Activation of the Mitogen-activated Protein Kinase Pathway by fMLP in the Absence of Lyn and Tyrosine Phosphorylation of SHC in Transfected Cells*

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The chemotactic peptide f-Met-Leu-Phe (fMLP) stimulates leukocyte functions through binding and activation of a specific G-protein-coupled formyl peptide receptor (FPR). Recent studies have shown that stimulation of neutrophils with fMLP induces the activation of two members of the mitogen-activated protein kinase (MAP kinase) family, ERK1 and ERK2, through mechanisms that are not completely understood but may involve the phosphorylation of the adapter protein SHC by the Src-related kinase Lyn. In this study, transfected fibroblasts expressing the rabbit FPR were used to investigate further the role of Lyn and SHC phosphorylation in fMLP-stimulated MAP kinase activation. Stimulation of transfected cells with fMLP resulted in the time- and dose-dependent increase in tyrosine phosphorylation and activation of ERK1 and ERK2 and the activation of MEK, the MAP kinase/ERK kinase. The activation of both ERKs and MEK was inhibited by pre-incubation of the cells with pertussis toxin, indicating that activation was dependent upon a Gαi-like protein that couples to the receptor. Our data also show that, unlike neutrophils, FPR-transfected fibroblasts do not express the Src-related kinase Lyn. In the absence of Lyn, fMLP stimulation did not result in an increased tyrosine phosphorylation of the adapter protein SHC, whereas it was still able to induce MAP kinase activation. These data suggest that Lyn and SHC are not the only upstream signals for activation of the MAP kinase/ERK pathway by fMLP and demonstrate the potential application of the FPR-transfected cells for the delineation of additional signaling mechanisms stimulated by fMLP.

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§ The abbreviations used are: fMLP, N-formyl-methionyl-leucyl-phenylalanine; ERK, extracellular-signal-regulated kinase; FPR, formyl nuclear neutrophils (PMN), bind to seven transmembrane helical G-protein-coupled receptors on the surface of these cells (1). Early responses induced by fMLP are mediated by activation of the functionally associated G-protein(s), which in turn activate effectors such as phospholipase Cβ for the generation of inositol 1,4,5-trisphosphate and diacylglycerol. Mobilization of Ca2+ from intracellular stores and activation of various isoforms of protein kinase C are then observed (2, 3). Recently, evidence was provided that additional signaling pathways involving kinases other than protein kinase C are triggered by fMLP (4, 5). We and others (6–9) demonstrated that fMLP stimulates the increase in tyrosine phosphorylation of several proteins. Two of these proteins that became rapidly and transiently activated were identified as members of the family of mitogen-activated protein (MAP) kinases or extracellular signal-regulated kinases (ERKs) (7, 8, 10). Activation of the MAP kinases requires dual phosphorylation on tyrosine and threonine by a family of multifunctional MAP kinase kinases or MEKs (11). In several cell types, including in neutrophils (12), activation of the MAP kinase cascade has been shown to occur through the GTP-binding protein, p21Ras (13). The molecular mechanisms leading to Ras activation are best understood with tyrosine kinase growth factor receptors (RTK) such as that for epidermal growth factor or nerve growth factor and have been shown to involve the participation of adapter proteins such as SHC (14) and Grb2 (15). This novel class of molecules lacks enzymatic activity but bears src homology (SH) domains that confer them the ability to bind phosphotyrosine (SH2) or proline-rich region (SH3), resulting in coupling to other signaling molecules (16). Binding of a ligand to the RTK induces dimerization and transphosphorylation of the RTK, resulting in the tyrosine phosphorylation of SHC isoforms and their concomitant association with the activated RTK (17). Tyrosine-phosphorylated SHC isoforms subsequently bind to the SH2 domain of Grb2, which is itself constitutively associated with the guanine nucleotide exchange factor, Sos (18, 19). This results in the formation of a SHC-Grb2-Sos complex at the plasma membrane where Sos catalyzes the Ras guanine nucleotide exchange (17). The activation of the MAP kinase pathway by Gαi-protein-coupled receptors is less well understood and has been shown to occur through Ras-dependent (11, 12, 20) and -independent pathways (21, 22). Several studies identified the Gαs, subunits of the hetero-

