Activation of MAP Kinase-Activated Protein Kinase 2 in Human Neutrophils After Phorbol Ester or fMLP Peptide Stimulation

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In response to extracellular stimulation, one of the earliest events in human neutrophils is protein phosphorylation, which mediates signal transduction and leads to the regulation of cellular functions. Mitogen-activated protein (MAP) kinases are rapidly activated by a variety of mitogens, cytokines, and stresses. The activated MAP kinases in turn regulate their substrate molecules by phosphorylation. MAP kinase-activated protein (MAPKAP) kinase 2, a Ser/Thr kinase, has been shown to be phosphorylated by p38 MAP kinase both in vivo and in vitro. Phosphorylation of the Thr-334 site of MAPKAP kinase 2 results in a conformational change with subsequent activation of the enzyme. To better define the role of MAPKAP kinase 2 in the activation of human neutrophils, its enzymatic activity was measured after stimulation by either a phorbol ester (phorbol myristate acetate [PMA]), a potent protein kinase C activator, or the tripeptide fMLP, which is a chemotactic factor. The in vitro kinase assays indicate that both PMA and fMLP stimulated a transient increase in the enzymatic activity of cellular MAPKAP kinase 2. The induced kinase activation was concentration-dependent and reached a maximum at 5 minutes for PMA and 1 minute for fMLP. To identify potential substrate molecules for MAPKAP kinase 2, a highly active kinase mutant was generated by mutating the MAP kinase phosphorylation site in the C-terminal region. The replacement of threonine 334 with alanine resulted in a marked augmentation of catalytic activity. Analysis of in vitro protein phosphorylation in the presence of the active kinase indicates that a 60-kD cytosolic protein (p60) was markedly phosphorylated and served as the major substrate for MAPKAP kinase 2 in human neutrophils. Based on the MAPKAP kinase 2 phosphorylation site of Hsp27, a competitive inhibitory peptide was synthesized. This competitive inhibitory peptide specifically inhibited MAPKAP kinase 2 enzymatic activity, as well as the in vitro and in vivo kinase-induced p60 phosphorylation. To assess the contribution of MAPKAP kinase 2 in neutrophil function, the oxidative burst response after manipulation of endogenous kinase activity was measured. Intracellular delivery of the competitive inhibitory peptide into human neutrophils reduced both PMA- and fMLP-stimulated superoxide anion production. Thus, the results strongly suggest that MAPKAP kinase 2 is involved in the activation of human neutrophils.

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Human neutrophils play a critical role in host defense against infection, as well as being implicated in a number of allergic and nonallergic tissue-damaging inflammatory reactions. Neutrophils are activated in response to various stimuli, including chemotactic factors, cytokines, and immune complexes. After stimulation, neutrophils engage in phagocytosis, antibody-dependent cell-mediated cytotoxicity, release of specific granules, and production of superoxide anions. One of the early events in neutrophil activation is the rapid induction of protein phosphorylation, which plays an essential role in the regulation of cellular functions. The activation of multiple kinases, including tyrosine kinases, protein kinase C (PKC), mitogen-activated protein (MAP) kinases, calcium/calmodulin-dependent protein kinases, ribosomal protein S6 kinases, and histone H4 protein kinase, has been observed in human neutrophils after stimulation.

Recent studies indicated that the MAP kinase-mediated signal transduction pathway is a conserved eukaryotic signaling cascade that converts receptor signaling into a variety of cellular functions. MAP kinases are dual-specificity protein kinases activated through Thr/Tyr phosphorylation by the MAP kinase kinases, which are themselves activated by the MAP kinase kinase kinases. Currently, three distinct mammalian MAP kinases, each with apparently unique signaling pathways, have been identified. The ERK group of MAP kinases is activated by growth factors via a Ras-dependent signal transduction pathway. The SAPK group of MAP kinases (also designated INK) phosphorylates and activates c-Jun, an oncogene product. A mammalian homologue of HOG1, p38 MAP kinase, represents the third group of MAP kinases. In response to environmental stresses, mitogens, and proinflammatory cytokines, p38 MAP kinase is activated and, in turn, stimulates MAP kinase-activated protein (MAPKAP) kinase 2.
To detect enzymatic activity, a synthetic peptide derived from N-terminus of glycogen synthase (KKLNRTLSVA-amide) was used. Enzymatic activity was evaluated by the amount of incorporation of 32p into protein kinase assay mixture containing 100 pmol/L substrate peptide. The reaction was allowed to proceed for 10 minutes at 30°C. Duplicate samples were blotted onto P81 phosphocellulose (Whatman, Inc, IL) and the induced phosphorylation was detected by autoradiography. To examine the effects of various protein kinases on Hsp27 phosphorylation, commercially available recombinant human Hsp27 (StressGen Biotechnologies Corp, Victoria, British Columbia, Canada) was phosphorylated in vitro by activated MAP kinase (p44/42 MAPK; Upstate Biotechnology Inc, Lake Placid, NY), cAMP-dependent protein kinase (Sigma, St Louis, MO), or purified active MAPKAP kinase 2 (mutant T334A).

