Chemotactic Peptide N-formyl-Met-Leu-Phe Activation of p38 Mitogen-activated Protein Kinase (MAPK) and MAPK-activated Protein Kinase-2 in Human Neutrophils*

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Activation of polymorphonuclear leukocytes (PMN) by chemotactic peptides initiates a series of functional responses that serve to eliminate pathogens. The intermediate steps that link engagement of the chemoattractant receptor to the microbicidal responses involve protein kinases that have yet to be identified. In this study we detected in human PMN the presence of p38 mitogen-activated protein kinase (MAPK), which became rapidly tyrosine phosphorylated and activated in response to the chemotactic peptide N-formyl-methionyl-leucyl-phenylalanine (fMLP). Pretreatment of PMN with Wortmannin, a phosphatidylinositol 3-kinase inhibitor, or bis-indolylmaleimide, a protein kinase C antagonist, resulted in partial inhibition of p38 phosphorylation upon fMLP stimulation. Similarly, phosphorylation of p38 was only partially inhibited when the fMLP-induced cytosolic calcium transient was prevented. Stimulation of PMN by the chemoattractant also resulted in the rapid phosphorylation and activation of MAPK-activated protein kinase-2 (MAPKAPK-2), which was completely inhibited by the specific p38 inhibitor, SB203580. The physical interaction of p38 with MAPKAPK-2 was studied by coimmunoprecipitation. These two kinases were found to be associated in unstimulated PMN but dissociated upon activation of the cells by fMLP. Together these findings demonstrate the activation of p38 by chemotactic peptides in human PMN by a process involving phosphatidylinositol 3-kinase, protein kinase C, and calcium. p38, in turn, is an upstream activator of MAPKAPK-2.

Polymorphonuclear leukocytes (PMN) respond rapidly to invading microorganisms or tissue injury by activation of numerous effectors, including the generation of superoxide anions, secretion of lytic enzymes, and phagocytosis of particles. These responses serve to neutralize and destroy the invading pathogens (1). The recruitment of PMN to sites of bacterial infection and their subsequent activation are initiated by binding of chemoattractants to specific cell surface receptors, which are coupled to heterotrimeric G proteins (2). Receptor engagement triggers a complex cascade of biochemical events which culminates in the activation of the microbicidal responses. Many of these intervening steps have yet to be defined.

Increased phosphorylation of several proteins has been found to correlate with the stimulation of PMN effectors, thereby suggesting a causal role in the activation process (3–6). Additional support for a central role of phosphorylation was provided by the finding that pharmacological agents that interfere with protein kinases and phosphatases are also potent modulators of PMN responsiveness (7–10). Indeed, there is abundant evidence that protein kinase C isoforms are essential to the microbicidal response (11, 12), and activation of tyrosine phosphorylation seems to be equally important (7, 9). Studies in PMN stimulated with the chemotactic peptide N-formyl-methionyl-leucyl-phenylalanine (fMLP) led to the identification of Erk-1 and Erk-2 as major targets of tyrosine phosphorylation (13–15). These Ser/Thr kinases are members of a family of mitogen-activated protein kinases (MAPK) which are characterized by dual phosphorylation on tyrosine and threonine residues during activation. Other members of the MAPK family include p38 (16, 17) and JNK (18–20), which can be activated by physico-chemical stress, lipopolysaccharides, and by some cytokines (21). These kinases were identified more recently, and their presence and regulation in PMN have not been explored.

p38 was originally cloned from Saccharomyces cerevisiae (termed HOG1) (22), but homologs were subsequently found in Xenopus (mpk2) (23), murine (p38) (16), and human tissues (CSBP1 and CSBP2) (17, 24). In view of its conservation and wide distribution, it would appear that p38 serves an important function in cellular responses. Indeed, the development of an inhibitor of p38 has pointed to its involvement in the synthesis of interleukin-1 and tumor necrosis factor in monocytes (17), interleukin-8 in peripheral blood mononuclear cells (25), and in platelet aggregation and secretion (26). Phosphorylation of a downstream substrate is believed to mediate the aforementioned effects of p38, but the identity of these putative target(s) remains unclear. One possible effector may be MAPK-activating protein kinase-2 (MAPKAPK-2), a Ser/Thr kinase that positively activated kinase; RAFT, rapamycin and FKBP12 target.
senses the ability to phosphorylate the small heat shock protein HSP27 (23, 27). HSP27 has, in turn, been suggested to modulate actin microfilament dynamics and cellular thermoresistance (28, 29).