peptide receptor; MAP kinase, mitogen-activated protein kinase; MBP, myelin basic protein; MEK, MAP kinase/ERK kinase; PAGE, polyacrylamide gel electrophoresis; PI, phosphatidylserine; PMN, polymorphonuclear neutrophils; PY, phosphotyrosine; PT, pertussis toxin; SH, src homology domain; V, vector.
trimeric G proteins as the primary mediator of Ras activation (11, 23, 24), and, recently, the tyrosine phosphorylation of SHC through a tyrosine phosphorylation event mediated by the G protein subunits and formation of the SHC-Grb2-Sos complex were also implicated in Ras activation by G protein-coupled receptors (25-27). Expression of a specific G protein, i.e. a G protein, binding peptide derived from βARK1 (23, 28), reduced MAP kinase activation and abolished SHC tyrosine phosphorylation stimulated by the α2-adrenergic receptor (25). The Gαs-mediated activation of MAP kinase was also blocked by disruption of the SHC-Grb2-Sos complex and by tyrosine kinase inhibitors (23, 25). In addition, thyrotropin-releasing factor and endothelin-1 induced the tyrosine phosphorylation of SHC and SHC-Grb2-Sos complex formation (26, 27), and the α2-thrombin receptor activated p100 tyrosine kinase (29). Recent studies in neutrophils demonstrated that fMLP induced the activation of Lyn and the association and tyrosine phosphorylation of SHC (30), suggesting that SHC and Lyn might play a role in the activation of the Ras/MAP kinase pathway.

Transfected cells have recently been used by others (23, 24, 31-33) for the identification of signaling pathways that are otherwise difficult to characterize in the native receptor-expressing cells. The rabbit fMLP receptor (FPR) has been cloned and transfected into mouse fibroblasts. Stably transfected cells displayed high affinity fMLP binding, Ca2+ mobilization, sensitivity to pertussis toxin (PT), and homologous desensitization, demonstrating that the transfected FPR could mediate transmembrane signaling and was coupled to a G-like protein (34, 35). In this report, we used these cells to determine whether fMLP triggered the increased tyrosine phosphorylation and activation of the MAP kinase pathway and to investigate the role of Lyn and SHC phosphorylation in activation of this pathway. We show that ERK1, ERK2, and MEK are activated through a G-protein-coupled pathway that does not involve the Src-related kinase Lyn and the adapter protein SHC.

**EXPERIMENTAL PROCEDURES**

Cell Culture, Preparation of Neutrophils, and Treatment with Pertussis Toxin—Formyl peptide receptor (FPR) and vector (V)-transfected mouse fibroblasts were maintained in the presence of 175 μg/ml G418 (Genetecin, Life Technologies, Inc.) (34). PMN were purified from the peripheral blood of normal volunteer adults as approved by the Committee on Human Investigation at Children's Hospital Los Angeles, as described (7). FPR cells were incubated for 16 h in medium containing 100 ng/ml PT (List Biological Laboratories, Campbell, CA) while PMN (5 × 10^6/ml) were treated with 1 μg/ml PT for 2 h at 37°C.

Preparation of Cell Extracts and Immunoblotting—Confluent cells were harvested with trypsin-free dissociation buffer (Life Technologies, Inc.), washed in phosphate-buffered saline and used directly. FPR and V cells or PMN (1 × 10^6/ml) were suspended in Krebs-Ringer phosphate buffer, warmed to 37°C, and stimulated for various times. The reaction was stopped by a quick spin, and total cellular extracts were prepared as described (7). Proteins (30 μg) were subjected to SDS-polyacrylamide gel electrophoresis (PAGE), electrotransferred to nitrocellulose membranes, and analyzed by immunoblotting with anti-phosphotyrosine antibody (PY) (4G10, Upstate Biotechnology, Inc., Lake Placid, NY) and MAP kinase (SC-94, Santa Cruz Biotechnology Inc., Santa Cruz, CA) antibodies, as described (7).

