Characterization of the MEK5-ERK5 Module in Human Neutrophils and Its Relationship to ERK1/ERK2 in the Chemotactic Response*

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The role of the extracellular signal-regulated kinase (ERK) 1 and ERK2 in the neutrophil chemotactic response remains to be identified since a previously used specific inhibitor of MEK1 and MEK2, PD98059, that was used to provide evidence for a role of ERK1 and ERK2 in regulating chemotaxis, has recently been reported to also inhibit MEK5. This issue is made more critical by our present finding that human neutrophils express mitogen-activated protein (MAP) kinase/ERK kinase (MEK)5 and ERK5 (Big MAP kinase), and that their activities were stimulated by the bacterial tripeptide, formyl methionyl-leucyl-phenylalanine (fMLP). Dose response studies demonstrated a bell-shaped profile of fMLP-stimulated MEK5 and ERK5 activation, but this was left-shifted when compared with the profile of fMLP-stimulated chemotaxis. Kinetics studies demonstrated increases in kinase activity within 2 min, peaking at 3–5 min, and MEK5 activation was more persistent than that of ERK5. There were some similarities as well as differences in the pattern of activation between fMLP-stimulated ERK1 and ERK2, and MEK5-ERK5 activation. The up-regulation of MEK5-ERK5 activities was dependent on phosphatidylinositol 3-kinase. Studies with the recently described specific MEK inhibitor, PD184352, at concentrations that inhibited ERK1 and ERK2 but not ERK5 activity demonstrate that the ERK1 and ERK2 modules were involved in regulating fMLP-stimulated chemotaxis and chemokinesis. Our data suggest that the MEK5-ERK5 module is likely to regulate neutrophil responses at very low chemoattractant concentrations whereas at higher concentrations, a shift to the ERK1/ERK2 and p38 modules is apparent.

The role of the MEK5-ERK5 module in human neutrophils and its relationship to ERK1/ERK2 in the chemotactic response remains to be identified since a previously used specific inhibitor of MEK1 and MEK2, PD98059, that was used to provide evidence for a role of ERK1 and ERK2 in regulating chemotaxis, has recently been reported to also inhibit MEK5. This issue is made more critical by our present finding that human neutrophils express mitogen-activated protein (MAP) kinase/ERK kinase (MEK)5 and ERK5 (Big MAP kinase), and that their activities were stimulated by the bacterial tripeptide, formyl methionyl-leucyl-phenylalanine (fMLP). Dose response studies demonstrated a bell-shaped profile of fMLP-stimulated MEK5 and ERK5 activation, but this was left-shifted when compared with the profile of fMLP-stimulated chemotaxis. Kinetics studies demonstrated increases in kinase activity within 2 min, peaking at 3–5 min, and MEK5 activation was more persistent than that of ERK5. There were some similarities as well as differences in the pattern of activation between fMLP-stimulated ERK1 and ERK2, and MEK5-ERK5 activation. The up-regulation of MEK5-ERK5 activities was dependent on phosphatidylinositol 3-kinase. Studies with the recently described specific MEK inhibitor, PD184352, at concentrations that inhibited ERK1 and ERK2 but not ERK5 activity demonstrate that the ERK1 and ERK2 modules were involved in regulating fMLP-stimulated chemotaxis and chemokinesis. Our data suggest that the MEK5-ERK5 module is likely to regulate neutrophil responses at very low chemoattractant concentrations whereas at higher concentrations, a shift to the ERK1/ERK2 and p38 modules is apparent.

Neutrophils, while playing an important role in host defense by killing microbial pathogens, are also responsible for tissue destruction in inflammatory conditions such as rheumatoid arthritis (1) and cystic fibrosis (2). A crucial initial step in this is the recruitment of neutrophils to sites of infection and inflammation by a process known as chemotaxis. A number of studies have reported that the extracellular signal-regulated protein kinase (ERK)1 and ERK2 modules are involved in regulating neutrophil chemotaxis (3–7). These studies showed that PD98059, a specific inhibitor of mitogen-activated protein (MAP) kinase/ERK kinase (MEK)1 and MEK2, the immediate upstream regulators of ERK1 and ERK2, inhibited chemotaxis in response to fMLP or IL8. However, PD98059 and another specific MEK1/MEK2 inhibitor, U0126, have recently been reported to also inhibit MEK5, the upstream regulator of ERK5 (8, 9). This therefore creates some doubt as to whether the previous conclusion is valid and whether the MEK5-ERK5 module could also be involved in regulating the chemotactic response.

