP-conjugated donkey anti-rabbit or goat anti-mouse Abs (Amersham Biosciences). Immunoreactivity was detected using the ECL Western blotting detection reagent (ECL; Amersham Biosciences). In experiments in which phosphorylation of specific proteins was detected with anti-phospho specific Abs, membranes were stripped for 30 min at 50°C in 62.5 mM Tris (pH 6.8), 2% SDS, and 100 mM 2-ME before incubation with anti-protein Abs to detect total protein loading. Abs used in this study were as follows. Total tyrosine phosphorylated proteins were detected with a mixture of the anti-phosphotyrosine clone 4G10 (Upstate Biotechnology) and clone PY99 (Santa Cruz Biotechnology). Anti-phosphospecific Ab directed against Akt (Ser^{473}), p38 MAPK (Thr^{180}/Tyr^{182}), ERK1/2 MAPK (Thr^{202}/Tyr^{204}), PAK1(Thr^{423})/PAK2(Thr^{402}), and JNK (JNK/stress-activated protein kinase (SAPK); Thr^{183}/Tyr^{185}) were obatined from Cell Signaling Technology. Anti-protein Abs directed against Akt, p38, PAK1/2, and JNK/SAP were obtained from Cell Signaling Technology. Anti-protein Abs directed against ERK1/2 were obtained from Santa Cruz Biotechnology. Anti-Vav Abs from Santa Cruz Biotechnology or Upstate Biotechnology were used for immunoprecipitations and immunoblotting, respectively. Anti-phosphospecific Ab directed against Vav (Y160) was obtained from BioSource International.

Rac activation assay

Human PMNs were stimulated, and the stimulation was stopped, as described above (see *Immunoprecipitation and immunoblotting*). After 10 min on ice, cells were lyzed in 2× NP-40 buffer (100 mM Tris-HCl (pH 7.4), 20 mM $MgCl_2$, 200 mM NaCl, 20%glycerol, 2% NP-40, 20 μg/ml PAO, and 2 mM Na_3VO_4, and 2 mM benzamidine (Sigma-Aldrich)). After rotation for 5 min at 4°C, lysates were cleared by centrifugation at 13,000 rpm for 10 min. Supernatant aliquots were saved for examining total Rac and protein content. A total of 7.5×10^6 cell equivalents was incubated for 30 min at 4°C under rotation with GST-PBD

prebound to glutathione-Sepharose 4B (Amersham Biosciences). After three washes with 1× NP-40 buffer, bound proteins were eluted with 4× SB and boiled for 5 min. Samples were subjected to electrophoresis in 12% SDS-PAGE gels, blotted on nitrocellulose, and probed with an anti-Rac2-specific Ab (Upstate Biotechnology).

Results

Superoxide anion generation and actin polymerization in response to fMLP is markedly reduced in double KO hck$^{-/-}$fgr$^{-/-}$ PMN

Previous studies demonstrated that fMLP-induced degranulation requires Src family kinases (17, 18). Because fMLP triggers a wide array of responses in PMN, we examined whether superoxide anion generation is affected by deficiency of Fgr or Hck. As shown in Fig. 1*A*, *hck*$^{-/-}$*fgr*$^{-/-}$ PMNs generated markedly lower amounts of superoxide anion in response to 1 μM fMLP compared with WT cells, and this marked difference was detected in conditions in which the response to fMLP was maximized by the priming agent CB. In the assay conditions reported in Fig. 1*A*, super-oxide anion generation by both WT and mutant PMNs was undetectable in the absence of CB. Even increasing the fMLP concentration up to 10 μM, mutant PMNs were still much less responsive (Fig. 1*B*). As seen with other PMNs responses (18, 30), *hck*$^{-/-}$ or *fgr*$^{-/-}$ single KO PMNs demonstrated normal super-oxide generation in response to fMLP (data not shown). The reduced response to fMLP of double mutant PMNs did not reflect alterations in the NADPH oxidase activity. In fact, as previously reported in adhesion assays (30), *hck*$^{-/-}$*fgr*$^{-/-}$ and WT PMNs responded equivalently to PMA (Fig. 1*C*). PP2, a Src family kinase inhibitor (29), effectively blocked the response to fMLP in both mouse (Fig. 1*D*) and human (data not shown) PMNs.

In previous studies, we demonstrated that activation of PMN respiratory burst in adherent conditions, exploiting assays originally described by Nathan (35), and in response to TNF-α and fMLP, depends on expression and activity of Fgr and Hck (30). However, we can exclude that signals generated by adhesion contributed to the response to fMLP. First, fMLP-induced superoxide generation was rapid and reached a plateau within 2 min (Fig. 1*A*), whereas adhesion-dependent responses require a long lag time before ensuing (35). Secondly, CB totally suppresses adhesion-dependent responses (33, 35), whereas it was required to detect a rapid response to fMLP (Fig. 1*A*). We conclude that superoxide anion generation induced by fMLP requires Fgr and Hck, independently of their established role in integrin signaling (36, 37).

To strengthen the finding that Fgr and Hck are implicated in signal transduction by fMLP receptors, we examined F-actin formation in response to different doses of fMLP (Fig. 2*A*) and after different times from the challenge (Fig. 2*B*). We found that the response of mutant PMNs was much lower than that of WT cells, confirming the involvement of these kinases in early responses to fMLP. Notably, triple mutant (*hck*$^{-/-}$*fgr*$^{-/-}$*lyn*$^{-/-}$) PMNs do not exhibit alteration in their migratory ability both in Transwell assays in vitro, and in a chemical peritonitis model in vivo (38). Additionally, *hck*$^{-/-}$*fgr*$^{-/-}$ PMNs displayed the same migratory response to CXCL1/MIP-2 as WT cells both in vitro and in vivo (23). We therefore asked whether the decrease in actin polymer-ization we found in *hck*$^{-/-}$*fgr*$^{-/-}$ PMNs resulted in alteration in cell migration in response to fMLP (Fig. 3). Confirming

previous studies (38), we could not detect any defect in the chemotactic response to fMLP of both *hck*$^{-/-}$*fgr*$^{-/-}$ PMNs or human PMN treaded with PP2 when assays were performed with 3-μm pore size Transwells (Fig. 3, *A* and *B*). However, we found that Fgr and Hck deficiency in mouse PMNs and Src family kinase inhibition in human PMNs resulted in a marked inhibition in migration through pores of 1-μm diameter. These interesting findings suggest that Src family kinases unlikely regulate cell polarization or migration, but may be implicated in cytoskeleton dynamics regulating cell deformability and, possibly, transmigration through cell barriers.

