Activation of MEKK by Formyl-methionyl-leucyl-phenylalanine in Human Neutrophils

MAPPING PATHWAYS FOR MITOGEN-ACTIVATED PROTEIN KINASE ACTIVATION*

(Received for publication, June 28, 1996, and in revised form, October 4, 1996)

Natalie J. Avdiš, Brent W. Winston§, Marijane Russel§§, Scott K. Young¶, Gary L. Johnson§§, and G. Scott Worthen‡‡‡‡

From the Departments of §Medicine and ¶Pediatrics and the ‡Division of Basic Sciences, National Jewish Center for Immunology and Respiratory Medicine and the Departments of ‡‡Medicine and ¶¶Pharmacology, University of Colorado School of Medicine, Denver, Colorado 80206

Mechanisms of neutrophil activation in response to chemoattractants remain incompletely understood. We have recently reported a Ras-mediated c-Raf pathway leading to the activation of mitogen-activated protein (MAP) kinase in human neutrophils stimulated with the chemoattractant formyl-Met-Leu-Phe (FMLP). However, concern that Raf activation may not fully account for the early FMLP-mediated human neutrophil responses prompted us to investigate the activation of MAP kinase/ERK kinase (MEK) by MEK kinase (MEKK). In cell lysates we identified protein species at 180, 160, 110, 72, and 54 kDa with a monoclonal antibody to MEKK. Activation of MEKK was determined on immunoprecipitates from FMLP-stimulated neutrophils by in vitro kinase assay, which utilized both MEK1 and MEK2 as substrates. It was rapid, detectable at 30 s and reaching a plateau at 5 min, and it was inhibited in a dose-dependent fashion by a specific phosphatidylinositol 3-kinase inhibitor, wortmannin. Partial inhibition by pertussis toxin was observed. We were unable to show inhibition of the MEKK response by GF 109203X, a protein kinase C-specific inhibitor. These data indicate that in neutrophils activation of MEKK in addition to Raf may underlie stimulation of MAP kinase and other MAP kinase homologues by FMLP.

The neutrophil plays a pivotal role in the inflammatory response and forms an important component of host defense (1). In response to inflammatory stimuli, neutrophils are seques-
tered in the microvasculature, transvascular barriers, and converge on inflammatory foci. Extensive studies have shown that chemoattractants, including formyl-Met-Leu-Phe (FMLP)1 (a formylated tripeptide derived from bacterial cell walls) (2), induce numerous neutrophil functional responses including cytoskeletal rearrangement, chemotaxis, granule secretion, release of superoxide, and phagocytosis (reviewed in Ref. 3). Although the signal transduction events pertaining to these specific functions remain elusive, it has been shown that FMLP-induced neutrophil stimulation is initiated by ligand binding to a seven-transmembrane-spanning (STMS) receptor coupling through the heterotrimeric G-protein, Gα2 (4). Activation of cellular functions has been ascribed to generation of inositol phosphates, Ca2+ transients, and activation of phospholipase C-β (5), but the activation of PI 3-kinase (6, 7) and phospholipase D (8) have also been shown to be induced by FMLP. Recently, FMLP and other stimuli acting through the STMS receptors have been shown to regulate a pathway involving activation of Ras, Raf, and MEK, culminating in the phosphorylation and activation of MAP kinase (9, 10).

A variety of MAP kinase activation cascades have been delineated in yeast, each of which appears to serve a unique functional role (11–13), so far largely linked to mitogenesis (14) and cellular differentiation (15). Recent evidence indicates the potential for involvement of MAP kinase cascades in functions distinct from growth and division, including stress and osmo-regulatory responses in mammalian cells (16–20). Furthermore, MAP kinase activation occurs in neutrophils (9, 21), a cell incapable of mitogenesis. These data suggest that the array of functions ascribed to MAP kinases is broader than currently invoked, and hence the mechanisms responsible for activation are of considerable importance.