Preparation of the competitive inhibitory peptide for MAPKAP kinase 2. To generate a specific competitive peptide inhibitor, an 18-amino acid peptide, AFHRAFNRQLANGVAEIR-amide, was designed based on the MAPKAP kinase 2 phosphorylation site of Hsp27 with the following mutations: Ser29 to Asn, Ser44 to Ala, Ser64 to Asn, and Ser89 to Ala (see Fig 4A). The competitive inhibitory peptide was synthesized by solid-phase peptide synthesis, purified with cation exchange reverse-phase chromatography, and characterized using analytical high performance liquid chromatography (Research Genetics, Huntsville, AB). To test the inhibitory effect on MAPKAP kinase 2, recombinant kinase was preincubated at 30°C with various concentrations of the competitive inhibitory peptide as indicated in the appropriate figures for 1 minute before the initiation of the kinase reaction by the addition of substrate peptides and ATP. The relative enzymatic activity was evaluated by the amount of 32p incorporation into the substrate peptide, as described above.

The potential cellular functions of the kinase, the respiratory burst response of activated neutrophils was analyzed after the cells had been loaded with a competitive inhibitory peptide against MAPKAP kinase 2.

MATERIALS AND METHODS

Cell preparation. Neutrophils were isolated from whole human blood using Ficoll/Hypaque gradients, as described elsewhere. The remaining erythrocytes were lysed by a hypotonic shock (45 seconds). The resulting neutrophils represented at least 97% of the cells. Cell viability estimated by Trypan Blue exclusion was 98%. The neutrophils were resuspended in Hank’s Balanced Salt Solution (HBSS) with 1 mmol/L calcium before further treatment.

Enzymatic assay of MAPKAP kinase 2. To examine the enzymatic activity of endogenous MAPKAP kinase 2, in vitro kinase assays were performed. Isolated human neutrophils (2 x 10^6 cells) were stimulated with 20 ng/mL PMA, 0.5 x 10^-4 mol/L FMLP, or buffer only at 30°C over a time course of 0 to 15 minutes. Cells were washed once with 0.5 mL cold HBSS, resuspended in 170 μL lysis buffer (100 mmol/L HEPES, pH 7.5, 0.05% Triton X-100, 5 μmol/L sodium pyrophosphate, 5 μmol/L sodium orthovanadate, 1 μmol/L okadaic acid, 1 mmol/L diisopropylfluorophosphate [DFP], 1 mmol/L benzamidate, 2 μmol/L aprotinin, 1 μmol/L pepstatin A, 10 μmol/L H-7, and lysed with mild sonication at 4°C for 8 seconds. The cell lysates were then centrifuged at 13,000 g for 30 minutes, and the resulting supernatant was used for the kinase assay.

To detect enzymatic activity, a synthetic peptide derived from N-terminus of glycogen synthase (KKLNRTLTSVA-amide) was used as the specific substrate for MAPKAP kinase 2. Cell lysates prepared from 2.5 x 10^7 human neutrophils were added into a final 40 μL kinase assay mixture containing 20 mmol/L HEPES (pH 7.3), 10 mmol/L MgCl_2, 1 mmol/L EGTA, 5 μmol/L sodium orthovanadate, 2 μmol/L okadaic acid, 2 mmol/L dithiothreitol (DTT), 10 mmol/L H-7, 40 μmol/L [γ-32P]ATP (4.4 x 10^4 cpm/μmol), and 20 μmol/L substrate peptide. The reaction was allowed to proceed for 10 minutes at 30°C. Duplicate samples were blotted onto P81 phosphocellulose papers, washed with water, and allowed to dry. The relative enzymatic activity was evaluated by the amount of 32p incorporation into the substrate peptide using a liquid scintillation counter.