MAP Kinase Immunoprecipitation and MBP Kinase Activity—MAP kinases were immunoprecipitated under native conditions with agarose-conjugated peptide antibodies to either ERK1 (SC-93) or ERK2 (SC-154) (Santa Cruz Biotechnology Inc., Santa Cruz, CA). After stimulation, cells were harvested on ice for 20 min (50 μg/ml Tris-HCl, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 0.1% SDS, 0.5% sodium deoxycholate, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 mM Na3VO4, 100 mM NaF, 25 mM β-glycerophosphate, 10 μg/ml leupeptin and apronitin) and centrifuged (15,000 × g, 20 min), and the cleared lysates, adjusted for protein content (400–600 μg), were incubated for 2 h with the antibodies. The agarose beads were subsequently washed with high salt (2 M NaCl) and lysis buffers. The ERK immune complexes were either released with 2 × sample buffer (36) for analysis by SDS-PAGE and immunoblotting with PY or MAP kinase antibodies as above or used to determine kinase activity with myelin basic protein (MBP) as substrate (37). After washing in kinase buffer (10 mM Tris-HCl, pH 7.5, 1 mM dithiothreitol, 10 mM MgCl2), agarose beads were incubated for 30 min at 30°C with 50 μl of kinase buffer containing 1 mg/ml MBP, 50 μM ATP, 10 mM p-nitrophenyl phosphate, and 5 μCi of [γ-32P]ATP (10 μCi/μl). The reaction was stopped by addition of 5 × Laemmli buffer and boiling for 5 min. The [γ-32P]MBP was resolved on 16% SDS-PAGE, and gels were exposed for autoradiography or the MBP protein band was excised from the gels and incorporated radioactivity was measured by Cerenkov counting.

MEK Immunoprecipitation and Kinase Activity—Cells were stimulated and lysed on ice as above, and cleared lysates (500 μg of protein) were incubated with 10 μl of MEK1 antibody (Transduction Laboratories, Lexington, KY) at 4°C for 2 h prior to incubation with 30 μl of protein A-Sepharose (Pharmacia Biotech Inc.) for 1 h. Immune complexes were recovered by centrifugation, washed several times with lysis buffer, and released in 2 × sample buffer for analysis by immunoblotting or used for kinase activity measurement using kinase-inactive recombinant ERK1 protein (K71A, ERK1, rERK1, Upstate Biotech., Inc., Lake Placid, NY) as substrate (38). Immune complexes were incubated for 30 min at 30°C with 20 μl of agarose-conjugated rERK1 and 50 μl of kinase buffer (50 mM Tris-HCl, pH 8, 10 mM MgCl2, 5 mM dithiothreitol, 1 mM EGTA, 1 mM Na3VO4, 1 mM NaF, 25 μM ATP, and 10 μCi of [γ-32P]ATP) with frequent and strong agitation. The reaction was stopped by addition of 5 × Laemmli buffer, and the amount of [32P]incorporated into rERK1 was determined after SDS-PAGE by autoradiography and Cerenkov counting.

SHC Immunoprecipitation—PMN or FPR cells were lysed as above after stimulation with fMLP for 1 or 5 min, respectively, and the cleared lysates were incubated overnight with 15 μl of polyclonal SHC antibody (Transduction Laboratories) and 1 h with 30 μl of protein A-Sepharose. Immune complexes were recovered by centrifugation, washed with lysis buffer, and analyzed by SDS-PAGE and immunoblotting with monoclonal antibodies to PY (Upstate Biotechnology, Inc.), SHC, Grb2, and Lyn (Transduction Laboratories).