ERK5 or Big MAP kinase (BMK) is a recently described stress-activated MAP kinase (10, 11). This kinase is similar in many aspects to ERK1 and ERK2, but has a unique loop 12 domain within the kinase region, which is followed by an unusually long C terminus. Although murine tissues have been reported to express three forms of ERK5 protein species, generated by alternative splicing (12), human tissues contain only one form of the protein, despite alternative splicing in the 5′-non-coding region (11). The activity of ERK5 is up-regulated through the phosphorylation of the TEY activation motif by MEK5. Alternative splicing at the 5′-end of MEK5 yields MEK5α and MEK5β and alternative splicing between the kinase subdomain IX-X region of each MEK5 species can potentially generate two further MEK5 species (13). Kinases such as MEK kinase (MEKK)2, MEKK3, tumor progression locus (Tpl)-2/cancer osaka thyroid (Cot) and mixed lineage kinase (MLK)-like MAP triple kinase have all been reported to serve as MAP kinase kinases of the ERK5 module (8, 10, 14). Recent studies have identified the kinase WNK (with no lysine) 1 to be an upstream regulator of the MEK5-ERK5 module, acting via MEKK2 and MEKK3 (15).

ERK5 is activated by stresses such as H2O2 and shear stress (8, 10, 16). However, unlike the p38 and JNK stress MAP kinases, ERK5 is not activated by UV irradiation or anisomycin (8). Depending on the cell type, the activity of ERK5 can be stimulated by agents such as serum, epidermal growth factor, 1,25 dihydroxyvitamin D3 and phorbol 12-myristate 13-acetate (8, 17). Substrates that have been identified for ERK5 include transcription factors such as Ets-1, MEF2C, and Sap1a (8, 17, 18). Recent studies have demonstrated that deletion of ERK5 results in angiogenic failure and death of the embryo (18). Both

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The abbreviations used are: ERK, extracellular signal-regulated protein kinase; MAP kinase, mitogen-activated protein kinase; MEK, MAP kinase/ERK kinase; BMK, Big MAP kinase; PI 3-kinase, phosphatidylinositol 3-kinase; MEKK, MEK kinase; Tpl, tumor progression locus; Cot, cancer osaka thyroid; MLK, mixed lineage kinase; WNK, with no lysine; fMLP, formyl methionyl-leucyl-phenylalanine.
MEK5 and ERK5 have sequences that suggest that these kinases may interact with cytoskeletal elements such as actin (13, 19).

The presence of MEK5 and ERK5 in neutrophils has not been described, and their roles in neutrophils remain unknown. The aims of this study were to characterize the expression and activation of MEK5 and ERK5 in human neutrophils, and to investigate whether this kinase module could regulate neutrophil migration. We have found for the first time that human neutrophils express MEK5 and ERK5 and the activity of the MEK-ERK5 module was stimulated by fMLP. Activation of the MEK-ERK5 module was blocked by wortmannin, an inhibitor of phosphatidylinositol 3-kinase. Investigations with inhibitors of phosphatidylinositol 3-kinase. Investigations with inhibitors of MEK-ERK5 module was stimulated by fMLP. Activation of MEK5 and ERK5 have sequences that suggest that these kinases are involved in regulating the chemotactic response.

**EXPERIMENTAL PROCEDURES**

**Materials—** Myelin basic protein, protein A-Sepharose, lucigenin, and fMLP were purchased from Sigma-Aldrich (St. Louis, MO). Myelin basic protein, protein A-Sepharose, lucigenin, and fMLP were purchased from Sigma-Aldrich (St. Louis, MO). Myelin basic protein, protein A-Sepharose, lucigenin, and fMLP were purchased from Sigma-Aldrich (St. Louis, MO). By Yan et al. (12) to amplify murine ERK5 were designed to the human ERK5 sequence. These were: sense primer ACAGGATCGAGCTCATGAGACG, and antisense primer GGTGATGAGATAGGATAGG. These are designed to amplify a 125-bp fragment (nucleotides 238–362) from the human ERK5 mRNA sequence (GenBank™ accession number HSU25278). The 5′ primer is located at nucleotide 537 and the 3′-primer at nucleotide 662 of the same intron (intron-exon junction at bp 16709 of GenBank accession number AC124066). These primers therefore define a PCR product of 774 bp (GenBank™ accession number AC124066, base 17723–16950) that can be amplified from human genomic DNA. This intron corresponds to the murine sequence reported as being alternatively spliced by Yan et al. (12). PCR amplification was performed in 50-μl reactions using 100 μl of each primer, and 3.5 units of Expand High Fidelity polymerase in a final 1× concentration of the recommended buffer plus 1× Q Buffer (Qiagen Pty. Ltd., Clifton Hill, Australia). Thirty cycles of amplification was performed with each cycle consisting of a denaturation of 94 °C for 30 s, annealing at 60 °C for 30 s and an extension of 68 °C for 30 s. PCR products were resolved on an agarose gel with pUC19/HpaII and Spel/EcoRI DNA markers (500 ng).

**Western Blot—** Proteins in the lysates were separated by 8 or 12% SDS-PAGE as appropriate and transferred to nitrocellulose membrane. Even transfer of proteins between the lanes was confirmed by staining SDS-PAGE gels with pUC19/HpaII and Spel/EcoRI DNA markers (500 ng).