Preparation of recombinant kinases. To generate recombinant MAPKAP kinase 2, a glutathione S-transferase (GST) fusion protein system expressed in Escherichia coli was used. Wild-type (WT) MAPKAP kinase 2 and the mutant NCT339 were prepared as described previously. Mutants T334S and T334A were constructed by point mutations of NCT339 Thr-334 to serine or alanine residues, respectively. All prepared expression plasmids were confirmed by DNA sequencing. Recombinant kinases were purified by glutathione-affinity columns, concentrated to 150 μg/mL protein, and stored in kinase buffer (20 mmol/L Tris-HCl, pH 7.4, 1.0 mmol/L EDTA, 50 mmol/L KCl, and 1.0 mmol/L DTT) containing 50% glycerol at -20°C until needed. The purified kinases were analyzed by 9% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and their enzymatic activities were examined using an in vitro kinase assay with the substrate being the synthetic peptide, as described above. The highly active kinase, mutant T334A, was used for all subsequent in vitro protein phosphorylation studies.

In vitro protein phosphorylation analysis. To identify potential substrate molecules for MAPKAP kinase 2 in human neutrophils, cellular proteins were fractionated and phosphorylated in vitro using the highly active mutant T334A kinase. Human neutrophils (1.3 x 10^6 cells) were resuspended in 4 mL of fractionation buffer (10 mmol/L HEPES, pH 7.3, 11.5% sucrose, 1 mmol/L EDTA, 1 mmol/L EGTA, and 1 mmol/L DFP), homogenized for 2 minutes, and spun at 3,000g for 20 minutes at 4°C. The supernatants were then centrifuged at 120,000g for 30 minutes at 4°C. The cytosolic fraction from the resulting supernatant was collected. The pellet containing the membrane fraction was resuspended in fractionation buffer with the assistance of mild sonication. All fractionated proteins were stored at -80°C until needed. For protein phosphorylation analysis, cellular fractions (5 μL) were incubated with 300 ng mutant T334A kinase in 40 μL phosphorylation reaction containing 20 mmol/L HEPES (pH 7.3), 10 mmol/L MgCl_2, 1 mmol/L EGTA, 5 μmol/L sodium orthovanadate, 5 μmol/L okadaic acid, 2 mmol/L DTT, 20 μmol/L H-7, and 0.2 mmol/L [γ-32P]ATP (10^5 cpm/μmol). The phosphorylation reaction was performed at 30°C for 10 minutes, unless otherwise indicated. Proteins were then separated on SDS-PAGE and the induced phosphorylation was detected by autoradiography.
The pellet fraction of the neutrophil lysates was resuspended in 1× SDS sample buffer with the assistance of a mild sonication. Finally, the protein phosphorylation of in vivo-labeled human neutrophils was analyzed using 11% SDS-PAGE and autoradiography.

To examine the effects of the synthetic competitive inhibitory peptide on MAPKAP kinase 2, the peptide was introduced into human neutrophils according to the protocol described by Hendey et al., with some modification. Briefly, to load the competitive inhibitory peptide into human neutrophils, cells were preincubated in 32P04 labeling buffer containing 30 μmol/L peptide at 30°C for 1 hour. Cells were washed with cold HBSS twice and exposed to a short hypotonic shock in distilled water for 15 seconds. This treatment stimulates the intracellular delivery of the competitive inhibitory peptide taken up via the endocytotic pathway during the incubation from the endosomal compartment into the cytoplasm. The cells were then resuspended in HBSS containing 1 mmol/L MgCl2, 1 mmol/L CaCl2, and 10 mmol/L glucose and incubated on ice for 10 minutes to allow for cellular recovery from the hypotonic shock before further treatment. Finally, the cells were incubated at 30°C for 10 minutes followed by stimulation with 20 ng/mL PMA or 0.5 × 10⁻⁸ mol/L fMLP and the changes in the induced protein phosphorylation were examined by 11% SDS-PAGE and autoradiography, as described above.