Tyrosine phosphorylation of several proteins is defective in hck$^{-/-}$fgr$^{-/-}$ PMNs

Reduced superoxide anion generation and F-actin formation in *hck*$^{-/-}$*fgr*$^{-/-}$ PMNs prompted examination of tyrosine phosphorylation signals in mutant cells. As shown in Fig. 4*A*, both basal and fMLP-stimulated tyrosine phosphorylation of several proteins was markedly defective in *hck*$^{-/-}$*fgr*$^{-/-}$ PMNs. In particular, phosphorylation of some of the proteins indicated by arrows in Fig. 4*A*, i.e., proteins of ~145, 125, 95, 85, 72, and ~66 kDa and a broad band between 58 and 62 kDa were hardly detectable in both basal and stimulated conditions in mutant cells. In contrast, a doublet of ~42/44 kDa, whose phosphorylation increased in response to fMLP, was phosphorylated at comparable levels in WT and mutant PMNs. Reprobing anti-phosphotyrosine blots with Abs directed against specific proteins or using anti-phosphospecific Abs indicated that p125, p95, p85, p72, p66, and the p42/p44 doublet migrated at the same level of c-Cbl, Vav1, cortactin, Syk, paxillin, and ERK1/2, respectively, whereas anti-Hck and anti-Fgr Abs detected proteins migrating at the level of the broad band between 58 and 61 kDa. Assays with human PMNs (Fig. 4*B*) strengthened the conclusion that Src family kinases play a dominant role in tyrosine phosphorylation signals in these cells because PP2, a Src family-specific inhibitor (29), suppressed both the basal and fMLP-stimulated response. Detailed examination of Src family kinase substrates in PMNs was beyond the scope of this investigation and we did not address this issue further. It must be noted that tyrosine phosphorylations signals (Fig. 4 and see below), as well as other signaling pathways (Figs. 5 and 6 and see below), were examined in assay conditions (CB treatment) that were used to assay super-oxide production (see also figure legends). To exclude the fact that treatment with CB affected signal transduction, we compared phosphorylation of a few proteins, i.e., p38, Vav1, PAKs, and AKT, in PMNs stimulated in the absence or presence of CB, but did not find any substantial difference in fMLP-induced responses (data not shown).

Deficiency of Hck and Fgr does not affect generation of intracellular calcium transients or phosphorylation of Akt

Among chemoattractant-induced signals, activation of phosphoinositide-specific PLC and the γ isoform of PI3K (PI3KIB) have been characterized as key features of the PMNs response that depend on the G$\beta\gamma$ subunits of trimeric G proteins (3, 8). We therefore asked whether Fgr and Hck are implicated within PLC- and PI3KIB-dependent signal transduction pathways, examining events downstream of these two enzymes, i.e., generation of intracellular calcium transients and phosphorylation of the Ser/Thr kinase Akt/PKB.

As shown in Fig. 5, intracellular Ca^{2+} concentration increased at comparable levels in WT and mutant PMNs in response to fMLP, thus excluding the fact that Fgr and Hck regulate fMLP-induced variation of cytoplasmic calcium.

As shown in Fig. 6*A*, fMLP effectively increased Akt phosphorylation in both mouse and human PMNs as repeatedly reported in different studies (see, for example, Refs. 7, 24, 25). Importantly, whereas the PI3K inhibitor wortmannin totally suppressed Akt phosphorylation, the deficiency of Fgr and Hck did not decrease this response to fMLP. To strengthen the notion that fMLP-induced Akt phosphorylation is independent of Src family kinases, we also examined human PMNs treated with the Src family kinase inhibitor PP2 (Fig. 6*B*). Although treatment of human PMNs with 10 μM PP2 markedly inhibited superoxide generation (data not shown), it had a negligible effect on fMLP-induced Akt phosphorylation. These findings are in accord with the notion that chemoattractants regulate primarily the γ isoform of PI3K via the G$\beta\gamma$ subunits of trimeric G proteins and independently of tyrosine phosphorylation signals (7). Reduced superoxide anion generation (Fig. 1) in *hck*$^{-/-}$*fgr*$^{-/-}$ PMNs in the presence of a normal activation of the PI3K-dependent signaling pathway (Fig. 6) may seem to conflict with the evidence that inhibition of PI3K activity by wortmannin or deficiency of PI3KIB results in an impairment of chemoattractant-induced respiratory burst (7, 39, 40). Indeed, in both mouse and human PMN, wortmannin effectively inhibited fMLP-induced superoxide generation, and in *hck*$^{-/-}$*fgr*$^{-/-}$ PMNs the drug had a totally suppressive effect (Fig. 7). We conclude that, in respiratory burst activation by fMLP, PI3K and Src family kinases play independent albeit coordinated roles. Signaling along both the PI3K and Src-dependent pathways is required for full development of oxidative burst in response to fMLP (see *Discussion*).

fMLP-stimulated phosphorylation of MAPKs in hck$^{-/-}$fgr$^{-/-}$ PMNs

Chemoattractant stimulation of PMNs results in phosphorylation and activation of distinct MAPK families (see, for example, Refs. 3, 17, 41–43 and references contained therein). We therefore compared early phosphorylation of ERK1/2, p38, and JNK/SAPKs in response to fMLP in WT and mutant murine PMNs or in human PMNs treated with the Src family kinase inhibitor PP2 (Figs. 8 and 9). We did not detect any consistent variations of fMLP-induced ERK1/2 phosphorylation in both *hck*$^{-/-}$*fgr*$^{-/-}$ and PP2-treated human PMNs (Fig. 8, *A* and *B*). These findings are in accord with previous reports with mouse PMNs defective of Src family kinases and human PMNs treated with PP1, another Src family inhibitor (17, 24). Also, phosphorylation of p38 was not consistently different in *hck*$^{-/-}$*fgr*$^{-/-}$ PMNs (Fig. 8*A*). However, PP2 decreased p38 phosphorylation in human PMNs (Fig. 8*B*). Because p38 phosphorylation is markedly reduced in fMLP-stimulated *hck*$^{-/-}$ *fgr*$^{-/-}$*lyn*$^{-/-}$ PMNs (17), these findings suggest that Lyn, and possibly other kinases, but not Hck and Fgr, are implicated in p38 phosphorylation. In marked contrast with the data obtained with ERK1/2 and p38 MAPKs, phosphorylation of JNK/SAPK was markedly decreased in both basal and fMLP-stimulated conditions in *hck*$^{-/-}$*fgr*$^{-/-}$ PMN, and independently of the time of stimulation (Fig. 9*A*). Additionally, PP2 markedly reduced JNK/SAPK phosphorylation in human PMNs (Fig. 9*B*).

Hck and Fgr are required for tyrosine phosphorylation of Vav1 and threonine phosphorylation of PAK1 and PAK2

Because NADPH oxidase activation in both human and murine PMNs by fMLP requires the small GTPase Rac (see Refs. 44–50 and reference contained therein) and phosphorylation of JNK, which occurs downstream of Rac activation (51, 52), is defective in $hck^{-/-}fgr^{-/-}$ PMNs (Fig. 9*A*), we addressed whether the Rac pathway was affected in the $hck^{-/-}fgr^{-/-}$ PMNs. Pull-down assays with the Rac binding region of PAK fused with glutathione *S*-transferase (53) on lysates of murine PMN, did not give results consistent and reproducible enough to conclude that activation of Rac is impaired in $hck^{-/-}fgr^{-/-}$ PMNs. However, in human PMNs treated with PP2, which inhibits all Src family kinases, we found a decreased loading of GTP on Rac2 (Fig. 10). To understand whether a Rac-dependent pathway requires expression of Hck and Fgr, we therefore addressed whether deficiency of these kinases affects upstream Rac activator or downstream Rac targets (Figs. 11 and 12).