In the current study we investigated whether p38 and/or MAPKAPK-2 is involved in the signaling process of human PMN activated by FMLP. Using immunoprecipitation, fractionation, and in vitro kinase assays we found that these enzymes are present as an inactive complex in unstimulated cells. Chemotactic stimulation promoted the activation and dissociation of p38 and MAPKAPK-2 by a pathway involving phosphatidylinositol 3-kinase (PI 3-kinase) and protein kinase C (PKC).

**EXPERIMENTAL PROCEDURES**

**Materials—**FMLP, phorbol 12-myristate 13-acetate (PMA), and thapsigargin were purchased from Sigma. The acetoxyethyl ester of BAPTA was from Molecular Probes. Ficoll-Paque, dextran T-500, protein A-Sepharose, and Mono Q were from Pharmacia Biotech (Québec, Canada). Bis-indolylmaleimide or GF 109203X (BIM) was from Biosearch Technologies, Inc. (Novato, CA). Glutathione S-transferase-tagged ATF-2 was obtained for Santa Cruz Biotechnologies (Santa Cruz, CA), and recombinant human HSP27 was from StressGen (Victoria, BC, Canada). Monoclonal phosphotyrosine antibody (clone 4G10) was purchased from Upstate Biotechnology Inc. (Lake Placid, NY). Rabbit polyclonal p38 antibody was kindly provided by Dr. Brent Zanke (Ontario Cancer Institute, Princess Margaret Hospital, Toronto, Canada). Rabbit polyclonal MAPKAPK-2 antibodies were raised against a peptide encoding the COOH-terminal domain (SRVLKEDK-PEWEDVKGC). The p38 inhibitor SB203580 was generously given by SmithKline Beecham (King of Prussia, PA).

**PMN Isolation—**Human PMN were isolated from blood freshly drawn by venipuncture from healthy donors essentially as described previously (30). Briefly, whole blood was sedimented on dextran T-500 to remove red cells, and the resulting supernatant was overlayed onto Ficoll-Paque cushions and centrifuged. Contaminating red cells in the pellet were removed by hypotonic lysis. The purified PMN were resuspended in HEPES-buffered (25 mM, pH 7.4) bicarbonate-free RPMI 1640 medium at 10⁶ cells/ml and stored at room temperature on a rotator until use. For immunoprecipitation and cell fractionation experiments, the cell suspension was treated with 2.5 mM diisopropyl fluorophosphate for 15 min at room temperature, sedimented, and resuspended in fresh HEPES-buffered RPMI 1640 before use. For the experiments, PMN were resuspended in HEPES-buffered saline (140 mM NaCl, 1 mM CaCl₂, 1 mM MgCl₂, 3 mM KCl, 10 mM glucose, and 10 mM HEPES, pH 7.4). Unless indicated otherwise, all experiments were performed at 37 °C.

**Ca²⁺ Depletion and Buffering—**Where indicated, intracellular Ca²⁺ stores were depleted by treating a PMN suspension with 100 nM thapsigargin for 5 min at 37 °C in Ca²⁺-free HEPES-buffered saline. Alternatively, intracellular Ca²⁺ was buffered by treating suspended PMN with 10 μM BAPTA-acetoxymethyl ester for 30 min at 37 °C in regular (Ca²⁺-containing) HEPES buffer. Following this incubation period, the cells were resuspended in nominally Ca²⁺-free HEPES-buffered saline and incubated an additional 1 min before stimulation with FMLP.