**Oxidative burst assay.** In an attempt to define the cellular function of MAPKAP kinase 2, the effects of regulating endogenous MAPKAP kinase 2 activity on the neutrophil respiratory burst were examined. The synthetic competitive inhibitory peptide was introduced into human neutrophils by incubating the cells with 30 μmol/L peptide for 40 minutes at 30°C in HBSS containing 1 mmol/L MgCl2, 1 mmol/L CaCl2, and 10 mmol/L glucose before being exposed to a short hypotonic shock to stimulate the intracellular delivery of the peptides, as described above. To measure the respiratory burst response, neutrophils (5 × 10⁶ cells/mL) loaded with or without peptides were resuspended in HBSS containing 145 mmol/L NaCl, 2 mmol/L sodium azide, 2 mmol/L CaCl2, and 2.4 mmol/L MgCl2 and equilibrated to 37°C. Neutrophils (4.5 × 10⁵) were dispensed into prewarmed microtiter plates and superoxide anion production was initiated by stimulating neutrophils with 20 ng/mL PMA or 0.5 × 10⁻⁸ mol/L fMLP. The reaction allowed to proceed at 37°C for 20 minutes. Superoxide production was measured kinetically by the reduction of cytochrome C at 550 nm using a THERMOsor, kinetic microplate reader (Molecular Devices, Menlo Park, CA).

**RESULTS**

**Activation of MAPKAP kinase 2 in stimulated human neutrophils.** To investigate the regulation of MAPKAP kinase 2, human neutrophils were stimulated by 20 ng/mL PMA or

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**Fig 1.** Activation of MAPKAP kinase 2 in human neutrophils. (A) Enzymatic assay of MAPKAP kinase 2 activity. After isolation, human neutrophils were stimulated with 20 ng/mL PMA, 0.5 × 10⁻⁸ mol/L fMLP, or control buffer at 30°C for various times as indicated. The cells were washed, resuspended in lysis buffer, and lysed by a short sonication. Cellular lysates were centrifuged at 13,000g for 20 minutes and the resulting supernatants were used as the kinase assay. The enzymatic activity of neutrophil MAPKAP kinase 2 was measured using a peptide derived from glycogen synthase as the substrate. The kinase activities are shown as the amount (cpm) of 32P incorporation into the substrate peptide as described in the Materials and Methods. (B) Dose-dependent effects of PMA on the activation of MAPKAP kinase 2. Neutrophils were stimulated with the noted concentrations of PMA for 10 minutes at 30°C. The relative enzymatic activities were evaluated and are shown as a percentage of the control. Maximal activation was arbitrarily set as 100%. The results are representative of five separate experiments. (C) Effects of fMLP on the activation of MAPKAP kinase 2. Neutrophils were stimulated with the noted concentrations of fMLP for 5 minutes at 30°C. The relative enzymatic activities were evaluated and are shown as a percentage of the control. Maximal activation was arbitrarily set as 100%. The results are representative of five separate experiments.
The threonine 334 residue is critical for enzymatic activity of MAPKAP kinase 2. Previous functional domain analysis of human MAPKAP kinase 2 indicated that, in the C-terminal region, amino acid residues 339 through 353 are acting as an autoinhibitory domain (Fig 2A). The region composed of amino acids 327 through 338, which contains a MAP kinase phosphorylation site (Thr-334), is indispensable for maintenance of high enzymatic activity. To understand the role of the MAP kinase phosphorylation site in MAPKAP kinase 2 enzymatic activity, the threonine 334 residue was changed to serine or alanine by a point mutation of the truncated kinase mutant NCT339 (Fig 2A). The relative enzymatic activities of the generated kinases were evaluated using an in vitro kinase assay, as described in the Materials and Methods. Mutation of Thr-334 to serine (mutant T334S) had little effect on kinase activity (Fig 2B). However, replacement of Thr-334 with alanine (mutant T334A) resulted in a marked increase of MAPKAP kinase 2 enzymatic activity. Mutant T334A had a very high enzymatic activity, with an eightfold increase over wild-type kinase (WT) and a 2.6-fold increase over mutant NCT339 being noted. These results suggest that the MAP kinase phosphorylation site (Thr-334) is critical to the enzymatic activity of MAPKAP kinase 2.