Vav1 belongs to a family of GEFs that catalize the exchange of GDP for GTP on Rho GTPases (54). Importantly, recent studies with $vav1^{-/-}$ PMNs established that Vav1 regulates fMLP-induced responses such as respiratory burst activation and F-actin formation (55). As shown in Fig. 11*A*, we detected a strong tyrosine phosphorylation signal in Vav1 immunoprecipitates from lysates of WT murine PMNs, and this signal slightly increased after fMLP stimulation. However, Vav1 tyrosine phosphorylation was very weak in both resting and stimulated conditions in $hck^{-/-}$ $fgr^{-/-}$ PMNs. In accord with these findings and in analogy with data obtained with PP1 (55), treatment with PP2 totally inhibited Vav1 tyrosine phosphorylation in control and fMLP-stimulated human PMNs (Fig. 11*B*). To strengthen these findings, we used a phosphospecific anti-Vav Ab recognizing a tyrosine residue located in the Vav acidic domain whose phosphorylation is implicated in Vav activation (56, 57) (Fig. 11*C*). Although phosphorylation of the Y160 residue did not increase consistently following fMLP stimulation, we confirmed that Hck and Fgr deficienty or treatment with PP2 resulted in a marked reduction of Y160 phosphorylation in mouse and human PMNs, respectively. As outlined above and illustrated in Fig. 11, *A*–*C*, we reproducibly detected a robust anti-phosphotyrosine signal in anti-Vav immunoprecipitates from lysates of both mouse and human PMNs even in the absence of fMLP stimulation. We cannot exclude that this may depend on partial activation of cells during the purification steps. However, this signal was clearly Src family kinase dependent. We conclude that Hck and Fgr are essential for Vav1 tyrosine phosphorylation.

The best established proximal targets of activated Rac proteins are the PAKs, and they have been implicated in activation of the NADPH oxidase (58). As shown in Fig. 12, fMLP increased phosphorylation of both PAK1 and PAK2 in murine and human PMNs. However, both the deficiency of Hck and Fgr in murine or the treatment of human PMNs with PP2 almost totally abrogated this response. Hence, both PAK (Fig. 12) and JNK phosphorylation (Fig. 9), which are placed downstream of Rac activation, are defective in $hck^{-/-}fgr^{-/-}$ PMNs.

Discussion

In the last 20 years, fMLP has perhaps been the most widely used agonist of cell responses in the field of phagocyte biology. Besides triggering cytoskeleton rearrangements, cell polarization, and cell migration, fMLP activates other neutrophil-specific responses including integrin-binding capacity, degranulation, and respiratory burst. These responses are directly or indirectly related to PMN recruitment in the inflammatory site because they mediate integrin-dependent leukocyte arrest to the inflamed endothelium and trans-endothelial migration, but also, via the release of granule constituents or reactive oxygen products, endothelial activation, and vascular permeability (59, 60).

Signal transduction by fMLP receptors, which belong to the family of heptahelical trimeric G protein-coupled receptors, has been investigated in great detail, and studies in the field established the paradigm that chemoattractant receptors activate phosphatidylinositol-specific PLC and calcium/diacylglycerol-dependent PKC inducing generation of calcium transients and phosphorylation of specific targets (3, 4). More recently, also activation of the γ isoform of PI3K (PI3KIB/PI3Kγ) has been established as a key feature of signal transduction by chemoattractant receptors that depends on the G$\beta\gamma$ subunits of trimeric G proteins (7, 8). Finally, accumulating evidence has implicated tyrosine phosphorylation signals in regulation of at least some neutrophil responses to chemoattractants.

Detailed investigations in both human and mouse neutrophils have established that signal transduction by fMLP receptors converges in activation of the Rho family GTPase Rac (49, 50). Importantly, whereas both Rac1 and Rac2 have been implicated in regulation of phagocytic cell migration, Rac2 also regulates activation of neutrophil respiratory burst (41, 44, 45, 61, 62), an action which likely results from its capability to coordinate the translocation of the $p67^{phox}$-$p47^{phox}$ complex to the flovo-cytochrome b558, as well as to regulate electron transfer to oxygen (46–48). The GTP-bound, active form of Rac is generated by GEFs that catalyze the release of GDP from inactive Rac. Vav1 and P-Rex1 are two well-established GEFs involved in Rac2 activation (55, 63, 64). Importantly, deficiency of Vav1 and P-Rex-1 results in a very similar neutrophil phenotype consisting in a selective defect in fMLP-induced respiratory burst, but in a minor impairment of fMLP-induced chemotaxis. Vav protein activity is regulated by tyrosine kinases including Src family kinases and Syk (38, 54), and Src family kinase inhibition results in a reduced Vav tyrosine phosphorylation in response to fMLP (55). In contrast, P-Rex1 is activated in a synergistic fashion by PtdIns(3,4,5)P3 and the $\beta\gamma$ subunits of trimeric G proteins (65). A comparison between the phenotype of neutrophils obtained from *rac2*$^{-/-}$ and *vav1*$^{-/-}$ or *P-rex1*$^{-/-}$ mice suggests that a partial reduction of Rac2 activation due to deficiency of either Vav1 or P-Rex1 is not sufficient to cause an inhibition of F-actin formation robust enough to reduce neutrophil chemotaxis toward fMLP, resulting only in a mild reduction of cell speed. In contrast, activation of NADPH oxidase seems to require full activation of Rac because both Vav1- and P-Rex1-deficient neutrophils display a marked defect in the generation of reactive oxygen intermediates in response to fMLP (Fig. 13). Interestingly, a very recent study showed that fMLP-induced activation of both Rac1 and Rac2 is reduced by 70% in PMNs deficient of DOCK2, a CDM family member Rac GEF, and DOCK2 deficiency results in a marked inhibition of cell polarity and translocation speed (66). Additionally,

similarly to *vav1*$^{-/-}$ or *P-rex1*$^{-/-}$ PMNs, *dock2*$^{-/-}$ PMNs are markedly defective in NADPH oxi-dase activation.

One important conclusion derived from our findings is that Hck and Fgr play an essential role in the regulation of Vav1 tyrosine phosphorylation, and this results in a neutrophil phenotype similar to that of Vav1$^{-/-}$ or P-Rex1$^{-/-}$ PMNs. Indeed, *hck*$^{-/-}$*fgr*$^{-/-}$ neutrophils display a partial reduction of F-actin generation but are able to polarize and chemotact toward an fMLP gradient (Figs. 2 and 3 (38); in contrast, fMLP-induced superoxide anion generation is markedly reduced in *hck*$^{-/-}$*fgr*$^{-/-}$ PMNs. It is important to note that the role played by Hck and Fgr in regulating these cell responses is totally independent of any possible alteration in generation of phospholipid-derived signals. In fact, as indicated by a normal generation of intracellular calcium transients and phosphorylation of AkT/PKB (Figs. 5 and 6), both activation of phosphatidylinositol-specific PLC and PI3Kγ are normal in Hck/Fgr-deficient neutrophils. Consistent with the evidence that NADPH oxidase activation requires the synergistic action of Vav1, P-Rex1, and DOCK2 GEFs (55, 63, 64, 66), in assay conditions revealing defective fMLP-induced superoxide anion generation by *hck*$^{-/-}$ *fgr*$^{-/-}$ neutrophils, a PI3Kγ inhibitor markedly reduced this response in WT neutrophils and totally suppressed it in KO cells (Fig. 7). Our findings, combined with those reported in studies with Vav1-, P-Rex1-, and DOCK2-deficient neutrophils, make plausible the model reported in Fig. 13 to envision signal transduction by fMLP, and possibly other chemoattractant receptors, leading to NADPH oxidase activation. This model implies that only full activation of Rac via both a Src-kinase- and Vav1-dependent, and a PI3K- and P-Rex1/DOCK2-dependent pathway results in NADPH oxidase activation. Whereas submaximal stimulation of Rac via the Vav1- or the P-Rex1-dependent pathway is sufficient to support F-actin formation to a level high enough to permit cell migration, DOCK2 deficiency results in a decrease in Rac activation high enough to result in impairment of cell migration. In this context, it is of interest that we detected a marked reduction in activation of the Rac downstream target PAK1 and, albeit to a lower extent, PAK2 in mouse Hck/Fgr-deficient and human PP2-treated neutrophils (Fig. 12). In fact, these kinases have been reported to be activated in chemoattractant-stimulated neutrophils and regulate NADPH oxidase activation (58). Hence, also optimal activation of PAKs might require full Rac activation, and it is tempting to speculate that PAK activation should be decreased in P-Rex1- and DOCK2-deficient neutrophils.