**Immunoprecipitation and Immunoblotting—**Following stimulation of PMN, the incubation was stopped by adding of 2 volumes of ice-cold HEPES-buffered saline and rapidly sedimenting the cells in a microcentrifuge. The cell pellet was solubilized in Nonidet P-40 buffer and the debris removed by centrifugation at 14,000 × g for 5 min. The supernatant (1 mg of protein) was fractionated on a Mono Q column (1-ml bed volume) equilibrated with buffer A (12.5 mM MOPS, pH 7.2, 12.5 mM β-glycerophosphate, 0.5 mM EDTA, 7.5 mM MgCl₂, and 1 mM diithiothreitol). The column was eluted with a 10-ml linear 0−0.8 M NaCl gradient in buffer A using the Pharmacia fast protein liquid chromatography system. Fractions (250 μl) were collected and assessed for HSP27 kinase activity and analyzed by SDS-PAGE and immunoblotting.

**RESULTS**

Detection of p38 in Human PMN and Activation by FMLP—

Immunoblotting of lysates of human PMN with a p38-specific polyclonal antibody revealed a single band of approximately 42 kDa (not shown). To determine whether treatment with FMLP induces activation of p38, PMN were stimulated for increasing lengths of time, and the kinase was immunoprecipitated. As shown in Fig. 1A, FMLP induced a time-dependent, transient tyrosine phosphorylation of p38. Phosphotyrosine precipitation was apparent at the earliest time analyzed, 0.5 min, at maximal level at 1 min, and returned to near basal levels between 5 and 10 min. Fig. 1B demonstrates that equal amounts of the MAPK were immunoprecipitated at all times. The phosphorylation of p38 was also demonstrated by immunoprecipitating tyrosine-phosphorylated proteins from PMN lysates. Blotting these immunoprecipitates with a p38-specific antibody also revealed a 42-kDa protein in stimulated, but not in resting (control) cells (Fig. 1C). We next determined whether the kinase activity of p38 was also stimulated by the chemotactic peptide. Only a marginal amount of ATF-2 phosphorylation was detected in p38 immunoprecipitates obtained from unstimulated PMN. By contrast, a sizable activity was consistently observed in precipitates from FMLP-stimulated cells (Fig. 1D). Together these results demonstrate the presence p38 in human PMN and the ability of FMLP to phosphorylate and activate this kinase.

Role of PI 3-kinase in p38 Activation—We next proceeded to investigate the signaling pathway leading to the activation of p38. Activation of PI 3-kinase, which catalyzes the formation of phosphatidylinositol 3,4,5-phosphate, is one of the earliest responses of PMN to chemoattractants (31). Although the activation of PI 3-kinase in human PMN has been associated with functional events (32−34), the downstream events have yet to be defined. We therefore considered the possibility that p38 may be a target of the activation of PI 3-kinase. We tested the
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Effect of Two Structurally Different Inhibitors of PI 3-Kinase on the Activation of p38 by fMLP. As shown in Fig. 2A, pretreatment of PMN for 10 min with 100 nM wortmannin resulted in a partial inhibition (60%) of the tyrosine phosphorylation of p38 in response to fMLP. Unexpectedly, a similar pretreatment of PMN with 100 μM LY294002, a second antagonist of PI 3-kinase, failed to inhibit the fMLP-induced phosphorylation of p38. In fact, pretreatment with this antagonist alone, in the absence of fMLP, induced a pronounced activation of p38, which was enhanced by the subsequent addition of the chemotactant (Fig. 2A). Identical conclusions were reached when the kinase activity of p38 was measured using ATF-2 as a substrate (Fig. 2B). To determine whether this effect of LY294002 was specific to p38, PMN were pretreated with either wortmannin or LY294002, and the phosphorylation of Erk-1 and -2 in response to fMLP was assessed using an antibody that recognizes only the phosphorylated form of these kinases. As shown in Fig. 2C, both wortmannin and LY294002 inhibited the phosphorylation of these MAPKs to a similar extent. Therefore, the stimulatory effect of LY294002 is unique to p38. Although the underlying mechanism remains obscure, it is clear that LY294002 should be used with caution when assessing the effects of PI 3-kinase in PMN.

Fig. 2D illustrates that, contrary to what was proposed earlier (13–15), Erk-1 and -2 are not the main ~40–42-kDa tyrosine-phosphorylated proteins in stimulated PMN. In this experiment, cells were pretreated with wortmannin or LY294002 and then stimulated with fMLP. Immunoblotting of whole cell lysates with a phosphotyrosine-specific antibody shows that the fMLP-induced tyrosine phosphorylation of the 42-kDa band was reduced by wortmannin but not by LY294002, which in fact increased phosphorylation even in the absence of the chemotactant. This profile parallels the effects of LY294002 on p38 and differs from its effects on Erk (cf. Fig. 2, A and C).