Identification of the potential substrate molecules for MAPKAP kinase 2 in human neutrophils. Cytosolic proteins derived from human neutrophils were phosphorylated in vitro using the highly active recombinant MAPKAP kinase 2, mutant T334A. The phosphorylated proteins were separated on SDS-PAGE, and the protein phosphorylation was detected by autoradiography. As shown in Fig 3A, the phosphorylation of a 60-kD protein (p60) was considerably enhanced in the presence of mutant T334A kinase. Kinetic analysis showed that p60 phosphorylation increased within 5 minutes after the addition of the active recombinant MAPKAP kinase 2, mutant T334A, and reached a maximum at 15 minutes (Fig 3A, lanes 6 through 9). The phosphorylation of p60 was not detectable in the membrane fraction derived from human neutrophils (data not shown). These observations suggest that p60 is a major cytosolic substrate molecule for MAPKAP kinase 2 in human neutrophils.

MAPKAP kinase 2 has been shown to be the enzyme that phosphorylates Hsp27/27 both in vivo and in vitro. To detect if MAPKAP kinase 2 induces Hsp27 phosphorylation in human neutrophils, a longer exposure of the autoradiograph was performed. Figure 3B shows that the phosphorylation of a 27-kD cytosolic protein (p27) was stimulated by

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Fig 2. Mutational analysis of the MAP kinase phosphorylation site in human MAPKAP kinase 2. (A) Construction of the recombinant kinases. The recombinant kinases were generated as GST fusion proteins, as described previously. The wild-type (WT) kinase contains the full-length sequence of human MAPKAP kinase 2. Mutant NCT339 is composed of amino acid residues 48 through 338; both potential SH3 domain-binding sites in the N-terminus as well as the autoinhibitory domain in the C-terminal region have been deleted. Mutants T334S and T334A were generated by point mutation of threonine 334 to serine or alanine, respectively, using mutant NCT339 as a template. Threonine 334 lies within the MAP kinase phosphorylation site of MAPKAP kinase 2. (B) Enzymatic analysis of the recombinant kinases. The relative enzymatic activities of the mutants were examined by the in vitro protein kinase assay described in Fig 1. The kinase reaction was performed at 30°C for 10 minutes with 300 ng purified recombinant kinase protein. GST vector protein served as the background control. The percentages of enzymatic activity as compared with the WT kinase (100%) are shown in the graph. The results shown are representative of six separate experiments.

0.5 × 10⁻⁸ mol/L fMLP. The induced kinase activity in cell lysates was evaluated by an in vitro kinase assay using the specific substrate peptide derived from glycogen synthase as described under the Materials and Methods. Treatment of neutrophils with PMA, a mitogen that activates neutrophils by stimulating cellular PKC, induced a transient activation of MAPKAP kinase 2 (Fig 1A). PMA stimulation resulted in an increase of kinase activity, which reached a maximum at 5 minutes that was sevenfold higher than control cells. Treatment with fMLP, a chemotactic factor that activates neutrophils through a Ras-mediated signal transduction pathway, increased MAPKAP kinase 2 activity to about fivefold higher than control cells within 1 minute (Fig 1A). The PMA-induced kinase activation was found to be concentration-dependent (Fig 1B), as was the activation of MAPKAP kinase 2 by the addition of fMLP, which reached saturation at 0.5 × 10⁻⁸ mol/L (Fig 1C). These findings indicate that stimulation of human neutrophils by PMA or fMLP induces the activation of MAPKAP kinase 2.