Recent evidence suggests that an alternate route to MAP kinase or other family members is through activation of the MEK kinase (MEKK) family (22). MEKK-1, a recently cloned mammalian homologue of the yeast protein kinases Byr2 and Ste11 (23), phosphorylates and activates MEK1 in a Raf-independent fashion in macrophages in response to stimulation with tumor necrosis factor α (24) and in PC12 cells, in response to nerve growth factor (through tyrosine kinase signaling) (25), leading to MAP kinase activation. MEKK also phosphorylates and activates Jun kinase kinase, thus effecting activation of MAP kinase homologues such as Jun kinase (26). We have previously shown that FMLP induces Gα2-dependent activation of Ras and Raf in human neutrophils, in a PKC-independent manner (9). We therefore questioned whether FMLP also activates MEKK and whether pathways leading to MAP kinase

* This work was supported by Specialized Center for Research in Atherosclerosis (in adult respiratory distress syndrome) Grant HL-40784, Grant HL-34303, and National Institutes of Health Grant GM-30324. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1724 solely to indicate this fact.

†† To whom correspondence should be addressed: Dept. of Medicine, National Jewish Center for Immunology and Respiratory Medicine, 1400 Jackson St., Denver, CO 80206. Tel.: 303-398-1171; Fax: 303-398-1851.

1 The abbreviations used are: FMLP, formyl-methionyl-leucyl-phenylalanine; MAP, mitogen-activated protein; MAPK, MAP kinase; MAPK-Rac, recombinant wild type MAPK; MAPK-Kb, recombinant kinase-inactive MAPK; MEK; MAPK/ERK kinase; MEK1-Ka, recombinant wild type MEK1; MEK2-Ka, recombinant wild type MEK2; MEK1-Kb, recombinant kinase-inactive MEK1; MEKK, MEK kinase (MAP kinase kinase); PI 3-kinase, phosphatidylinositol 3-kinase; PMA, phorbol 12-myristate 13-acetate; EGFR, epidermal growth factor receptor; PKC, protein kinase C; ERK, extracellular signal-regulated kinase; HSA, human serum albumin; ER, extraction buffer; PAGE, polyacrylamide gel electrophoresis; PAN, Pipes buffer; STMS, seven-transmembrane-spanning; BSA, bovine serum albumin fraction V; KRPD, Krebs-Ringer phosphate dextrose; PMN, polymorphonuclear leukocyte.
activation could also result from MEKK activation by a STIMS, G-protein-linked receptor in primary human cells.

**EXPERIMENTAL PROCEDURES**

**Materials—**Endotoxin-free reagents and plastics were used throughout the experiments. Neutrophils were resuspended in Krebs-Ringer phosphate buffer, pH 7.2, with 0.2% dextrose (5% dextrose in 0.2% sodium chloride, injectable; Abbott Laboratories, North Chicago, IL). Salts used in the buffer were purchased from Mallinckrodt Specialty Chemicals (Paris, KY). KRPD components were diluted in endotoxin-free saline (0.9% saline for irrigation; Abbott Laboratories) prepared with 5% bovine serum albumin (HSA) from Intergen (Purchase, NY). Reagents for incubation were stored for 30 min at 37°C and then stimulated with 10 nM of ATP (28) were resuspended at 20 mg/mL of MAP kinase column fraction activity curve was quantified using the β2 antagonist ATRA were added to the beads. EGFR-(662–681) phosphorylation was determined as above for the MAP kinase assay. To determine MEKK and c-Raf-1 immunoprecipitation. EGFR-(662–681) phosphorylation was determined as above. 

**MEKK Antibodies—**Full-length MEKK1 appears to represent a 180-kDa kinase with a large NH2-terminal regulatory domain and a C-terminal kinase domain. The original MEKK clone encoded an apparent 80-kDa fragment (referred to here as 80-kDa MEKK1), which lacks 100 kDa of the NH2 terminus of full-length MEKK1. The antibodies used in this study were directed against this 80-kDa fragment and described as follows. 80-kDa MEKK1 (1–301) refers to the NH2-terminal 301 amino acids of the 80-kDa MEKK1. Two antibodies (43-Y, a rabbit polyclonal anti-human MEKK, and 1C-9A, a mouse monoclonal anti-human MEKK) were directed against the NH2 terminus amino acids 1–301, and a rabbit polyclonal anti-human MEKK (C-22) raised against carboxyl-terminal amino acids 663–684 (the immunogen referred to as 80-kDa MEKK1-(663–684)) were all obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA).

**Other Antibodies—**Anti-ERK1 (C-16), anti-ERK2 (C-14), and anti-c-Raf-1 were obtained from Santa Cruz Biotechnology Inc. Anti-MEK1 and anti-MEK2 monoclonal antibodies were obtained from Transduction Laboratories (Lexington, KY). Rabbit IgG was from Pierce. PD98059 and anti-MEK2 monoclonal antibodies were obtained from Transduction Laboratories (Lexington, KY). Rabbit IgG was from Pierce. PD98059 was a gift from Dr. A. Sulte.