**RNA Isolation and RT-PCR—** Total RNA was isolated from normal human monocytes and neutrophils using TRIzol® reagent as per the manufacturer’s instructions. cDNA was synthesized from total RNA using the manufacturer’s instructions. cDNA was synthesized from total RNA using the manufacturer’s instructions. cDNA was synthesized from total RNA using the manufacturer’s instructions. cDNA was synthesized from total RNA using the manufacturer’s instructions. cDNA was synthesized from total RNA using the manufacturer’s instructions. cDNA was synthesized from total RNA using the manufacturer’s instructions. cDNA was synthesized from total RNA using the manufacturer’s instructions. cDNA was synthesized from total RNA using the manufacturer’s instructions. cDNA was synthesized from total RNA using the manufacturer’s instructions. cDNA was synthesized from total RNA using the manufacturer’s instructions. cDNA was synthesized from total RNA using the manufacturer’s instructions. AX-bacterial cultures were used as a negative control for these reactions. PCR products were resolved on an agarose gel with pUC19/HpaII and Spel/EcoRI DNA markers (500 ng).

**Preparation of Cell Lysates—** The cells were lysed in buffer A (20 mm Hepes, pH 7.4, 0.5% Nonidet P-40 [v/v], 100 μm NaCl, 1 μm EDTA, 2 mm Na3VO4, 2 mm diithiothreitol, 1 mm phenylmethylsulfonyl fluoride, and 10 μg/ml each of leupeptin, aprotinin, pepstatin A, and benzamidine) for 2 h (4 °C) with constant mixing (24). Cell debris was sedimented (12,000 × g for 30 s), and the protein content of the soluble fraction was measured. Samples were stored at −20 °C for up to 2 weeks with no apparent loss of kinase activity being detected within this period.

**Kinase Activity Assay—** The assay was started by adding 30 μl of a 1% v/v solution of a 1% v/v solution of a 1% v/v solution of a 1% v/v solution of a 1% v/v solution of acid buffer (30% containing 10 μCi of (γ-32P)ATP, 3.8 μg p-nitrophenyl phosphate, and 15 μg of myelin basic protein. After 20 min, the assay was terminated by the addition of Laemmli buffer and boiling the samples for 5 min at 100 °C. Phosphorylated myelin basic protein was placed in a humidified atmosphere of 5% CO2 in air at 37 °C for 90 min. In this assay, neutrophils continuously migrate out of the wells in every direction, albeit with the majority of the migrating cells displaying directional migration toward the chemotactant-containing well (23). The leading neutrophils form a distinct migrating front. To quantify migration, we measured the distance between the edge of the central well and the migrating front moving toward either the FMLP (chemotaxis) or MeSO (random migration) containing well using an inverted Leitz microscope fitted with a grid eyepiece graticule. The plates were sealed and stored at 4 °C until photomicrographed.

**Kinase Activity Assay—** The assay was started by adding 30 μl of an antibody for the assay buffer (30% containing 10 μCi of (γ-32P)ATP, 3.8 μg p-nitrophenyl phosphate, and 15 μg of myelin basic protein. After 20 min, the assay was terminated by the addition of Laemmli buffer and boiling the samples for 5 min at 100 °C. Phosphorylated myelin basic protein was placed in a humidified atmosphere of 5% CO2 in air at 37 °C for 90 min. In this assay, neutrophils continuously migrate out of the wells in every direction, albeit with the majority of the migrating cells displaying directional migration toward the chemotactant-containing well (23). The leading neutrophils form a distinct migrating front. To quantify migration, we measured the distance between the edge of the central well and the migrating front moving toward either the FMLP (chemotaxis) or MeSO (random migration) containing well using an inverted Leitz microscope fitted with a grid eyepiece graticule. The plates were sealed and stored at 4 °C until photomicrographed.
the kinase activity in the ERK5 immunoprecipitates was un-
tected the dual phosphorylation of ERK1/ERK2, suggesting that
in SDS gels with an
we reported that ERK5 in COS-1 monkey kidney cells migrated
basic protein as a substrate. Consistent with the data in other
immunoprecipitates (Fig. 1). In contrast, H2O2 did not stimu-
results shown in
were determined as described under “Experimental Procedures.” The
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H2O2. Since H2O2 has
expression of ERK5 in neutrophils, we first subjected neutro-
migrates at 100 or higher (16, 28, 29). To characterize the
were obtained in three separate experiments.
were a representative digital radiogram from the
Statistical analyses were performed using Stu-
are a representative digital radiogram from the
expression level. Human placenta has previously been re-
genomic DNA in the neutrophil cDNA sample (Fig.
Some of the bands that had been ascribed to ERK5 degradation products
were generated by alternative splicing in the 5
as compared to the anti-ERK5 antibody. The faster migrating species are likely to be degra-
Expression of ERK5 and MEK5 in Neutrophils—
We next investigated the expression of MEK5 in neutrophils.
resolved by 16% SDS-polyacrylamide gel electrophoresis and was de-
tected and quantitated using an Instant Imager (Packard Instruments).