Role of PKC and Intracellular Calcium in p38 Activation—Several isoforms of PKC coexist in PMN and are known to be activated by chemotactic peptides. To evaluate whether PKC isoforms mediate the effect of fMLP on p38, we used a reasonably specific inhibitor, namely BIM (35). Pretreatment of PMN with 5 μM BIM for 10 min had little effect by itself but resulted in partial inhibition (65%) of the tyrosine phosphorylation of p38 induced by fMLP (Fig. 3A). To ensure that BIM was effectively inhibiting PKC, this kinase was stimulated directly with phorbol ester. Incubation of PMN with 100 nM PMA for 5 min induced a low tyrosine phosphorylation of p38. This PMA-induced tyrosine phosphorylation of p38 was fully inhibited by 5 μM BIM. These results suggest that the concentration of BIM used sufficed to inhibit PKC thoroughly. Because BIM inhibited p38 activation by fMLP only partially, and since maximally stimulatory doses of PMA yielded a response smaller than that elicited by the chemotactant, we conclude that the response to fMLP is only partially dependent on PKC.

The activation of PMN by fMLP triggers an increase in cytosolic Ca$^{2+}$ concentration ([Ca$^{2+}$]), which originates both from release of intracellular stores and from extracellular Ca$^{2+}$ influx. The role of the cation in the activation of p38 is investigated next, using two different strategies aimed at minimizing the elevation in [Ca$^{2+}$]. In the first series of experiments, PMN were stimulated with 100 nM fMLP in the absence of extracellular Ca$^{2+}$. As shown in Fig. 3B, tyrosine phosphorylation of p38 proceeded normally under these conditions. Because release from intracellular stores is the predominant component of the early response of [Ca$^{2+}$], to fMLP, omission of external Ca$^{2+}$ has comparatively small effects on this response. To analyze more effectively the role of [Ca$^{2+}$], experiments were undertaken using cells that had their internal stores depleted prior to addition of the chemotactant. The stores were depleted using thapsigargin, an inhibitor of the endo-

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**Fig. 1. Tyrosine phosphorylation and activation of p38 in fMLP-stimulated PMN.** Panel A, time course of p38 tyrosine phosphorylation induced by fMLP. PMN were stimulated with 100 nM fMLP for the indicated periods, then immediately lysed in 1% Nonidet P-40-containing buffer, and p38 was immunoprecipitated (IP). The immunoprecipitates were separated by SDS-PAGE, transferred to PVDF membranes, and blotted with a phosphotyrosine-specific monoclonal antibody. Results shown are representative of three separate experiments.

Panel B, the blot shown in panel A was stripped and reprobed using a p38-specific antibody to show the amounts of p38 precipitated. Panel C, immunoprecipitation of tyrosine-phosphorylated p38. PMN were stimulated with 100 nM fMLP for 1 min or its diluent (dimethyl sulfoxide, 0.2% v/v; control), then lysed in 1% Nonidet P-40-containing buffer. Tyrosine-phosphorylated proteins were immunoprecipitated from PMN lysates using a phosphotyrosine-specific monoclonal antibody, the resulting immunoprecipitates were subjected to SDS-PAGE, transferred to PVDF membranes, and blotted with a p38-specific antibody. Panel D, PMN were stimulated for 1 min with 100 nM fMLP or its diluent (control), and p38 was immunoprecipitated. The kinase activity of the immunoprecipitates was assayed using 3 μg of glutathione S-transferase-tagged (GST)-ATF-2 as a substrate in the presence of 5 μCi of γ-32P]ATP. Samples were then subjected to SDS-PAGE, and radioactivity was analyzed with a PhosphorImager. Results shown are representative of three separate experiments.
membrane Ca\(^{2+}\)-ATPase. Parallel experiments using indo-1 demonstrated that, following pretreatment with 100 nM thapsigargin for 5 min in Ca\(^{2+}\)-free medium, stimulation with fMLP failed to increase [Ca\(^{2+}\)] \(_{i}\), (not illustrated; see Ref. 36). Using a comparable protocol, the effect of Ca\(^{2+}\) depletion on the phosphorylation of p38 was assessed by immunoprecipitation and blotting. The pretreatment with thapsigargin reduced, but did not eliminate, the tyrosine phosphorylation of p38 (see Fig. 5). It is noteworthy that the transient elevation in [Ca\(^{2+}\)] \(_{i}\), which accompanies inhibition of the ATPase by thapsigargin in Ca\(^{2+}\)-free medium had no effect on p38 phosphorylation.