**Histidine-tagged Recombinant Proteins—**Kinase-inactive MEKK1 (MEK1kd), wild type MEKK1 (MEK1wt), wild type MEK2 (MEK2wt), kinase-inactive p42/p44 MAPK (ERK2(MAPKkd)), wild type p42/p44 MAPK (ERK2(MAPKwt)), and NH2-terminal MEKK1 were expressed in E. coli. Lysates were purified as described previously (9, 10). Supernatants were separated by 10% SDS-PAGE and transferred to nitrocellulose (29). The membrane was washed several times in Tris-buffered saline containing 20 mM Tris, pH 7.5, 137 mM NaCl, with 0.5% (v/v) Tween 20 (TTBS) and then blocked in 3% milk/TTBS for anti-MEKK and 5% BSA/TTBS for anti-c-Raf-1 immunoblotting. Antibody (19A-2C), a monoclonal anti-human MEKK and anti-MEK2 were used to probe for MEKK. Secondary antibodies were horseradish peroxidase-linked, and proteins were identified by ECL. For immunoprecipitates, cells were stimulated, and lysates were Protein A-Sepharose-immunoprecipitated in the presence of either anti-MEKK (45-Y) anti-c-Raf-1 or rabbit IgG, and beads were washed, as described for kinase reactions. Laemmli sample buffer was added to the immunoprecipitates, which were subjected to SDS-PAGE and immunoblotting as above.

**MEK Immunoprecipitation and Kinase Assay—**PMN were resuspended at 20 × 10⁶/ml in complete KRPD and preincubated for 30 min at 37°C prior to stimulation with 10⁻³ M FMLP for 2 min. Reactions were terminated with a 20-s centrifugation at 15,000 rpm, and cells pellet was resuspended in EB. Lysates were immunoprecipitated with monoclonal anti-MEKK or anti-MEK2 for 2 h. Beads were washed twice in EB and twice in PAN and resuspended in 60 µl of PAN. After the addition of 20 µl of in vitro kinase mixture containing 20 mM Pipes, pH 7.0, 10 mM MnCl₂, 100 ng of MAPKkd, and 20 µl of [γ-³²P]ATP, beads were incubated for 20 min at 30°C. Wild type MAP kinase (MAPKwt) was autophosphorylated in parallel as a marker to localize MAPKkd. Reactions were terminated with the addition of 5 × Laemmli sample buffer, subjected to 10% SDS-PAGE and autoradiography, and quantified by PhosphorImager analysis.

**Anti-c-Raf-1 Immunoprecipitation and Kinase Assay—**Neutrophils were immunoprecipitated and assayed for kinase activity as described previously (9, 10).

**MAP Kinase Assays: Mono Q Column Fractions—**MAP kinase activation was determined by Mono Q ion exchange chromatography in neutrophil lysates as described previously (9, 30). The area under the MAP kinase column fraction activity curve was quantified using the formula, (cpm + cpm - cpm × (fraction) - fraction - cpm) × 2, where cpm represents cpm of kinase activity for each fraction.
were resuspended at 20×10⁶/ml in complete KRPD and pretreated for 15 min at 37°C with either 25 nM, 50 nM or 100 nM wortmannin prior to stimulation. Neutrophils were similarly resuspended and then pretreated with 0.1% Me₂SO, 2 μM or 5 μM bisindolomaleimide (GF 109203X) for 30 min at 37°C prior to stimulation.

**Pertussis Toxin Pretreatments**—Neutrophils were resuspended at 10×10⁶/ml in 0.25% HSA/KRPD and pretreated with 500 ng/ml pertussis toxin for 2.0 h at 37°C. Cells were centrifuged, resuspended at a concentration of 20×10⁶ PMN/ml in complete KRPD, and further preincubated in the presence of 500 ng/ml pertussis toxin for 30 min at 37°C prior to stimulation. Lysates were immunoprecipitated with anti-MEKK (C-22), and MEKK activation was determined by the MEKK → EGFR(662–681) coupled assay.