The role played by the Rac GTPase in phosphorylation of the MAPKs ERK1/2, p38, and JNK has been investigated in some detail in *rac2*$^{-/-}$ neutrophils (41, 44, 45, 62). These studies showed that Rac2 deficiency results in a markedly reduced ERK1/2 and JNK phosphorylation, but only in a small decrease in p38 phosphorylation, in response to fMLP. Because we could not detect any difference in ERK1/2 phosphorylation either in mouse Hck/Fgr-deficient or human PP2-treated neutrophils (Fig. 8), Rac activation via the Src-kinase-/Vav1-independent pathway must be sufficient for optimal ERK1/2 phosphorylation. As found in *rac2*$^{-/-}$ neutrophils (41), p38 phosphorylation in response to fMLP was diminished only marginally in *hck*$^{-/-}$*fgr*$^{-/-}$ PMNs and to an higher extent in human cells treated with an inhibitor of all Src family kinases. These findings suggests that p38 phosphorylation accurs mostly via Rac2-independent pathways and, as previously suggested

(17), Lyn, the Src family kinase that is expressed, together with Hck and Fgr, at the highest level in neutrophils, is implicated in a signaling pathway leading to p38 phosphorylation. Both Hck/Fgr deficiency in mouse, and PP2 treatment in human, neutrophils result in a marked reduction of fMLP-induced JNK phosphorylation. Because JNK phosphorylation was reported to be markedly decreased in Rac2-deficient neutrophils stimulated with fMLP (41) it is likely that, as found for NADPH oxidase activation and PAK phosphorylation, only full Rac activation via both the Src-kinase- and Vav1-dependent and independent pathways result in JNK phosphorylation.

Both in vitro and in vivo studies excluded the fact that neutrophil chemotaxis toward chemoattractants or chemokines requires Src family kinases (23, 28, 38). However, in the LPS-induced systemic inflammatory reaction, $hck^{-/-}fgr^{-/-}$ PMNs accumulate in the blood and are impaired in their capability to migrate into the liver (67). In addition, Fgr deficiency results in a marked reduction in the accumulation of eosinophils in the lung in a murine model of allergic asthma (68). Noteworthy, mice expressing a constitutively active form of Hck or with the selective granulocyte inactivation of the Src family kinase inhibitor C-terminal Src kinase (Csk) develop an exaggerated pulmonary inflammation (69, 70).

Src family kinases, as well as their downstream targets cortactin, paxillin, c-Cbl, and Syk (see Ref. 36 and references contained therein), are relevant players in the integrin-dependent organization of actin-bound protein complexes. Despite the fact that regulation of integrin affinity by chemoattractants is not dependent on Hck and Fgr (71), firm adhesion to integrin ligands was recently reported to be strictly dependent on Hck and Fgr, as well as Vav proteins both in vitro and in vivo (71–73). Hence, one step of neutrophil recruitment that may be regulated by Src family kinases is certainly stabilization of adhesion. $hck^{-/-}fgr^{-/-}$ neutrophils might adhere less firmly to the vascular wall and, under flow, detach from the vessel wall before entering the inflammatory site. The opposite may be true for granulocytes expressing a constitutively active form of Hck or deficient of Csk (70, 71). They may be retained more firmly on the vascular wall and, by default, be recruited in higher numbers in the tissue. This study, together with previous findings implicating Src family kinases in degranulation (18, 21, 23), identifies other possible steps regulating neutrophil recruitment that may depend on Src family kinases. In fact, release of reactive oxygen intermediates or granule constituents may regulate expression of counterreceptors for leukocyte integrins by the vascular endothelium or tightness of the endothelial cell layer (59, 60). Therefore, defective release of reactive oxygen molecules in response to chemoattractants may result in reduced activation of endothelial cells and the consequent recruitment of neutrophils. In this context, it is worth noting that also neutrophils from P-Rex1$^{-/-}$ mice, which display only a mild alteration in their migratory ability in vitro, are recruited to a significantly lower extent in sites of inflammation in vivo (63). Our findings that $hck^{-/-}fgr^{-/-}$ PMNs chemotact normally through 3-μm pore size filters, but fail to go through narrower 1-μm pores, raise the intriguing possibility that Src family kinases regulate cell deformability and/or strength of leading edge protrusion through the endothelial cell layer. Thus, depending on the vascular wall structure, $hck^{-/-}fgr^{-/-}$ PMNs might migrate normally through the peritoneal vessel endothelium, but be inhibited in the capability to transmigrate into other tissues. In

theory, this process would make Src family kinases a target to regulate PMNs recruitment into specific tissues. This issue will certainly deserve further investigation.

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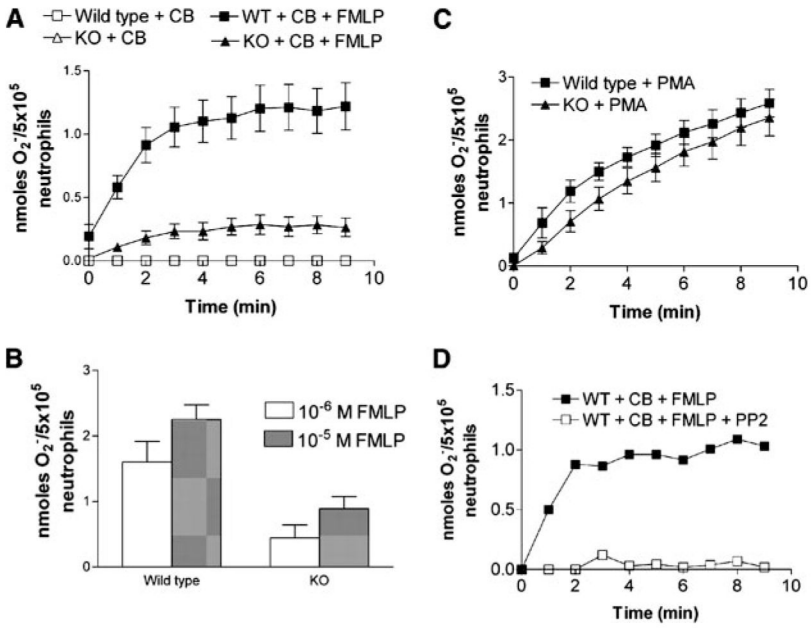

FIGURE 1.