RESULTS

H2O2 Stimulated the Activity of ERK5—Since H2O2 has widely been reported to stimulate the activity of ERK5 (8, 16, 17), we first investigated whether H2O2 was able to stimulate the ERK5 module in neutrophils. Neutrophils were incubated with H2O2, lysed, and lysates were subjected to immunoprecipitation with an anti-ERK5 antibody that was raised against residues 790–803 in the C terminus of human ERK5 (Sigma-
Aldrich Corp.) and had previously been used for this purpose
(15, 26, 27). ERK5 kinase activity was assayed using myelin basic protein as a substrate. Consistent with the data in other cell-types, H2O2 stimulated the kinase activity in the ERK5 immunoprecipitates (Fig. 1). In contrast, H2O2 did not stimu-
lated the dual phosphorylation of ERK1/ERK2, suggesting that the kinase activity in the ERK5 immunoprecipitates was unlikely to have been caused by contaminating ERK1 or ERK2.

Expression of ERK5 and MEK5 in Neutrophils—Previously, we reported that ERK5 in COS-1 monkey kidney cells migrated in SDS gels with an Mᵦ of greater than the top Mᵦ marker of 113,000 (17). This is consistent with other reports that ERK5 migrates at 100 or higher (16, 28, 29). To characterize the expression of ERK5 in neutrophils, we first subjected neutrophil lysates to Western blotting. The anti-ERK5 antibody de-
tected a number of bands that migrated in SDS gels with Mᵦ of
−97,000, −90,000−82,000 and −45,000. On prolonged expo-
sure of the film, a faint −120-kDa band could also be seen in the neutrophils (Fig. 2a and also Fig. 3b). In contrast, when the
lysates were immunoprecipitated with the anti-ERK5 antibody prior to Western blotting, the blots showed predominantly 2
bands of Mᵦ of −97,000 and −120,000 (Fig. 2b). No bands could be seen if the immunoprecipitation was carried out with an anti-β-actin (Fig. 2c) or ERK2 (Fig. 2d) antibody. This implies that the −97- and −120-kDa bands were specifically brought down by the anti-ERK5 antibody.

When lysates from human peripheral blood monocytes, hu-
man embryonic kidney HEK293T cells, and HL60 cells (not shown) were subjected to Western blotting with the anti-ERK5 antibody, the blots showed the presence of predominantly the
−120-kDa band (Fig. 2e), although some monocyte samples also contained faster migrating bands (see Fig. 3b). These data imply that the −120-kDa band is ERK5. The presence of faster migrating anti-ERK5 immunoreactive bands in cell lysates is not unprecedented as others have previously reported that ERK5 blots sometimes contained multiple immunoreactive bands that had been ascribed to ERK5 degradation products (30). For comparison, lysates from HEK293T cells were also probed with an anti-ERK5 antibody from Santa Cruz Biotechnol-
ology. According to the manufacturer’s specification sheet, this antibody detects a −120-kDa ERK5 band. Akin to the antibody from Sigma-Aldrich, the Santa Cruz antibody also detected a −120-kDa band in HEK293T cells (Fig. 2e, right lane). Taken together, our data imply that the −120-kDa band is ERK5. The faster migrating species are likely to be degra-
dation products (30).

The expression of ERK5 in neutrophils was also investigated at the mRNA level. Human placenta has previously been re-
ported to contain three ERK5 transcripts (11). However, since these are generated by alternative splicing in the 5’-non-coding region, only one form of ERK5 is expressed at the protein level (11). In contrast, murine tissues have been reported to contain three isoforms of ERK5 protein, designated mERK5a, mERK5b and mERK5c, which are derived from differential splicing of the N terminus of mERK5 (12). The data in Fig. 3a demonstrate the presence of only one species of ERK5 mRNA in both peripheral blood monocytes and neutrophils (lanes 2 and 3, lower arrow). The presence of a PCR product corresponding to genomic DNA in the neutrophil cDNA sample (lane 3, upper arrow) demonstrates that the PCR would be capable of detecting larger, alternatively spliced, mRNA species analogous to those found in mice, if they were present. In the absence of reverse transcriptase RNA templates (lanes 4 and 5), only the product corresponding to genomic DNA was seen (lane 7) indicating some contamination of the RNA with DNA. In the absence of template no product was detected (lane 6). Although it is not possible to exclude the possibility that contaminating mononuclear leukocytes could have contributed to the PCR product in the neutrophil preparations, template dilution ex-
periments with mononuclear leukocyte samples to 1/50 of the amount of RNA present in neutrophil samples, corresponding to the maximum level of mononuclear leukocyte contamination, suggest that this contamination would only have contributed to a proportion of the neutrophil PCR signal (not shown). More importantly and consistent with the data in Fig. 3a, when lysates from mononuclear leukocyte were Western-blotted at a protein load of 2 µg (Fig. 3b, lane 3) as opposed to 100 µg of neutrophil lysate protein (Fig. 3b, lane 1), no immunoreactive bands could be detected (see also kinase activity data in Fig. 5c). In contrast, the same mononuclear leukocyte fraction loaded at 100 µg displayed a strong band at −120-kDa band and a faster migrating band (lane 2). A very faint −120-kDa band in addition to the −97-kDa band could be detected in the neutrophil lysates. The above data imply that faint −120-kDa and −97-kDa bands seen in the neutrophils could not have come from contaminating mononuclear leukocytes.