**Inhibition of Kinase Activity and Superoxide Release by the MEK Inhibitor PD98059**—For the MEK and MAPK activity assays, neutrophils were preincubated with 10 μM PD98059 for 1 h at 37°C and stimulated with FMLP (10⁻⁷ M, 1 min). MEK activity (MEK in vitro kinase assay using the substrate MAPK₁₃) and MAPK activity (MAPK in vitro kinase immunoassay using the substrate EGFR(662–681)) were described above. For the functional assay, superoxide release was determined as described previously (31) with the following modifications. Neutrophils, resuspended at 2.5×10⁶/ml in KRPD containing 0.25% HSA, were pretreated with 10 μM PD98059 for 1 h, 37°C, and then cytochalasin B-treated (5 μg/ml, 5 min, 37°C) prior to stimulation with 10⁻⁸ M FMLP for 10 min in the presence of cytochrome c, at 37°C. Reactions were stopped with a 20-s centrifugation at 15,000 rpm, supernatants were transferred to a 96-well plate, and the absorbance was determined in a microtiter plate reader at 550 nm.

**RESULTS**

**Activation of MEKK by FMLP Stimulation of Human Neutrophils**—We have previously shown MAP kinase to be activated within 30 s of FMLP stimulation in neutrophils, reaching a peak between 1 and 2 min, and declining in activity after 5 min (9, 21). MEK₁ was found to be similarly activated by FMLP (9, 32). MEKK activity was assessed at varying times following FMLP stimulation, by immunoprecipitation of MEKK from Triton-soluble neutrophil lysates. Activity of the immunoprecipitates was measured by the incorporation of [γ³²P]ATP into recombinant kinase-inactive MEK₁ (MEK₁kd) substrate. Auto-phosphorylated wild type MEK₁ (MEK₁wt) was used as a marker for the position of MEK₁kd. The autoradiograph in Fig. 1A is a representative experiment depicting the time course of MEKK activation by FMLP. The phosphorylation of MEK₁kd appears to be biphasic with an initial rise at 30 s and a subsequent increase at 2 min. The graph depicted in Fig. 1B represents mean data from four such experiments, quantified by PhosphorImager analysis. We observed an initial increase in activity at 30 s, which apparently plateaued at 60 s, and then a second increase in MEK₁ activity occurred at 2 min, reaching a plateau between 5 and 10 min after stimulation. The maximal extent of activation was 2–3-fold above baseline.

**Detection of MEKK in Whole Cell Lysates and Immunoprecipitates**—Immunoblotting (Fig. 2) of Triton-soluble neutrophil lysates with a monoclonal antibody (C-22) to 80-kDa MEKK₁ (1–301) revealed major immunoreactive protein species at 54, 72, 110, 160, and 180 kDa. Bands at 180, 160, 110, and 54 kDa (the latter perhaps corresponding to a band observed in adipocytes (33)) were also observed in murine bone marrow-derived macrophages (Fig. 2) and a human fibroblast cell line (data not shown). In order to determine which of these species was likely to explain the MEK₁ activity observed, neutrophil lysates were immunoprecipitated with polyclonal antibody (43-Y) directed against 80-kDa MEKK₁ (1–301). Immunoblotting with the monoclonal amino terminus antibody detected predominantly the 54-kDa species (Fig. 2), suggesting that this form (which may include several species) is likely to account for at least some of the observed MEKK activity.

**Specificity of Immunoprecipitation for MEKK and c-Raf-1**—To ensure that the activity of immunoprecipitated MEKK was due to MEKK itself and not merely that of Raf co-immunoprecipitation with MEKK, we undertook two further sets of experiments. The first consisted of immunoprecipitating MEKK (with antibody 43-Y) from neutrophil lysates and immunoblotting for the presence of c-Raf-1. We also confirmed the presence of a c-Raf-1 species in whole cell lysates from neutrophils, murine bone marrow-derived macrophages, and fibroblasts. As observed in Fig. 3A, neutrophil whole cell lysates, when probed with anti-c-Raf-1 antiserum, revealed the presence of a 33-kDa band, believed to represent an immunoreactive fragment of the 72-kDa c-Raf-1 kinase that has been degraded in human neutrophils. No c-Raf-1 was identified in the MEKK immunoprecipitates. However, in order to ascertain that the immunoprecipitated form of c-Raf-1 can actually be
detected with anti-c-Raf-1, we undertook a further step in which neutrophil lysates were immunoprecipitated with either anti-c-Raf-1 or anti-MEKK (43-Y) and then probed for the presence of c-Raf-1 in both cases. As shown in Fig. 3B, a band at 33 kDa, corresponding to the same band seen in neutrophil whole cell lysates in Fig. 3A, is observed in the anti-c-Raf-1 immunoprecipitates but not in the anti-MEKK immunoprecipitates, thus validating the use of this antibody to probe for and identify c-Raf-1 species in MEKK immunoprecipitates.