The role of [Ca\(^{2+}\)] \(_{i}\) was also analyzed in cells loaded with BAPTA, an effective Ca\(^{2+}\)-chelating agent. Under the conditions used, BAPTA virtually eliminates the [Ca\(^{2+}\)] \(_{i}\), transient elicited by fMLP, as measured with indo-1 (not shown). As in the case of thapsigargin, obliteration of the [Ca\(^{2+}\)] \(_{i}\), transient with BAPTA only partially inhibited (70%) the chemoattractant-induced tyrosine phosphorylation of p38. Together, these observations indicate that elevated [Ca\(^{2+}\)] \(_{i}\) is not sufficient, nor is it absolutely necessary, to activate p38.

**Activation of MAPKAPK-2 by fMLP in Human PMN—**p38 was proposed to be an upstream activator of MAPKAPK-2. This suggestion is based on the ability of p38 to reactivated dephosphorylated, inactive MAPKAPK-2 (37). Because p38 was found to be activated by fMLP, we next determined whether MAPKAP-2 is also present and activated by chemoattractants in PMN. A single highly immunoreactive band of 50 kDa was detected in PMN lysates by the MAPKAPK-2 antibody (Fig. 4). Upon stimulation with fMLP, a progressive decrease in the electrophoretic mobility of MAPKAPK-2 was noticed. The shift was detectable within 30 s, reached maximal levels around 1 min, and persisted for up to 10 min.

To define whether MAPKAPK-2 is indeed phosphorylated and activated upon fMLP stimulation of PMN, the kinase was immunoprecipitated from cells treated with or without the chemoattractant. The immune complexes were tested for kinase activity using HSP27 as a substrate (Fig. 5A). Stimulation of the cells with fMLP induced a marked increase in the ability of MAPKAPK-2 to phosphorylate HSP27. A second phosphorylated band of ~50 kDa was also noticeable; in fMLP-stimulated samples, which became clearly apparent upon longer exposure of the radiograms (Fig. 5B). Although fainter than the HSP27 band, the 50-kDa band was consistently observed and likely represents autophosphorylated MAPKAPK-2 (37).

Independent evidence that MAPKAPK-2 is activated by fMLP and is responsible for the HSP27 kinase activity in stimulated PMN was obtained by subjecting cell lysates to Mono Q chromatography. In chemoattractant-stimulated PMN, an
crease in HSP27 kinase activity was detected in fractions 21–23 (Fig. 5C). The collected fractions were also analyzed by SDS-PAGE, and the distribution of MAPKAPK-2 was immunoprecipitated as described under “Experimental Procedures.” The kinase activity of the immune complexes was assayed by incubation with 3 μCi of HSP27 and 5 μCi of [γ-32P]ATP. Following SDS-PAGE, phosphorylation of HSP27 was analyzed with a PhosphorImager. Panel B, the autoradiogram shown in panel A was exposed for a longer period of time to reveal the autophosphorylation of a 50-kDa band, likely MAPKAPK-2. For clarity, only the mid portion of the radiogram is illustrated. Panel C, PMN that were either unstimulated (open squares) or stimulated with 100 nM fMLP for 1 min (solid squares) or 2 min (triangles) were lysed in 1% Nonidet P-40 buffer. The soluble fraction was then fractionated using a Mono Q column. Fractions were collected and assayed for HSP27 kinase activity as described under “Experimental Procedures” (top panel). Collected fractions were also subjected to SDS-PAGE and blotted with a polyclonal MAPKAPK-2-specific antibody (bottom panel; the fraction numbers are indicated below the blot). Results shown are representative of three separate experiments.