We next investigated the expression of MEK5 in neutrophils.
MEK5α and MEK5β migrate in SDS gels with a Mr of ~50,000 and 40,000 respectively (13). To confirm that neutrophils express MEK5, we probed neutrophil lysates with an antibody that was raised against residues 59–74 of MEK5α. MEK5β lacks this sequence (13). Fig. 4a shows that neutrophil lysates contained an immunoreactive protein that migrated on SDS gels with an Mr of ~50,000. When the lysates were subjected to immunoprecipitation and Western blotting with the anti-

MEK5α antibody, a band of the expected Mr was also seen (Fig. 4b). Although interaction between MEK5 and ERK5 has been demonstrated in cells overexpressing epitope-tagged MEK5 and ERK5, such interaction has not been reported for the endogenous kinases. To investigate this, the above blot was stripped and probed with the anti-ERK5 antibody. MEK5 immunoprecipitates contained little or no ERK5 (Fig. 4c).

**Fig. 2. Expression of ERK5 protein in neutrophils, monocytes, and HEK293T cells.** Cells (3 × 10^7 for neutrophils and monocytes, 1 × 10^6 for kidney cells) were lysed, and aliquots containing equal amounts of proteins were subjected to either Western blotting (WB) or immunoprecipitation (IP) followed by Western blotting using the indicated antibodies. The anti-ERK5 antibody was from either Sigma Aldrich (a–e) or Santa Cruz (e). a, WB of neutrophil lysates after an overnight exposure; b, duplicate neutrophil lysates (1 mg of lyase protein each) immunoprecipitated and Western-blotted with the anti-ERK5 antibody; c, neutrophil lysates immunoprecipitated with a monoclonal anti-β-actin antibody and Western-blotted with the anti-ERK5 antibody; d, neutrophil lysates immunoprecipitated with a goat anti-ERK2 antibody and Western-blotted with the anti-ERK5 antibody; e, monocyte and HEK293T cell lysates Western-blotted with the Sigma or Santa Cruz (HEK, right lane) anti-ERK5 antibody. ERK5 migrated in SDS gels with a Mr of ~120,000. ERK5 immunoprecipitates from neutrophil samples contained predominantly the ~120- and ~97-kDa bands. When neutrophil lysates were subjected to immunoprecipitation with the anti-β-actin (c) or anti-ERK2 (d) antibody, no anti-ERK5 antibody reactive bands were detected. The blots (c and d) were stripped and reprobed with the anti-β-actin (c) or anti-ERK2 (d) antibody to show that each antibody recognized its cognate antigen (right panels).

**Fig. 3. PCR analysis of ERK5 mRNA expression in human neutrophils and monocytes and a comparison of ERK5 protein expression between the two cell-types.** RT-PCR analysis of total RNA from monocytes and neutrophils (a) was performed as described under “Experimental Procedures.” The arrows to the left of the figure indicate the hERK5 PCR products amplified from genomic DNA (774 bp) and cDNA (125 bp). Lane 1, Spl/EcoRI size markers; lane 2, monocyte cDNA template; lane 3, neutrophil cDNA template; lane 4, monocyte RNA template; lane 5, neutrophil RNA template; lane 6, no template control; lane 7, human genomic DNA template; lane 8, pUC/HpaII size markers. b, lysates from neutrophils (100 µg of protein) (lane 1) and mononuclear leukocytes (lane 2, 100 µg of protein; lane 3, 2 µg of protein) were Western-blotted with the anti-ERK5 antibody. The blot was overexposed to show the faint ~120-kDa ERK5 band in the neutrophil lysate. Right panel shows lane 2 from a shorter exposure. The results are representative of two separate experiments.