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Fig 3. Identification of potential substrate molecules for MAPKAP kinase 2. (A) Analysis of the in vitro protein phosphorylation. Cytosolic proteins fractionated from human neutrophils were phosphorylated in vitro in the presence of the highly active MAPKAP kinase 2, mutant T334A (kinase +, lanes 6 through 9), or GST vector alone (kinase -, lanes 2 through 5) at 30°C for 5 to 20 minutes, as indicated at the top of each lane. Phosphorylated proteins were separated using 11% SDS-PAGE and detected by autoradiography as described in the Materials and Methods. Molecular weight standards are shown on the left. The position of mutant T334A kinase is marked by an asterisk on the right. The position of the MAPKAP kinase 2-phosphorylated protein, p60, is indicated by an arrow. Lane 1 contains the kinase alone (no cell lysate present) reacted for 20 minutes at 30°C. Densitometric analysis indicates that, in the presence of MAPKAP kinase 2, p60 phosphorylation (lanes 6 through 9) was 2.8-, 3.4-, 3.8-, or 3.7-fold higher than that of control cells (lanes 2 through 5) for 5, 10, 15, or 20 minutes, respectively. The autoradiography of (A) was overexposed and the region containing p27 is shown in (B). The MAPKAP kinase 2-phosphorylated protein, p27, is indicated by an arrow. (C) Characterization of p27. Human neutrophils fractionated into cytosol (lanes 1 and 2), membrane (lanes 3 and 4), or whole cell lysates (lanes 5 and 6) were phosphorylated in the presence of mutant T334A kinase (kinase +, lanes 2, 4, and 6) or GST vector alone (kinase -, lanes 1, 3, and 5) in vitro for 15 minutes at 30°C. Phosphorylated proteins were analyzed using SDS-PAGE followed by autoradiography. In addition, commercial recombinant Hsp27 (StruwsGen) was used as a control (lanes 7 and 8). Hsp27 was phosphorylated by mutant T334A kinase (lane 8) or GST vector alone (lane 7), separated on the same gel with neutrophil proteins, and detected by autoradiography. Lane 9 shows the kinase alone (no cell lysate present) for a control. Molecular weight is shown on the left. The position of p27/Hsp27 is indicated by an arrow on the right. (D) Effect of MAP kinase on Hsp27 phosphorylation. Commercially available recombinant human Hsp27 proteins were phosphorylated in the presence of control buffer (kinase -), active MAPKAP kinase 2 (mutant T334A), activated MAP kinase (p44/p42), or cAMP-dependent protein kinase (cAPK) in vitro. Hsp27 proteins were separated using SDS-PAGE and stained by Coomassie Blue (upper panel), and protein phosphorylation was detected by autoradiography (lower panel). The positions of Hsp27 proteins and phosphorylated Hsp27 (pHsp27) are indicated by arrows on the right.

the active recombinant MAPKAP kinase 2, mutant T334A. In addition, analysis of subcellular fractions showed that the p27 detectable in whole cell lysates of neutrophils existed primarily in the cytosolic fraction (Fig 3C, lanes 1 through 6). To ascertain if the p27 phosphorylated by the active recombinant MAPKAP kinase 2 mutant T334A is neutrophil Hsp27, commercial recombinant human Hsp27 was used as a control. As shown in Fig 3C, MAPKAP kinase 2 phosphorylates recombinant human Hsp27 (lanes 7 and 8) and neutrophil p27 (lanes 1 and 2), and both phosphorylated proteins migrate similarly when separated using SDS-PAGE (lanes 2 and 8). These results suggest that activated MAPKAP kinase 2 may phosphorylate Hsp27 in human neutrophils.

Specific inhibition of MAPKAP kinase 2-induced protein phosphorylation in vitro. Protein phosphorylation studies indicate that cAMP-dependent protein kinase (cAPK), calmodulin-dependent protein kinase II, PKC, or ribosomal protein S6 kinase II are not directly involved in the phosphorylation of Hsp27.25,26 To examine the effect of MAP kinase on Hsp27 phosphorylation, commercial recombinant Hsp27 was
phosphorylated by activated MAP kinase (p44MAPK) in vitro. Hsp27 proteins were then separated on SDS-PAGE and stained by Coomassie blue (Fig 3D, upper panel). Protein phosphorylation was detected by autoradiography (Fig 3D, lower panel). MAPKAP kinase 2 and cAPK were used as positive and negative controls, respectively. These findings reveal that MAP kinase is unable to directly phosphorylate Hsp27.

A powerful method in defining the cellular function of protein kinases is the intracellular delivery of specific peptide inhibitors/analogs. These may be sequences corresponding to regions within the pseudosubstrate, autoregulatory, or autoinhibitory domains of each kinases or phosphorylation sites of specific substrates for the kinases. To regulate MAPKAP kinase 2 activity, a competitive inhibitory peptide was designed from the MAPKAP kinase 2 phosphorylation site sequence of Hsp27, with mutation of all the serine residues into residues that cannot be phosphorylated (Fig 4A). Ser-78 and Ser-82 residues have been shown to be phosphorylated by MAPKAP kinase 2 in vitro. The effects of the competitive inhibitory peptide on MAPKAP kinase 2 enzymatic activity was examined with an in vitro kinase assay that used the active mutant T334A kinase. As shown in Fig 4B, the competitive inhibitory peptide repressed the enzymatic activity of MAPKAP kinase 2 in a concentration-dependent manner, with an IC50 of 20 μmol/L, suggesting that the competitive inhibitory peptide had an affinity for the kinase similar to the substrate peptide derived from glycogen synthase.