In the second set of experiments 80-kDa MEKK1-(1–301) was utilized as substrate for immunoprecipitated MEKK (43-Y) and c-Raf-1. It has been shown previously that MEKK phosphorylates the NH₂ terminus of recombinant wild type 80-kDa MEKK1, whereas c-Raf-1 is unable to phosphorylate this fragment (23). Hence, we compared the in vitro kinase phosphorylation of the 80-kDa MEKK1-(1–301) and MEK1kd by immunoprecipitated MEKK or c-Raf-1. As shown in Fig. 3C, immunoprecipitation of MEKK from FMLP-stimulated neutrophils led to the generation of activity that, when quantified, showed both 80-kDa MEKK1-(1–301) and MEK1kd to be phosphorylated to a similar extent. In contrast, c-Raf-1 phosphorylation of MEKkd was at least 400-fold greater than that of the NH₂ terminus. These results indicate that immunoprecipitated MEKK has properties fundamentally different from c-Raf-1.

Substrate Specificity of MEKK—To further determine whether one of the MEK species (MEK1 or MEK2) was a preferred substrate for active MEKK, MEKK was immunoprecipitated, using anti-MEKK (C-22) from unstimulated and FMLP-stimulated neutrophils. These immunoprecipitates were then subjected to in vitro kinase assays utilizing as substrate either 80-kDa MEKK1-(1–301) or MEK1kd, subjected to in vitro kinase assays utilizing as substrate either 80-kDa MEKK1-(1–301) or MEK1kd, separated by SDS-PAGE, and quantified by autoradiography. B, buffer (control); F, FMLP.

MEKK Activation by FMLP Is Partially Pertussis Toxin-sensitive—A variety of studies link ligand interaction with the FMLP receptor to activation of phospholipases by mechanisms that are sensitive to pertussis toxin and hence ascribed to involvement of G₁₂ (34). We have previously shown that FMLP induces G₁₂-dependent activation of Ras and Raf in neutrophils...
and thus questioned whether MEKK activation was similarly sensitive to pertussis toxin. FMLP-stimulated MEKK activation was determined on nonpretreated and pertussis toxin-pretreated (500 ng/ml, 2.5 h) neutrophils, using a MEKK → EGFR-662–681 coupled in vitro kinase assay. As seen in Fig. 5, pertussis toxin pretreatment inhibited FMLP-stimulated MEKK activation by 50% when MEK1wt/MAPKp/C/EGFR-662–681 was used as the kinase substrate and 80% with the combination MEK2wt/MAPKp/C/EGFR-662–681, indicating a pertussis-sensitive component (likely G12) on the pathway to MEKK. However, as shown, the MEKK activity was only partially inhibited by pertussis toxin, suggesting that an additional pathway upstream of MEKK may also involve pertussis-insensitive components.

Wortmannin, an Inhibitor of PI 3-Kinase, Inhibits Activation of Both Raf and MEKK—PI 3-kinase, a member of the phosphatidylinositol kinase family, has 85-kDa regulatory and 110-kDa catalytic subunits and has been found in association with activated tyrosine kinases (35). FMLP constitutes one of the most effective and rapid stimuli to PI 3-kinase activation (7). Since wortmannin has been suggested to inhibit MAP kinase activation in guinea pig neutrophils (36) and evidence for PI 3-kinase in Gβγ-associated MAPK pathways has been presented (37), we wished to determine the role of PI 3-kinase on MAP kinase activation and that of its upstream kinase activators in human neutrophils. A five-phase comparison was performed in which we determined wortmannin effects on the activation of c-Raf-1, MEKK, MEK1, MEK2, and MAP kinase. Neutrophils were pretreated for 15 min with varying doses of wortmannin prior to FMLP stimulation. Lysates were immunoprecipitated with anti-MEK1, anti-MEK2, anti-MEKK (43Y), or anti-c-Raf-1 and then subjected to in vitro kinase assays in the presence of the pertinent substrate for each immunoprecipitated kinase. MAP kinase activation was evaluated by Mono Q ion exchange chromatography, where column fractions were subjected to in vitro kinase reactions using the EGFR-662–681 as MAP kinase substrate. As shown in composite (Fig. 6), wortmannin causes a dose-dependent inhibition of activity in each of the kinases tested. A detailed analysis reveals that activation of MEKK (Fig. 6A) and c-Raf-1 (Fig. 6B) both exhibit between 30% and 50% inhibition at 25 nM and 50 nM wortmannin; in contrast, at these concentrations of wortmannin there is less than 30% inhibition of MEK1 and MEK2 (data not shown) and less than 20% inhibition of MAP kinase activity (Fig. 6C). 100 nM wortmannin inhibited MEKK and RAF by approximately 75% while inhibiting MEK (data not shown) and MAP kinase activity by about 50% (Fig. 6D).