**Fig. 4. Expression of MEK5 and interaction between MEK5 and ERK5 in neutrophils.** Neutrophils (3 × 10^7) were lysed, and aliquots were either Western-blotted with an anti-MEK5 (a), anti-ERK2 (right lane, d and f) antibody, or subjected to immunoprecipitation with anti-MEK5 (b–d), anti-ERK5 (e and f) antibody or without an antibody (e) followed by Western blotting with the indicated antibody. The blot in b was stripped and reprobed with anti-ERK5 antibody (shown in c). The anti-MEK5 and anti-ERK5 antibodies were raised in rabbits whereas the anti-ERK2 antibody was raised in goat. MEK5α migrated in SDS gels with a Mr of ~50,000. The MEK5 antibody pulled-down MEK5α (b) but not ERK5 (e) or ERK1/ERK2 (d). MEK5α (e, right panel) but not ERK1/ERK2 (f, left panel) was pulled down by the anti-ERK5 antibody. Right panel in d and f confirms that the anti-ERK2 antibody was able to detect ERK1 and/or ERK2. The data are representative of two (c and d) or 3 (a, b, e, and f) individual experiments.
Fig. 5. Dose-dependent stimulation of MEK5, ERK5, ERK1/ERK2, and p38 activities, and chemotaxis by fMLP. Cells were incubated with fMLP for 3 (a–c), 1 (d and e), 5 (f), or 40 (g) minutes. Kinase activation (a–f) and chemotaxis (g) were then determined. MEK5 (a), ERK5 (b and c) and p38 (f) activities were assayed after immunoprecipitation as described under “Experimental Procedures.” Western blots of replicate samples of the immunoprecipitates demonstrated that similar amounts of the ~120-kDa band were present in each of the immunoprecipitates. A representative anti-ERK5 blot of immunoprecipitated ERK5 from unstimulated and fMLP-stimulated neutrophils is shown in c. fMLP caused a slight retardation in the electrophoretic mobility of the ~120-kDa band (c). To assay for ERK1 and ERK2 activation, the lysates were split so that one aliquot was subjected to Western blotting using a phosphospecific antibody (a, upper panel shows phosphorylated ERK1/ERK2, and lower panel shows protein loading) whereas the other aliquot was subjected to immunoprecipitation and followed by enzymatic assay (e). Chemotaxis (g) was investigated using 3-μm polycarbonate filter chambers and the number of neutrophils that had migrated across the membrane over a 40-min incubation period and collected at the bottom of the wells were harvested and counted. In c, activation of ERK5 by fMLP (10−4 M) was compared between equal numbers (3 × 107) of neutrophils and mononuclear leukocytes. Kinase activity was stimulated in neutrophils but not in mononuclear leukocytes, exclude the possibility that the ERK5 activity seen in the neutrophil samples was due to contaminating mononuclear leukocytes. Control cells received vehicle at an amount that was equivalent to that present at 10−6 M fMLP. Results shown are means ± S.E. of 3–9 experiments. Significance of difference between control and fMLP-stimulated cells (a, b, e–g): *p < 0.05; **p < 0.01; ***p < 0.005 (Student’s unpaired t test or Tukey-Kramer multiple comparisons test as appropriate).

Stimulation of the MEK5-ERK5 Module by fMLP—Previous studies have demonstrated that the MEK1/MEK2 inhibitor, PD908059, inhibited fMLP-stimulated neutrophil chemotaxis (3–7). As discussed above, this and the structurally unrelated MEK inhibitor, U0126, have now been found to also inhibit MEK5. Thus, it is imperative that we investigate whether some of the effects of the MEK inhibitors on neutrophil responses could be due to inhibition of the MEK5-ERK5 module (8, 15). We first compared the activation of the MEK-ERK5 and ERK1/ERK2 modules by fMLP over a concentration range of 10−10 to 10−6 M. The data in Fig. 5 demonstrate that incubation of neutrophils (3 × 107/sample) with fMLP resulted in enhanced MEK5 (a) and ERK5 (b) activities that reached their peaks at around 10−7 M. The threshold was at around 10−10 M. Kinase activities showed a downward trend as the concentration of fMLP was increased further. Any contribution from contaminating mononuclear leukocytes can be excluded on the basis that fMLP (10−9 M) did not significantly alter ERK5 activity in samples prepared from an equivalent number (3 × 107) of mononuclear leukocytes (Fig. 5c). This implies that the kinase activities seen in the immunoprecipitates (Fig. 5, a and b) could only have originated from neutrophils. fMLP also caused a retardation in the electrophoretic mobility of the ~120-kDa ERK5 band in neutrophils (Fig. 5c). Because the MEK5 immunoprecipitates contained little or no ERK5 and no ERK1/ERK2 or p38, the data in Fig 5a represent MEK5 activity even though MEK5 could contribute to the kinase activity in the ERK5 immunoprecipitates (but see also Fig. 6).