Because p38 activity was affected differentially by wortmannin and LY294002, we used these agents to define whether this kinase or Erk is responsible for the phosphorylation and activation of MAPKAPK-2. As shown in Fig. 6A, pretreatment of PMN with wortmannin partially inhibited the electrophoretic mobility of MAPKAPK-2 induced by fMLP. Moreover, as observed for p38 in Fig. 3A, pretreatment with LY294002 alone induced the electrophoretic mobility shift of MAPKAPK-2. Subsequent stimulation with fMLP further potentiated the band shift. That the mobility shift was an indication of an active MAPKAPK-2 was confirmed with a kinase assay. Fig. 6B and C, shows that the autophosphorylation of MAPKAPK-2 and the phosphorylation of HSP27, respectively, were inhibited similarly by wortmannin and stimulated by LY294002, as observed previously for p38. These results indicate that the regulation of MAPKAPK-2 by PI 3-kinase closely resembles that of p38 and clearly differs from those of Erk-1 and -2.

That p38, and not Erk, is the upstream activator of MAPKAPK-2 was confirmed by pretreating PMN with a specific inhibitor of p38, namely SB203580 (17). Pretreatment of PMN for 20 min with 10 μM SB203580 completely eliminated the electrophoretic mobility shift of MAPKAPK-2 upon stimulation with fMLP (Fig. 7A). More direct evidence of the effect of SB203580 on the activity of MAPKAPK-2 was obtained by quantifying its autophosphorylation (Fig. 7B) and its ability to phosphorylate HSP27 in vitro (Fig. 7C). SB203580 consistently eliminated the activation of MAPKAPK-2 in PMN stimulated by fMLP.

Association of p38 with MAPKAPK-2 in Human PMN—Since the phosphorylation and activation of MAPKAPK-2 and p38 occur in parallel, we explored the possibility that these two kinases might physically associate in vivo. In the first series of experiments, MAPKAPK-2 was immunoprecipitated, and the presence of p38 in the immune complexes was assessed. The results of one such experiment are illustrated in Fig. 8A (left...
Stimulation of PMN by chemotactic peptides leads to the phosphorylation of several intracellular proteins that are believed to participate in the activation of the microbicidal function of these cells. In the present study we described the presence of p38 and MAPKAPK-2 in human PMN and their activation by fMLP, which was found to be rapid and transient. This result contrasts with the much slower activation of p38 induced by cytokines or environmental stresses, which has been found to peak after 15–30 min (21). Moreover, we provided evidence that these kinases are associated with each other in quiescent cells and that their dissociation is facilitated by chemotactic stimulation.

The kinase(s) directly responsible for the phosphorylation and activation of p38 in human PMN have yet to be determined. At least three dual specificity kinases, MKK3 (37), MKK4 (37, 38), and MKK6 (39), possess the ability to phosphorylate p38. However, their presence and role toward p38 activation in PMN remain to be defined. Recent studies have shown that p38 can be activated in cells transfected with constitutively active Rac or Cdc42 (40–42) possibly via a p21-activated kinase (PAK) (40). This notion stems from the demonstration that overexpression of a dominant-negative PAK prevented the activation of p38 induced by Rac or Cdc42. It is therefore attractive to suggest that one pathway leading to p38 activation by fMLP in PMN involves Rac or Cdc42, followed by PAK. Indeed, two isoforms of PAK have been detected in human PMN, where they can be activated by fMLP (43). Moreover, Knaus et al. (43) noted that PAK activation by fMLP includes a PI 3-kinase-dependent step, which may explain the inhibitory effect of wortmannin on p38 reported in the present study. It is clear, however, that an additional pathway(s) must contribute to the activation of p38, since only partial inhibition was obtained with a concentration of wortmannin known to block PI 3-kinase completely. That an additional pathway may involve PKC is suggested by the following observations. First, the activation of p38 was partially prevented by BIM, a PKC inhibitor. Second, PMA, a direct activator of PKC, also induced partial activation of p38. Finally, obliteration of the cytosolic Ca2+ transient triggered by fMLP resulted in a degree of inhibition comparable to that observed with BIM. It is thus tempting to speculate that Ca2+-sensitive isoforms of PKC, such as the α or β isoenzymes, may be involved in the activation of p38. These two conventional isoforms of PKC have, in fact, been detected in human PMN (44–46). That the PKC-dependent pathway of p38 activation is independent of PAK is suggested by the fact that PMA is unable to activate PAK in human PMN.