Superoxide generation by *hck*$^{-/-}$*fgr*$^{-/-}$ PMNs is defective in response to fMLP, but not PMA. WT or *hck*$^{-/-}$*fgr*$^{-/-}$ (KO) bone marrow PMNs were isolated and assayed as described in *Materials and Methods. A*, Time-course of superoxide generation in response to 1 μM fMLP. Cells were preincubated for 10 min at 37°C in the absence or presence of 2 μg/ml CB before challenge with fMLP. Mean results of duplicate assays of five independent experiments ± SEM are reported. *B*, Superoxide generation by CB-treated PMNs in response to two different doses of fMLP after 2 min of stimulation. Mean results of duplicate assays of two independent experiments ± SEM are reported. *C*, Su-peroxide generation by CB-treated PMNs in response to 50 ng/ml PMA. Mean results of duplicate assays of five independent experiments ± SEM are reported. *D*, Su-peroxide generation by CB-treated, WT PMNs preincu-bated for 10 min at 37°C in the absence or presence of 10 μM PP2.

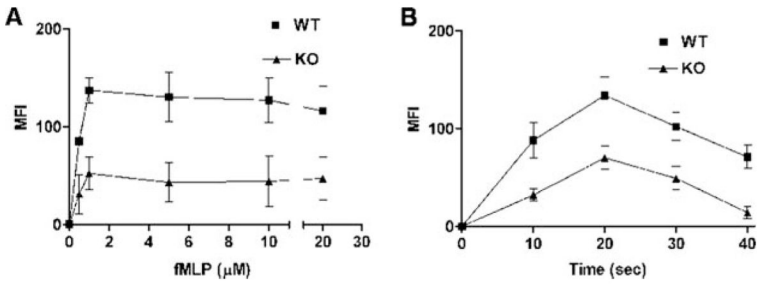

FIGURE 2.

F-actin formation in *hck*$^{-/-}$*fgr*$^{-/-}$ PMNs is defective in response to fMLP. WT or *hck*$^{-/-}$ *fgr*$^{-/-}$ (KO) bone marrow PMNs were isolated and assayed as described in *Materials and Methods. A*, F-actin formation after 25 s from stimulation with different doses of fMLP. *B*, F-actin formation at different times after stimulation with 1 μM fMLP. Mean results of duplicate assays of three independent experiments ± SEM are reported.

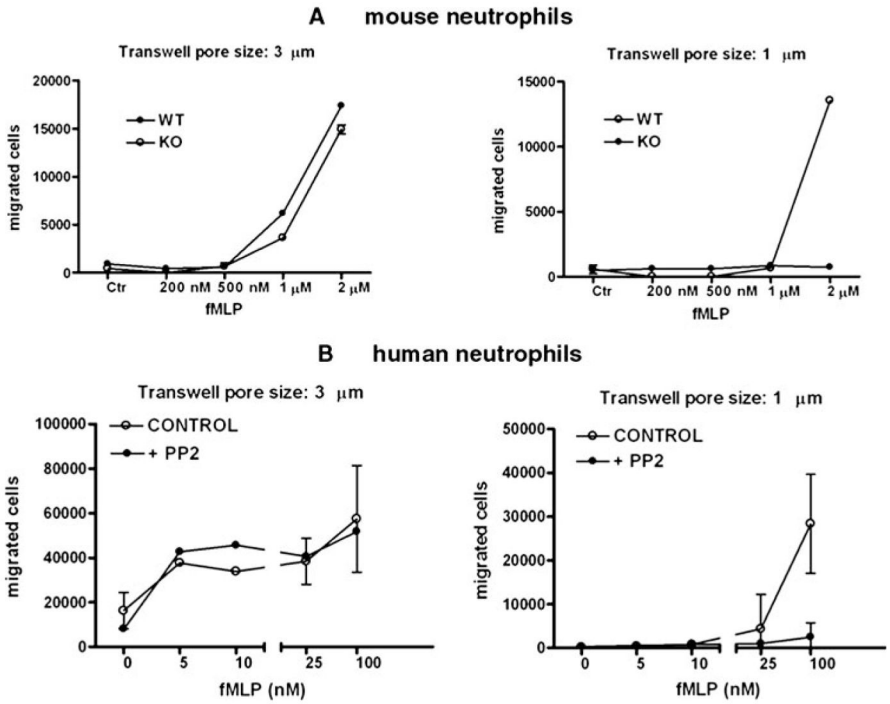

FIGURE 3.

hck$^{-/-}$*fgr*$^{-/-}$ mouse PMNs and human PMN treated with a Src family kinase inhibitor migrate normally in response to fMLP through 3-μm pore size Transwells, but are unable to migrate through 1-μm pore size Transwells. WT or *hck*$^{-/-}$*fgr*$^{-/-}$ (KO) bone marrow PMNs and human PMNs were isolated and assayed as described in *Materials and Methods*. *A*, Mouse PMNs; *B*, human PMNs. PP2 refers to PMNs preincubated for 10 min at 37°C in the presence of 10 μM PP2. Mean results of duplicate assays of three independent experiments ± SEM are reported.

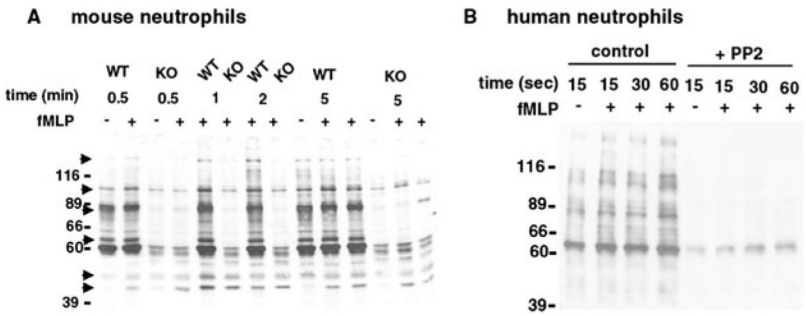

FIGURE 4.

Decreased protein tyrosine phosphorylation in resting and fMLP-stimulated conditions in *hck*$^{-/-}$*fgr*$^{-/-}$ bone marrow and PP2-treated human PMNs. *A*, WT or *hck*$^{-/-}$*fgr*$^{-/-}$ (KO) bone marrow PMNs were stimulated with 1 μM fMLP for the times indicated after preincubation for 10 min at 37°C in the presence of CB (see Fig. 1 legend). At the end of the incubation, the reaction was terminated and proteins were extracted and subjected to immunoblot analysis with anti-phosphotyrosine Abs as described in *Materials and Methods*. Numbers at the *left* indicate migration of m.w. markers. Arrow points indicate major proteins whose tyrosine phosphorylation increased upon treatment with fMLP or whose extent of phosphorylation was markedly different in WT vs *hck*$^{-/-}$*fgr*$^{-/-}$ PMNs. One of several experiments performed is reported. *B*, Human PMNs were preincubated with 10 μM PP2 for 10 min at 37°C and treated with CB as described in Fig. 1 legend for mouse PMNs. Cells were either left untreated or stimulated with 1 μM fMLP for the times indicated. Numbers at the *left* indicate migration of m.w. markers. One of three experiments performed is reported.

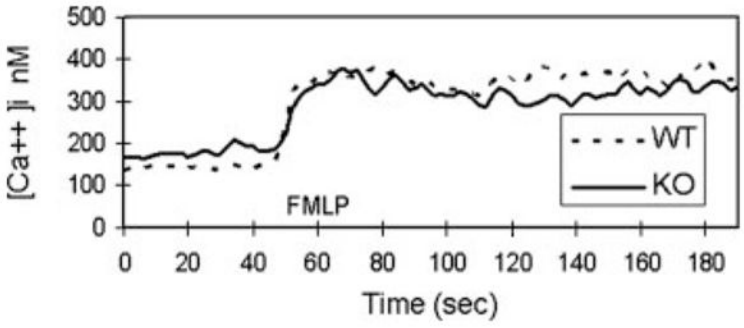

FIGURE 5.