**RESULTS**

**fMLP Induces the Pertussis Toxin-sensitive Activation of ERK1 and ERK2 in FPR Cells—Immunoblotting of whole protein extracts from FPR and V cells with an anti-phosphotyrosine antibody demonstrated that several protein bands were phosphorylated on tyrosine in the absence of stimulation. No substantial difference in the basal level of tyrosine phosphorylation was observed between FPR and vector cells, except for a protein band migrating slightly slower and not change with stimulation (compare Fig. 1, A and B). A slight increase in a 45-kDa protein band that also peaked at 5 min was observed, although it was more difficult to assess since this band was not always well resolved from a tyrosine-phosphorylated band that migrated slightly slower and did not change with stimulation (compare Fig. 1, A and B). Low levels of tyrosine phosphorylation of the p45 and p42 protein bands were noted in the absence of stimulation in both FPR and V cells that varied between experiments and increased with incubation at 37°C; however, these levels were consistently increased in FPR cells stimulated with fMLP but were not discernible change was ever observed in V cells under similar conditions (Fig. 1A). In contrast, two protein bands with similar molecular masses exhibited increased tyrosine phosphorylation after treatment of V cells with phorbol myristate acetate, supporting the notion that the lack of response of V cells to fMLP was due to the lack of the receptor. None of the protein bands previously reported to exhibit increased tyrosine phosphorylation after fMLP stimulation in PMN (6–9) were observed in fMLP-stimulated FPR cells, suggesting that these proteins are either only expressed in myeloid cells or that their...
Phosphorylation is dependent upon a myeloid-specific pathway. The increase in tyrosine phosphorylation of the 45- and 42-kDa proteins was dose-dependent and appeared to peak between $10^{-8}$ and $10^{-7}$ M fMLP (Fig. 1B). Based on our previous studies, these proteins were likely to be the two members of the MAP kinase family, ERK1 and ERK2. Immunoblotting with a MAP kinase antibody recognizing both isoforms showed the presence of two proteins co-migrating with the tyrosine-phosphorylated proteins and exhibiting a shift in migration after fMLP stimulation of FPR cells, more readily detected with ERK2 (Fig. 1A). No such shift was seen in V cells, unless the cells were stimulated with phorbol myristate acetate. Incubation with pertussis toxin (PT) inhibited the increase in tyrosine phosphorylation of the MAP kinase/ERKs, similarly in FPR cells as in PMN (Fig. 1C). This is consistent with previous reports showing inhibition of the fMLP-stimulated increase in intracellular Ca$^{2+}$ by PT (34, 39). To more clearly demonstrate that the 45- and 42-kDa proteins with increased tyrosine phosphorylation were the activated MAP kinase/ERKs, we performed immunoprecipitation with two antibodies that more specifically recognize either ERK1 or ERK2, as seen in Fig. 2A. Immunoblotting of the immunoprecipitates with the PY antibody showed low levels of...
tyrosine phosphorylation of ERK1 and ERK2 in control cells; however, both isoforms increased their tyrosine phosphorylation after treatment with fMLP with a time course similar to that observed with whole cell extracts (Fig. 2A). A shift in the electrophoretic mobility of ERK2 was also observed when the immunoprecipitates were probed with the MAP kinase antibody, consistent with phosphorylation and activation of the kinase activity of the MAP kinase/ERKs. To confirm this, we determined the kinase activity of the ERK1 and ERK2 immunoprecipitates, using MBP as substrate. No increase in $^{32}$P incorporation into MBP was observed in V cells while a time-dependent increase was detected after fMLP stimulation of FPR cells. The activity peaked at 5 min for both ERK1 and ERK2 and was greater in the ERK2 immunoprecipitates (Fig. 2, B and C). This time course of activation was slightly more prolonged than that observed in PMN where the activity peaked at 1 min and was close to basal levels by 5 min (7, 8). Thus, these data demonstrate that transfection of the FPR receptor can trigger the activation of the MAP kinase pathway.

Absence of Lyn Does Not Prevent MAP Kinase Activation—Recent studies showed that the Src-related kinase Lyn becomes activated upon PMN treatment with fMLP (30, 42) and associates with the adapter protein SHC (14), which undergoes tyrosine phosphorylation (30). It was suggested that the Lyn-SHC signaling pathway might provide a link between the FPR and the Ras/MAP kinase pathway. We compared the expression of Lyn and SHC in FPR cells and PMN. As expected, immunoblotting with an antibody against Lyn demonstrated the presence of Lyn in PMN as two strong protein bands migrating approximately at 54 and 56 kDa; however, no immunoreactivity was detected with two different antibodies in FPR lysates, indicating that Lyn is not expressed in these cells (Fig. 4A). Strong expression of the three SHC isoforms (p46, p52, and p66) was observed in FPR cells while PMN had very low level of expression of mainly p52 SHC (Fig. 4A). SHC was immunoprecipitated from PMN and FPR lysates with a polyclonal antibody, and the immunoprecipitates were probed with...
PY, SHC, Lyn, and Grb2 antibodies. Low amount of p52SHC was found in immunoprecipitates from PMN lysates. While consistent with the low level expression of SHC in PMN, the amount in the immunoprecipitates appears lower. This is due to the use of a monoclonal antibody to probe the blot that was not as efficient as the polyclonal antibody used for the immunoprecipitation and expression in lysates. The latter could not be used because of strong interference with the immunoglobulin heavy chain. A phosphotyrosine protein that appeared to co-migrate with p52 SHC and exhibited an increase in tyrosine phosphorylation after fMLP treatment was observed when immunoblotting the SHC immunoprecipitates with the PY antibody, indicating that fMLP stimulates the tyrosine phosphorylation of SHC in PMN. Another tyrosine phosphorylated protein, migrating at 44 kDa, was also detected when longer exposure times were used, and its identity has not been determined so far (Fig. 4B). In addition, Grb2, which associates with tyrosine-phosphorylated SHC through its SH2 domain (43), and Lyn were also detected in the SHC immunoprecipitates; however, recovery of either Grb2 or Lyn in the SHC immunoprecipitates was the same in control and stimulated PMN, suggesting that fMLP did not alter the extent of their association with SHC. Neither band of the Lyn doublet coincided with the p52 tyrosine-phosphorylated protein. Immunoblotting of the SHC immunoprecipitates from FPR lysates with the PY antibody showed a faint tyrosine-phosphorylated protein band that did not precisely co-migrate with any of the SHC isoforms and did not change its tyrosine phosphorylation after fMLP treatment, suggesting that this protein might not be SHC despite the large amount of SHC proteins immunoprecipitated from these cells (Fig. 4C). This protein did not react either with the Lyn antibody. Very little Grb2 was recovered in the SHC immunoprecipitates, consistent with the apparent lack of tyrosine phosphorylation of SHC. Thus, in the absence of Lyn, fMLP stimulation of FPR-transfected cells did not result in an increase in tyrosine phosphorylation of the SHC isoforms.