The specificity of wortmannin effects on MAP kinase activation was determined using a stimulus (PMA) that does not act through G-proteins. It has been shown in transiently transfected COS-7 cells that PMA activation of MAPK was unaffected by wortmannin (37). The effect of wortmannin on PMA-stimulated activation of Raf and MAP kinase is demonstrated in Fig. 7. We determined that in contrast to FMLP-stimulated activation of these kinases, phosphorylations of MEKKp/C by immunoprecipitated Raf kinase (Fig. 7A) and EGFR-662–681 by immunoprecipitated MAP kinase (Fig. 7B) were both unaffected by exposure of neutrophils to wortmannin prior to PMA stimulation. Similar results were obtained with PMA-stimulated activation of MEK1 and MEK2 (data not shown).

Role of PKC in Modulation of MEKK Activation—Evidence that more than one effector pathway may lead to Ras, Raf, and MEK activation may also be provided by examining the effects of another effector kinase, PKC. Phorbol ester activation of PKC leads to Ras activation in neutrophils (data not shown). PKC may also directly phosphorylate and activate Raf (38). We have previously reported that the PMA-stimulated c-Raf-1 activation of MEKK in human neutrophils was comparable in magnitude with that of FMLP (9). Additionally, we have found in previous studies that although neutrophils exhibit PMA-induced Raf activation, FMLP-stimulated Raf activation was independent of PKC. Accordingly, it was important to determine the effects of the bisindolemaleimide GF 109203X, a specific PKC antagonist, on MEKK, MEK, and MAP kinase activation (Fig. 7) and, additionally, whether PMA stimulated MEKK and MEK activation. Neutrophils were pretreated with either 0.1% MeSO4 (control) or with the inhibitor GF 109203X (2 μM and/or 5 μM) for 30 min at 37°C and then stimulated in the presence of either 10−7 M FMLP or 10 ng/ml PMA. MEKK, MEK1, and MEK2 activities were determined by in vitro kinase assays following immunoprecipitation, while MAP kinase activity was assessed by FPLC (on Mono Q) using an in vitro kinase assay on column fractions. PMA stimulation provided a positive control for PKC activation.

Fig. 8A depicts MEKK activity in FMLP- and PMA-stimulated neutrophils, in the presence and absence of GF 10923X. We observed that pretreatment of neutrophils with PKC inhibitor GF 10923X did not inhibit MEKK activation in FMLP-stimulated MEKK immunoprecipitates. In fact, we noted increased basal and FMLP-stimulated MEKK activity in the presence of GF 10923X, suggesting that PKC may exert complex effects on MEKK activation. In contrast, (Fig. 8B) 2 μM GF 109203X inhibited MEK1 activity by 16% and 5 μM GF 109203X inhibited it by 50%. MEK2 activity was inhibited by 56% with 2 μM GF 109203X and by 60% with 5 μM GF 109203X. We observed PMA-stimulated activation of both MEK1 and MEK2.

FMLP-stimulated MAP kinase activation (Fig. 8, D and E) was inhibited by 20% in the presence of 2 μM GF 109203X. In contrast, inhibition of PMA-stimulated MAP kinase activation by the same concentration of GF 109203X exceeded 60%. 5 μM GF 109203X inhibited FMLP-stimulated MAP kinase activation by close to 50% (data not shown).