Increase in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) in response to fMLP is not different in WT and *hck*$^{-/-}$*fgr*$^{-/-}$ PMNs. WT or *hck*$^{-/-}$*fgr*$^{-/-}$ (KO) bone marrow PMNs were pretreated with CB as described in Fig. 1 legend before stimulation with 1 μM fMLP. One representative of three experiments performed is reported.

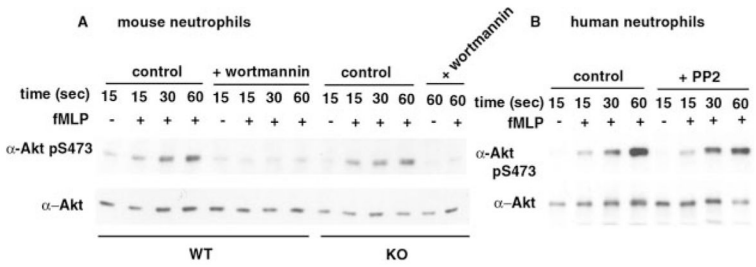

FIGURE 6.

Phosphorylation of Akt/PKB in response to fMLP is not defective in murine *hck*$^{-/-}$*fgr*$^{-/-}$ PMNs or human PMNs treated with the Src family inhibitor PP2. *A*, WT or *hck*$^{-/-}$*fgr*$^{-/-}$ (KO) bone marrow PMNs were preincubated for 30 min at room T in the absence or presence of 100 nM wortmannin and stimulated with 1 μM fMLP for the times indicated after preincubation for 10 min at 37°C in the presence of CB (see Fig. 1 legend). *B*, Human PMNs were preincubated with 10 μM PP2 for 10 min at 37°C and stimulated as described in Fig. 4 legend. At the end of the incubation, the reaction was terminated and proteins were extracted and subjected to immunoblot analysis with anti-phospho-Akt (Ser^{473}) Abs as described in *Materials and Methods*. After stripping, blots were incubated with anti-protein Abs. One representative experiment of three performed is reported.

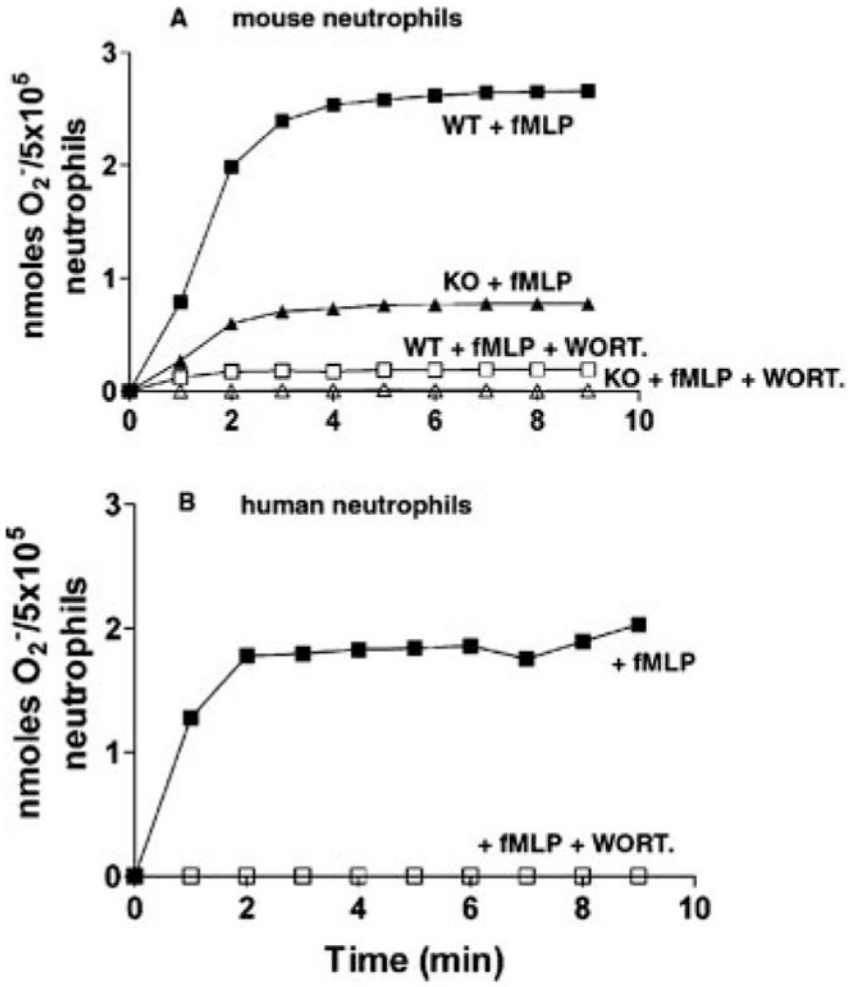

FIGURE 7.

Wortmannin inhibits superoxide generation in murine and human PMNs. WT or *hck*$^{-/-}$*fgr*$^{-/-}$ (KO) bone marrow PMNs (*A*) or human PMNs (*B*) were preincubated for 30 min at room T in the absence or presence of 100 nM wortmannin and stimulated with 1 μM fMLP for the times indicated in the conditions described in Figs. 1 and 4 legend. Mean results ± SEM of one representative of three experiments performed is reported.

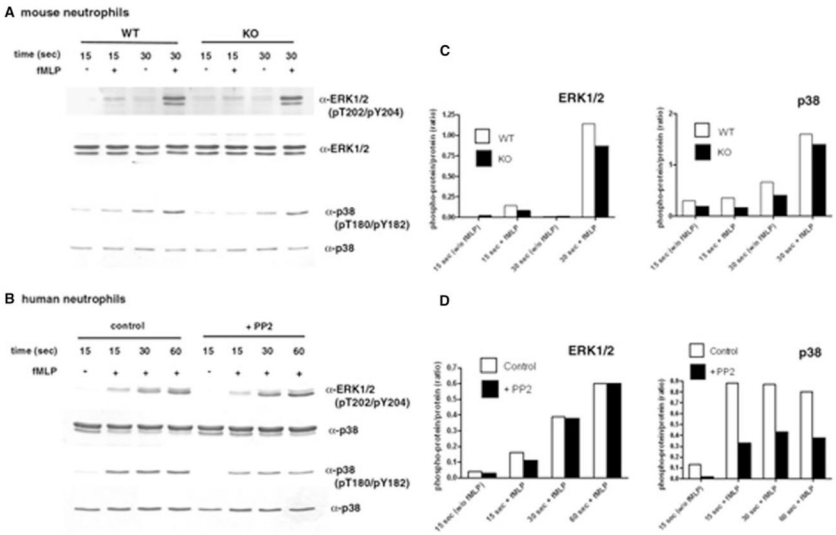

FIGURE 8.

MAPK phosphorylation in response to fMLP in murine *hck*$^{-/-}$*fgr*$^{-/-}$ PMNs or human PMNs treated with the Src family inhibitor PP2. *A*, WT or *hck*$^{-/-}$*fgr*$^{-/-}$ (KO) bone marrow PMNs were stimulated with 1 μM fMLP for the times indicated after preincubation for 10 min at 37°C in the presence of CB (see Fig. 1 legend). *B*, Human PMNs were preincubated with 10 μM PP2 for 10 min at 37°C and stimulated as described in Fig. 4 legend. At the end of the incubation, the reaction was terminated and proteins were extracted and subjected to immunoblot analysis with anti-phosphospecific Abs as described in *Materials and Methods*. After stripping, blots were incubated with anti-protein Abs. One representative of three experiments performed is reported. *C* and *D*, Densitometric analysis of the immunoblots shown in *A* and *B*, respectively.