DISCUSSION

We demonstrate here that stimulation with fMLP of fibroblasts transfected with the formyl peptide receptor results in the pertussis toxin-sensitive activation of ERK1, ERK2, and MEK in the absence of Lyn and tyrosine phosphorylation of SHC, suggesting that Lyn and SHC are not essential for activation of the MAP kinase pathway. These data support previous reports that documented the activation of the MAP kinase pathway by G-protein-coupled receptors (20, 22, 23, 44, 45) and suggest that more than one signal is necessary to induce maximal activation of the MAP kinase pathway by fMLP.

Previous studies have indicated that the Gβγ subunits might participate in the activation of the MAP kinase pathway (23, 25, 28). A possible scenario could be envisioned where Gβγ subunits activate an Src-like tyrosine kinase that phosphorylates SHC, allowing the formation of SHC-Grb2-Sos complex and the activation of the Ras/MAP kinase pathway. Our data, however, show that the lack of tyrosine phosphorylation of SHC by fMLP, which appears to be dependent upon the presence of a tyrosine kinase activity absent in FPR cells (presumably that of Lyn), did not prevent the fMLP-induced activation of ERKs and MEK in these cells. This suggests that SHC and Lyn are not the only upstream signals and that there exists an additional pathway for fMLP-induced activation of MAP kinase.

This notion is supported by the observation that MAP kinase activation was only reduced by blocking SHC phosphorylation (23, 25). Furthermore, expression of Gαq, alone produced only a 2-fold increase in MAP kinase activation compared with the severalfold increase induced by a2-adrenergic receptor (25). Similar observations were made when analyzing the activation of MAP kinase by the C5a receptor, another seven transmembrane domain receptor which couples to Gαq and Gα16, using HEK293-transfected cells (46).

While MAP kinase activation occurred in the absence of Lyn and SHC in the transfected FPR cells, we noted subtle differences in the extent and time course of activation of the MAP kinases in these cells compared with PMN, i.e. a similarly rapid activation but much slower return to basal levels and a less robust increase in tyrosine phosphorylation. This suggests a differential mode of regulation of the MAP kinase cascade and supports the notion that more than one pathway might be involved. An hypothesis may be proposed where activation of the MAP kinases could occur through a pathway in which Lyn, SHC phosphorylation, and the ensuing complex do not play a role, as observed in the FPR-transfected cells, but full activation would require Ras activation through SHC. Both pathways are likely to occur after activation of the G-proteins since PT inhibited MAP kinase activation both in FPR cells and in neutrophils.

Other candidate activators may include various isoforms of protein kinase C, p65PAK (47), which has also been described in neutrophils (48) and PI 3-kinase (49). The activation of the MAP kinase pathway through Gαq was shown to involve at least in part the release of its βγ subunits as well as activation of PI 3-kinase (50). Alternatively, differences at the level of the receptor and G-proteins might explain our data, even though similar deactivation was previously observed in these cells (34). At present, it is not clear whether the multifunctional phosphatases that specifically dephosphorylate the MAP kinases in some cells (51) might play a role here. Nevertheless, data presented above clearly indicate that the absence of Lyn and the lack of tyrosine phosphorylation of SHC do not abrogate the ability of the fMLP receptor to stimulate MAP kinase activation and suggest the presence of an as yet incompletely understood signaling pathway.

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