**FIG. 7.** Effect of SB203580 on the phosphorylation and activation of MAPKAPK-2 in fMLP-stimulated human PMN. PMN were stimulated with 100 nM fMLP or its diluent in the presence or absence of 10 μM SB203580 (SB). Panel A, whole cell lysates (106 cell equivalents) were subjected to SDS-PAGE, transferred to PVDF membranes, and blotted with a MAPKAPK-2-specific antibody. Panel B, stimulated cells were lysed, and MAPKAPK-2 was immunoprecipitated and incubated in the presence of 5 μCi of [γ-32P]ATP and 3 μg of HSP27. The region corresponding to the autophosphorylated form of MAPKAPK-2 is illustrated. Panel C, the phosphorylation of HSP27 is illustrated.

**FIG. 8.** Association of p38 with MAPKAPK-2 in human PMN. PMN were treated with or without 100 nM fMLP for 1 min, lysed, and used for immunoprecipitation of p38 or MAPKAPK-2 as described under “Experimental Procedures.” Panel A, immunoprecipitates of MAPKAPK-2 were subjected to SDS-PAGE, transferred to PVDF membranes, and blotted with a p38-specific antibody (left) or with a phosphotyrosine-specific antibody (right). Panel B, Left, the kinase activity of immunoprecipitates of MAPKAPK-2 was assayed using ATF-2 as a substrate. Right, the kinase activity of immunoprecipitates of p38 was assayed using HSP27 as a substrate. Results shown in panels A and B are representative of three separate experiments of each kind.
The ability of a member of the MAPK family to form heterodimeric complexes with other kinases in vitro is not without precedent. Haiao et al. (53) noted that inactive Erk-2 associates with p90 in Xenopus oocytes; upon activation the complex was found to dissociate. Moreover, McLaughlin et al. (54) observed that in its inactive form, MAPKAPK-2 associates with p38 and that the interaction is disrupted upon exposure of the cells to hypertonic sorbitol, a treatment known to activate stress kinases. These interactions resemble the association between MAPKAPK-2 and p38 reported here. In other systems multiple kinases appear to be maintained in elaborate complexes by specific docking proteins (55). It will be of interest to determine whether additional kinases and/or docking proteins are found complexed with MAPKAPK-2 and p38 in PMN.
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46. Smallwood, J. I., and Malawista, S. E. (1992) J. Leukocyte Biol. 51, 84–92
47. Powis, G., Bonjouklian, R., Berggren, M., Gallegos, A., Abraham, R., Ashendel, C., Zalkow, L., Matter, W. F., Dodge, J., Grindey, G., and Vlahos, C. J. (1994) Cancer Res. 54, 2419–2423
48. Vlahos, C. J., Matter, W. F., Hui, K. Y., and Brown, R. F. (1994) J. Biol. Chem. 269, 5241–5248
49. Sabatini, D. M., Pierchala, B. A., Barrow, R. K., Schell, M. J., and Snyder, S. H. (1995) J. Biol. Chem. 270, 20875–20878
50. Walker, B. A., Hagenlocker, B. E., Douglas, V. K., Tarapchak, S. J., and Ward, P. A. (1991) Lab. Invest. 64, 105–112
51. Stokoe, D., Campbell, D. G., Nakielny, S., Hidaka, H., Leevers, S. J., Marshall, C., and Cohen, P. (1992) EMBO J. 11, 3985–3994
52. Cuenda, A., Rouse, J., Doza, Y. N., Meier, R., Cohen, P., Gallagher, T. F., Young, P. R., and Lee, J. C. (1994) FEBS Lett. 364, 229–233
53. Hsiao, K.-M., Chou, S.-Y., Shih, S.-J., and Ferrel, J. E. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 5480–5484
54. McLaughlin, M. M., Kumar, S., McDonnell, P. C., Van Horn, S., Lee, J. C., Livi, G. P., and Young, P. R. (1996) J. Biol. Chem. 271, 8488–8492
55. Elion, E. A. (1995) Trends Cell. Biol. 5, 322–327