PD98059 Inhibition of MEK and MAP Kinase Activity and Superoxide Release—Recent evidence for the involvement of MAP kinase pathways in growth control has been observed in numerous cell systems. Hence, the MEK inhibitor, PD98059, was utilized in order to determine whether inhibition of the activation of a constituent of this pathway could influence neutrophil functions, thus ascribing biological significance to those pathways culminating in MEK-induced MAP kinase ac-
tivation in the neutrophil. Fig. 9 depicts the effect of PD98059 on FMLP-stimulated neutrophil kinase activities (MEK1 and MEK2; ERK1 and ERK2) compared with the superoxide release, a critical neutrophil function. Although it is evident that neither MEK1 nor MEK2 activity (as determined by the phosphorylation of MAPKkd) was completely ablated by PD98059, pretreatment of neutrophils with 10 μM PD98059 inhibited MEK1 activity by 45% and MEK2 activity by 57%. Similarly, this concentration of PD98059 inhibited ERK1 activation by 68% and ERK2 activation by 61%. The FMLP-stimulated release of superoxide anion from cytochalasin B-treated neutrophils as determined by cytochrome c reduction was also measured in the presence of this MEK inhibitor. Pretreatment of neutrophils with 10 μM PD98059 inhibited superoxide production by 41%. Secretion of myeloperoxidase by FMLP-stimulated neutrophils, in contrast, was not inhibited by PD98059, indicating that this compound is not a nonspecific inhibitor of neutrophil responses.

DISCUSSION

Recent evidence suggests that control and integration of cellular function by extracellular signals in yeast may be attributed to multiple kinase cascades (11, 13). Recognition of homologous kinases in mammalian systems is proceeding quickly, due in part to the highly conserved nature of these kinases among fungal, yeast, and metazoan species (39). Whereas the genetic approach to determination of function of these kinase cascades in yeast has allowed glimpses of discrete kinase cascades, each responsible for a unique function, determination of such functions in mammalian kinase systems is at present much more difficult than in the more genetically manipulable yeast (12).

Nonetheless, yeasts may serve as models that help us to understand mammalian systems. In Saccharomyces cerevisiae, activation of the mating pathway by STMS pheromone receptors is accompanied by activation of a MEKK whose function is essential in downstream signaling to a MAP kinase homologue (13). MEKK-1 represents one of a family of emerging MEKKs in mammalian cells, and while this kinase has been shown to be regulated by tumor necrosis factor (24), epidermal growth factor, and nerve growth factor (25), there have been no examples of regulation of this kinase by a STMS G-protein-linked receptor in mammalian systems. In this paper, we present evidence for both pertussis toxin-sensitive and -insensitive regulation of MEKK by the FMLP receptor, which in human neutrophils is a prototypical STMS receptor that couples pre-
dominantly to the heterotrimeric G-protein Gi2 (34). Our data suggest that in human neutrophils both MEK1 and MEK2 are activated by MEKK and that PI3-kinase may modulate MEKK activation and that of its two downstream components (MEK and MAPK). Furthermore, MAPK activation may also occur through a separate wortmannin-insensitive PKC-mediated pathway.

Again by analogy to yeast, MEKK-1 is expected to represent a family of MEKKs serving potentially different functional roles. The diversity and expression of MEKKs, however, remains incompletely understood. For instance, yeast cells expressing 80-kDa MEKK reveal a predominant band of 110 kDa (22) similar to that observed in neutrophils and macrophages. Rodent cell lines, by contrast, appear to express 98- and 82-kDa MEKK bands (23) The predominant band seen after immunoprecipitation of MEKK is, however, the 54-kDa band, suggesting that this species, probably a proteolytic product, may represent an active form of MEKK1. Further work will be required to define the full array of MEKK activities in the neutrophil, as the absence of the higher molecular weight forms in the immunoprecipitates makes assessment of their relative activities difficult.

Several lines of evidence suggest that the MEKK activity measured here represents an activity distinct from that of the Raf family. In the series of experiments performed, the antibodies we used for immunoprecipitation were directed against the NH2-terminal putative regulatory domain of MEKK, a region with no homology to corresponding domains in Raf family members (40, 41). Anti-c-Raf-1 immunoblots revealed an immunoreactive band both in c-Raf-1 immunoprecipitates and neutrophil lysates that ran at 33 kDa. No detectable c-Raf-1 was immunoprecipitated by anti-MEKK antibodies, and the MEKK immunoprecipitates, but not the c-Raf-1 immunoprecipitates, were able to phosphorylate the NH2-terminal fragment of MEKK. Further work will be required to define the full array of MEKK activities in the neutrophil, as the absence of the higher molecular weight forms in the immunoprecipitates makes assessment of their relative activities difficult.