In contrast, fMLP-stimulated activation of ERK1 and ERK2 as assessed by Western blot followed a different profile (Fig. 5d). Whereas phosphorylation of ERK1/ERK2 was just detectable at 10−9 M, the degree of phosphorylation increased dose-dependently to 10−7 M, and this was maintained at 10−6 M fMLP. Since the phospho-ERK data only suggested activation, we also investigated ERK1 and ERK2 activation by immunoprecipitation followed by enzymatic assay. Surprisingly, ERK1/
ERK2 kinase activity (Fig. 5c) showed a totally different dose response profile from that revealed by the Western blots. Thus, an increase in the activity of ERK1/ERK2 was detectable at $10^{-10}$ M, and kinase activity reached a plateau at $10^{-6}$ M fMLP. Activation was maintained at this level as the concentration of fMLP was increased to $10^{-6}$ M but declined as the concentration of fMLP increased to $10^{-6}$ M. In these experiments, each lysate was split into two aliquots so that the two different types of assays could be conducted on the same lysates. The possibility that ERK5 or MEK5 could have contributed to the kinase activity in the ERK2 immunoprecipitates was excluded on the basis that neither ERK5 nor MEK5 could be detected in the ERK2 immunoprecipitates (data not shown). Furthermore, immunoprecipitation of ERK1/ERK2 under more stringent conditions in the presence of SDS (0.1%) and sodium deoxycholate (1%) in addition to Nonidet P-40 did not alter the dose response profile of the kinase activity assays (data not shown). Given that the Western blot analysis does not measure enzymatic activity, the data from the immunoprecipitated ERK1/ERK2 are likely to reflect more accurately the status of cellular ERK1/ERK2 activity.

Another MAP kinase that regulates neutrophil responses such as chemotaxis is p38 (31, 32). Since p38 is activated by fMLP, we also compared the dose-response characteristics of fMLP-stimulated p38 activation with that of the MEK5-ERK5 module. The data in Fig. 5g demonstrate that while $10^{-9}$ M fMLP was ineffective at stimulating p38 activity, higher concentrations caused a 2-fold increase in kinase activity which peaked at $10^{-7}$ M and decreased at $10^{-6}$ M.

Because the above dose response curves all showed a bell-shaped profile that had been reported for fMLP-stimulated chemotaxis (31), it is important to also establish a dose response profile of fMLP-stimulated chemotaxis under our conditions to permit a direct comparison between the profiles using the same batch of fMLP. The data in Fig. 5g, obtained using the chamber assay, demonstrate that the profile for fMLP-stimulated chemotaxis most closely resembled that for p38, followed by ERK1/ERK2 and least by the MEK5-ERK5 module.

The kinetics of fMLP-stimulated MEK5 and ERK5 activation was investigated next. At 2 min, MEK5 activity (Fig. 6a) increased 2.2-fold whereas ERK5 activity (Fig. 6b) increased 1.9-fold. The increase in ERK5 activity was transient, reaching a peak at around 5 min after stimulation, whereas the increase in MEK5 activity reached a maximum between 2 and 3 min and persisted for the duration of the experiment. This difference suggests that MEK5 might probably only have contributed to a minor degree to the overall kinase activity seen in the ERK5 immunoprecipitates and that MEK5 and ERK5 have different inactivation mechanisms. For example, phosphorylation of MEK5 by ERK5 following the prior activation of ERK5 by MEK5 (30, 33) could contribute to the difference in kinetics. In contrast, fMLP-stimulated ERK2 activation reached a peak at around 1 min and declined thereafter (Fig. 6c).

The above data demonstrate that fMLP stimulated the activity of the MEK5-ERK5 module. Since the interaction between MEK5 and ERK5 (Fig. 4, c–f) were investigated in resting neutrophils, we also investigated whether activation affected the degree of interaction between the kinases. Neutrophils were incubated with fMLP ($10^{-9}$ M) for 3 min, and the lysates were subjected to immunoprecipitation and Western blot analyses as described in Fig. 4. The data (not shown) demonstrate that fMLP did not affect the degree or pattern of interaction between MEK5 and ERK5 or cause ERK1 and ERK2 to interact with ERK5 or MEK5.

Mechanism of fMLP-stimulated Activation of the MEK5-ERK5 Module: Role of Phosphatidylinositol 3-Kinase—Although H$_2$O$_2$ stimulated the activity of ERK5 in neutrophils, fMLP-stimulated activation of ERK5 did not require H$_2$O$_2$ because fMLP generally has little or no effect on the NADPH oxidase at $10^{-10}$ and $10^{-9}$ M. We previously reported that p21ras regulate the activation of ERK5 (17) and recently, p21ras was reported to also regulate the activity of PI 3-kinase (34). Since fMLP is known to stimulate p21ras (36), and fMLP-stimulated p38 activation is dependent on PI 3-kinase (37), it is possible that PI 3-kinase may lie upstream of the MEK5-ERK5 module. Although it is customary to investigate the effect of PI 3-kinase by using wortmannin and LY294002, two PI 3-kinase inhibitors that act via different mechanisms (38, 39), we focused primarily on wortmannin. This was because LY294002 per se had been reported to stimulate the activity of p38 in human neutrophils without affecting fMLP-stimulated p38 activity, thereby making data interpretation difficult (37). In contrast, wortmannin, while having no effect on the activity of p38 in the absence of fMLP, partially blocked fMLP-stimulated p38 activity (37).