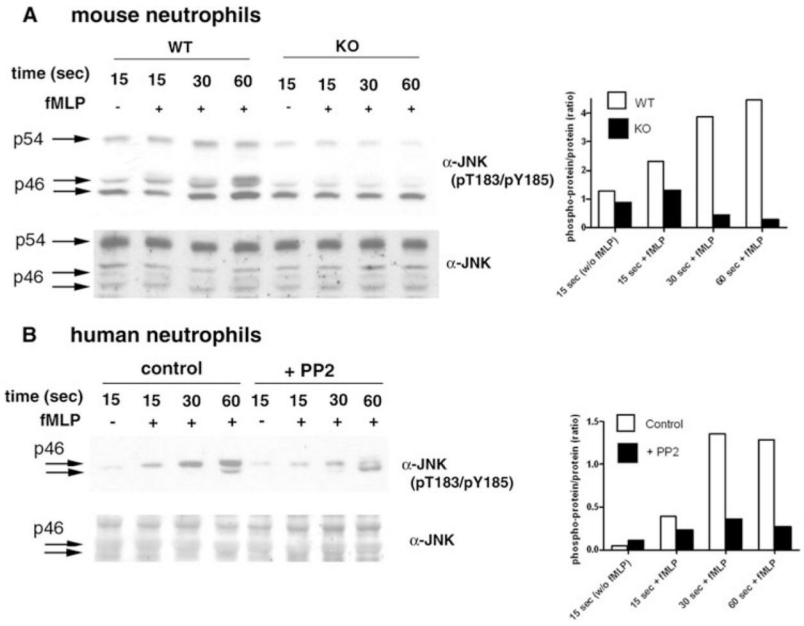

FIGURE 9.

Fgr and Hck deficiency in murine or treatment with PP2 in human PMNs results in decreased phosphorylation of JNK. *A*, WT or $hck^{-/-}fgr^{-/-}$ (KO) bone marrow PMNs were stimulated with 1 μM fMLP for the times indicated after preincubation for 10 min at 37°C in the presence of CB (see Fig. 1 legend). *B*, Human PMNs were preincubated with 10 μM PP2 for 10 min at 37°C and stimulated as described in Fig. 4 legend. At the end of the incubation, the reaction was terminated and proteins were extracted and subjected to immunoblot analysis with phosphospecific Abs directed agains JNK as described in *Materials and Methods*. After stripping, blots were incubated with anti-JNK Abs. One representative of three experiments performed is reported. Shown at the *right* are the densitometric analyses of the immunoblots.

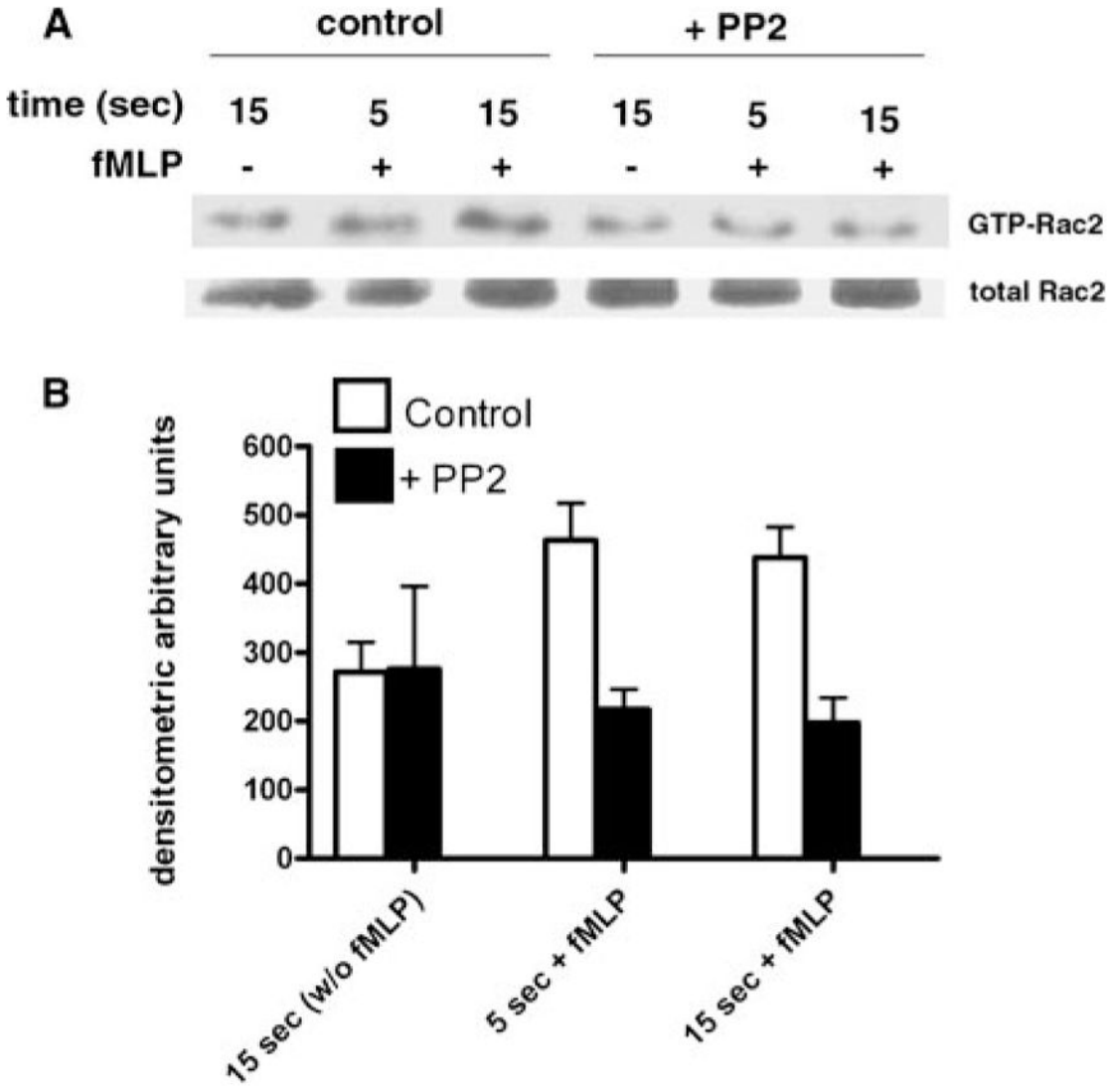

FIGURE 10.

PP2 inhibits fMLP-induced activation of Rac. Human PMNs were preincubated with 10 μM PP2 for 10 min at 37°C and stimulated as described in Fig. 4 legend. Cell lysates were incubated with GST-PBD and pull-down assays performed as described in *Materials and Methods. A*, Representative experiment showing the amount of GTP-bound Rac2 pulled down from the lysate or total Rac2. *B*, Mean results of densitometric analysis ± SEM of anti-Rac2 blots in three independent pull-down experiments.