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Fig. 10. Proposed scheme for activation of MAP kinase cascades in response to chemotactic agents in the human neutrophil.

was MEKK.

The complexity of determining which pathways culminate in MAP kinase is further evident when considering the inhibitory role of wortmannin, a fungal metabolite, which potently inhibits PI 3-kinase (42). Recent studies indicate that the Ras family member Rac is an effector of PI 3-kinase in platelet-derived growth factor signaling (43), and GTP-bound forms of Rac1 and Cdc42Hs have been shown to bind to p85 and elicit stimulation of PI 3-kinase activity (44). Similarly, Ras, which is linked to activation of both Raf and MEKK, interacts with the catalytic unit of PI 3-kinase (45). Wortmannin inhibition of Ras activation has been recently described in COS-7 and CHO cells transiently transfected with Gβγ (37). From our studies in the FMLP-stimulated neutrophil (Fig. 6) it appears that certain components of this MAPK pathway are wortmannin-sensitive, indicating possible PI 3-kinase modulation upstream of MEKK and Raf (37). The potential relationships between these pathways are shown in the scheme in Fig. 10. However, we show that wortmannin is a more potent inhibitor at the level of the Ras-dependent kinases, Raf and MEKK, and less active on MEK and MAP kinase, indicating that perhaps additional factors or pathways, leading from serpineptide receptors, may converge on MEK and thus modulate PI 3-kinase input, via Raf and MEKK, on MAP kinase activation.

While PI 3-kinase may regulate pathways leading to MAPK activation, it has also been implicated in the activation of neutrophil cellular responses (46–48), especially FMLP-stimulated secretion of O2·−. Wortmannin, however, may act proximal at the level of PI 3-kinase, whose activity is linked to Rac, itself implicated in the activation of the NADPH oxidase (49), making interpretation of the wortmannin data complex.

Accordingly, we have also used the recently described MEK inhibitor, PD98059 (50, 51) to determine the functional significance of MAP kinase activation in neutrophils. Low concentrations of PD98059 inhibited FMLP-stimulated MEK activities as well as both p42/ERK1 and p44/ERK2 and inhibited superoxide anion production by comparable amounts. In contrast, this inhibitor failed to inhibit either myeloperoxidase secretion or the FMLP-induced Ca2+ transient (data not shown), indicating that the effect of PD98059 is not due to nonspecific inhibitory effects. Thus, while the data using wortmannin and PD98059 indicate potential links between MAP kinase activation pathways and optimal activation of the NADPH oxidase, further work will be required to unequivocally determine the functional consequences of MAP kinase activation.

Activation of neither Raf nor MEKK is inhibited by GF 109203X, a PKC antagonist, although activation of both MEK and MAPK is partially inhibited by this antagonist. These data suggest the possibility of yet unknown inputs from PKC to MEK1 (and hence MAPK) upon FMLP stimulation in the neutrophil. Inhibition of PKC in unstimulated neutrophils results in apparent stimulation of MEKK activity. Hence, regulation of MEKK by PKC may reveal both positive and negative effects, and considerable further work will be necessary to determine whether this occurs and the mechanisms of its regulation. Additionally, since PMA and other PKC agonists (and their activation of MAP kinase pathway components) are insensitive to inhibition by wortmannin, to the extent that wortmannin effects are solely due to the inhibition of PI 3-kinase, these data suggest that direct activation by PKC may bypass a requirement for PI 3-kinase activation.

In summary, the ligand (FMLP) for a seven-transmembrane-spanning, G-protein linked receptor in primary human neutrophils induces activation of MEKK. Activation is partially sensitive to both pertussis toxin (indicating linkage to Gαi) and wortmannin (indicating a role for PI 3-kinase) and leads to activation of both MEK1 and MEK2. The data presented, taken together with previous studies (24, 25), suggest more than one route from chemotactic receptors to activation of MAP kinase in the neutrophil. It is apparent that the regulation of these downstream events is both complex and exciting, and deciphering the mechanisms by which pathways to MAPK activation are differentially and preferentially activated in FMLP-stimulated neutrophils warrants considerable further study.

Acknowledgment—We thank D. W. R. Riches for helpful discussions.

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