The data in Fig. 7 demonstrate that the ability of fMLP to stimulate MEK5 (a) and ERK5 (b) activities was dose-depend-
concentrations (40), studies with a recently described specific MEK inhibitor, PD184352, have demonstrated that this agent is able to distinguish between the MEK5-ERK5 and MEK1/MEK2-ERK1/ERK2 modules if used at 0.5 \( \mu M \).

\section*{Concentration-dependent Inhibitory Effects of PD184352 on fMLP-stimulated ERK5 and ERK1/2 Activity—Whereas PD98059 inhibits MEK1/MEK2 and MEK5 at similar concentrations (40), studies with a recently described specific MEK inhibitor, PD184352, have demonstrated that this agent is able to distinguish between MEK5 and MEK1/MEK2 (40, 41). Thus, whereas PD184352 totally inhibited the activation of ERK1 and ERK2 at around 0.5–1 \( \mu M \) (40, 41), in fMLP-stimulated cells: *, \( p < 0.05 \); **, \( p < 0.01 \) (Tukey-Kramer multiple comparisons test).

\section*{Inhibition of fMLP-stimulated Chemotaxis by PD184352—}

The above data imply that PD184352 could be used as an agent to define the roles ERK1 and ERK2 play in fMLP-stimulated chemotaxis. We first investigated the effect of PD184352 on neutrophil migration using the chamber method. The data in Fig. 9a demonstrate that PD184352 inhibited chemotaxis in a dose-dependent manner. At 0.5 \( \mu M \), the chemotactic response was inhibited by \(-25\%\). This result implies that the ability of fMLP to stimulate neutrophil migration was partially inhibited as a consequence of ERK1 and ERK2 inhibition and hence strengthens the conclusion that ERK1 and ERK2 are involved, at least in part, in regulating the chemotactic response. At 2 \( \mu M \), chemotaxis was inhibited by \(-40\%\) and at 10 \( \mu M \), the inhibitor caused a near total inhibition of neutrophil migration (Fig. 9a). However, data interpretation becomes difficult because 2 \( \mu M \) PD184352 also caused a partial inhibition of ERK5 activity, in conjunction with a near total inhibition of the activity of ERK1/ERK2 (Fig. 8). At 10 \( \mu M \) PD184352, it was likely that the activities of ERK1/ERK2 and ERK5 were totally inhibited (40).

While the above data demonstrate that PD184352 reduced the number of neutrophils that were capable of migrating across
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increases in the fMLP concentration and declined when higher concentrations were attained, especially at 10^{-6} \text{M}. They differed in that the MEK5 and ERK5 profiles were left-shifted, the ERK1/ERK2 profile was more normally distributed whereas the p38 profile was right-shifted. Human neutrophils express two classes of fMLP receptors, FPR and FPRL1 that represent the high and low affinity fMLP receptors, respectively (43). In neutrophils, FPR has a K_d for fMLP of around 10^{-9} \text{M} whereas the K_d of FPRL1 is several hundred-fold higher (43). It remains to be determined whether the dose response profiles reflect differential use of the two classes of fMLP receptors.

Previous studies have found that fMLP-stimulated p38 and ERK1/ERK2 activation in neutrophils required PI 3-kinase (37, 42). The data in the present study demonstrate that this dependence on PI 3-kinase can be extended to fMLP-stimulated activation of the MEK5-ERK5 module. Thus, the ability of fMLP to stimulate MEK5 and ERK5 activities were dose-dependedly inhibited by wortmannin. A relationship between PI 3-kinase (37, 42) and present data than the anti-phospho-ERK blots. Consistent with this suggestion, the dose-response profile of ERK1/ERK2 activity more closely reflected that of fMLP-stimulated chemotaxis. Both reached their high points at 10^{-8} \text{M} and declined at 10^{-6} \text{M} fMLP whereas the level of phosphorylated ERK1 and ERK2 showed very poor or no correlation with the chemotactic response.

Based on our results, we propose that there is a hierarchy in the utilization of MAP kinase modules by chemoattractants such as fMLP which also stimulates neutrophil responses pertinent to microbial killing. At low chemoattractant concentrations, MAP kinase activation is predominantly restricted to the MEK5-ERK5 and ERK1/ERK2 modules. As the concentration of the chemoattractant increases, there is a gradual shift from MEK5-ERK5 activation to p38 activation. Thus, the activity of the MEK5-ERK5 module declines as the activity of the p38 module is up-regulated by the chemoattractant. Further increases in the chemoattractant concentration eventually cause the ERK1, ERK2 and p38 to also decline. The MAP kinase modules therefore provide the neutrophils with one family of signaling pathways that cover a range of chemoattractant concentrations.

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