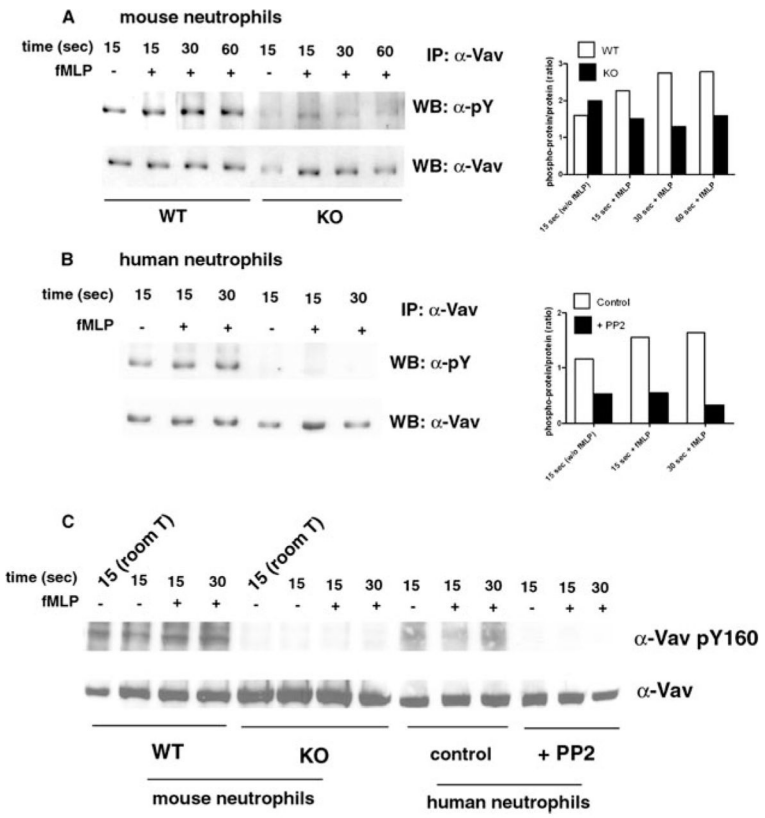

FIGURE 11.

Fgr and Hck deficiency in murine or treatment with PP2 in human PMNs results in decreased phosphorylation of Vav1. *A*, WT or $hck^{-/-}fgr^{-/-}$ (KO) bone marrow PMNs were stimulated with 1 μM fMLP for the times indicated after preincubation for 10 min at 37°C in the presence of CB (see Fig. 1 legend). *B*, Human PMNs were preincubated with 10 μM PP2 for 10 min at 37°C and stimulated as described in Fig. 4 legend. At the end of the incubation, the reaction was terminated and proteins were extracted and subjected to immunoprecipitation with anti-Vav1 Abs followed by immunoblotting with anti-phosphotyrosine Abs as described in *Materials and Methods*. After stripping, blots were incubated with anti-Vav-1 Abs. One representative of three experiments performed is reported. Shown at the *right* are the densitometric analyses of the immunoblots. *C*, WT or $hck^{-/-}fgr^{-/-}$ (KO) bone marrow PMNs or human PMNs were stimulated in the assay conditions described above. At the end of the incubation, the reaction was terminated and proteins were extracted and subjected to immunoblot analysis with anti-phosphospecific Abs directed against Vav as described in *Materials and Methods*. After stripping, blots were incubated with anti-Vav1 Abs. One representative of two experiments performed is reported.

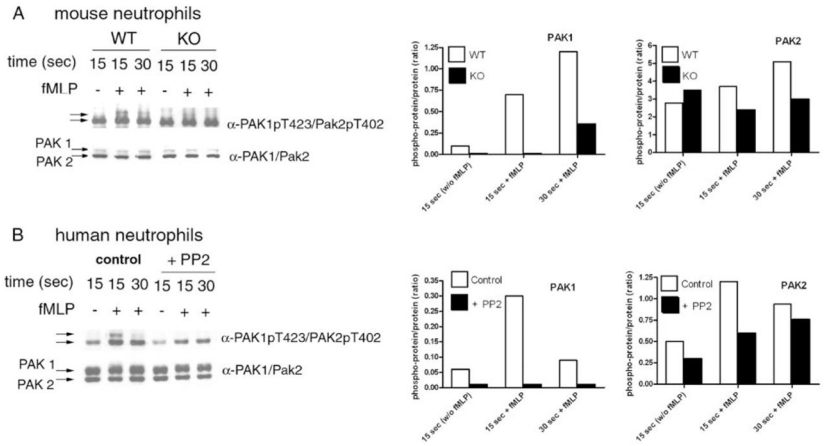

FIGURE 12.

Fgr and Hck deficiency in murine or treatment with PP2 in human PMNs results in decreased phosphorylation of PAKs. *A*, WT or $hck^{-/-}fgr^{-/-}$ (KO) bone marrow PMNs were stimulated with 1 μM fMLP for the times indicated after preincubation for 10 min at 37°C in the presence of CB (see Fig. 1 legend). *B*, Human PMNs were preincubated with 10 μM PP2 for 10 min at 37°C and stimulated as described in Fig. 4 legend. At the end of the incubation, the reaction was terminated and proteins extracted and subjected to immunoblot analysis with anti-phosphospecific Abs directed against PAK1 and PAK2 as described in *Materials and Methods*. After stripping, blots were incubated with anti-PAK1/2 Abs. One representative of two experiments performed is reported. Shown at the *right* are the densitometric analyses of the immunoblots.

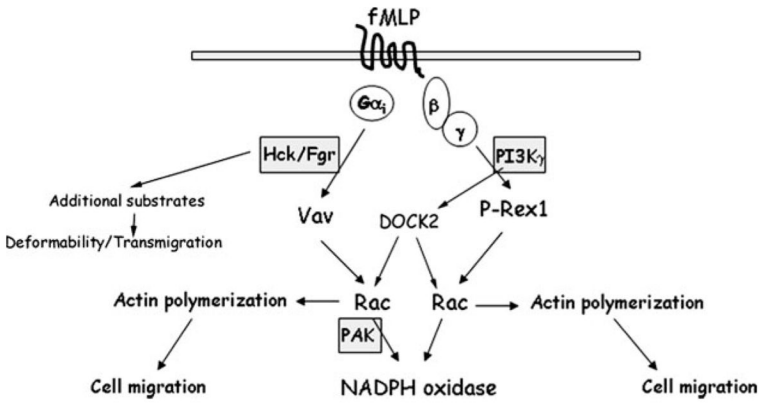

FIGURE 13.

Model for fMLP-induced activation of NADPH oxidase in PMNs. fMLP receptor(s) trigger activation of Vav, P-Rex1, and DOCK2 GEFs. P-Rex1 requires the $\beta\gamma$ subunits of trimeric G proteins and increased formation of phosphatidylinositol 3P by PI3K. Vav requires phosphorylation by Hck and Fgr and likely occurs downstream of G α_i. DOCK2 is also placed downstream of phosphatidylinositol 3P formation. P-Rex1-, Vav1-, and DOCK2-dependent Rac activation pathways are required to activate the NADPH oxidase. F-actin formation and cell migration are induced by either the Vav- or the P-Rex1-dependent pathway; deficiency of one of the two pathways results in a partial reduction in F-actin formation, which is however insufficient to decrease cell migration. In contrast, DOCK2 gives a major contribution to Rac activation, and its deficiency results in alteration of cell migration. (See the text for details and references.) Likely, via phosphorylation of additional substrates, Hck and Fgr regulate PMN deformability and possibly transmigration through cell barriers.