To test the effects of the competitive inhibitory peptide on MAPKAP kinase 2-induced neutrophil protein phosphorylation, various doses of the peptide (Fig 4C, lanes 2 through 9) were added to the in vitro protein phosphorylation reactions in the presence of mutant T334A kinase (Fig 4C, lanes 2 through 9). The phosphorylated proteins were analyzed using SDS-PAGE. Autoradiography showed that the competitive inhibitory peptide efficiently inhibited the MAPKAP kinase 2-induced phosphorylation of p60 and p27 in human neutrophils.
MAPKAP KINASE 2 ACTIVATION IN NEUTROPHILS

A

Peptide (-)                              Peptide (+)

kDa

80'                                        p60

49.5                                       p47phox

32.5

27.5

p27

1 2 3                                      4 5 6

Fig 5. Inhibition of fMLP or PMA-induced p60 phosphorylation in vivo by loading the competitive inhibitory peptide into neutrophils.

(A) Analysis of in vivo protein phosphorylation. Human neutrophils were prelabeled with $^{32}$PO$_4$, stimulated with PMA or fMLP, and lysed in the Triton-buffer, as described under the Materials and Methods. The cellular proteins were separated on SDS-PAGE and the induced protein phosphorylation was detected by autoradiography. Stimulation of neutrophils with either PMA or fMLP induced the phosphorylation of several cellular proteins, including p60 and p27 (Fig 5A and B, lanes 1 through 3). Both p60 and p27 of human neutrophils appeared to be major potential substrates for MAPKAP kinase 2 in the in vitro protein phosphorylation assay (Fig 3A and 3B). Densitometry analysis of p60 phosphorylation showed that stimulation of neutrophils with fMLP induced 4.7-fold higher than control cells (Fig 5A, lanes 1 and 2), whereas PMA resulted in a 4.8-fold increase (Fig 5A, lane 3), respectively. It is notable that PMA stimulation also induced the phosphorylation of p47 phox (Fig 5A), an essential component of the respiratory burst response and a major substrate for PKC in human neutrophils. Stimulation of neutrophils by PMA resulted in a 5.4-fold increase in p47 phox phosphorylation (Fig 5A, lane 3).

To study the effects of the competitive inhibitory peptide with its presumed downregulation of MAPKAP kinase 2 activity during in vivo protein phosphorylation, human neutrophils were preincubated with the peptide and then treated with a short hypotonic shock as described in the Materials and Methods. This shock results in the release of the peptide from the endosomal vesicles into the cytoplasm. The neutrophils were then stimulated with PMA or fMLP and protein phosphorylation events were analyzed using SDS-PAGE and autoradiography. Intracellular delivery of the competitive inhibitory peptide into human neutrophils repressed both PMA- and fMLP-induced protein phosphorylation of p60 and p27 (Fig 5A and B, lanes 4 through 6). Densitometry analysis indicated that loading of the competitive inhibitory peptide into neutrophils resulted in a marked decrease of both fMLP- and PMA-stimulated p60 phosphorylation, 65% and 64% inhibition, respectively (Fig 5A and B, lanes 4 through 6). In contrast, loading of the competitive inhibitory peptide had less than 10% inhibition on PMA-stimulated p47 phox phosphorylation (Fig 5A, lane 6), with a 5.0-fold increase being noted. These results suggest that the addition of the competitive inhibitory peptide specifically inhibits cellular MAPKAP kinase 2 activity and the kinase-induced protein phosphorylation that normally occurs in human neutrophils.

Inhibition of PMA and fMLP-stimulated superoxide production in human neutrophils. That stimulation of neutrophils by PMA or fMLP increases MAPKAP kinase 2 enzymatic activity and the protein phosphorylation of two major potential substrates for MAPKAP kinase 2, p60 and p27, strongly suggest that MAPKAP kinase 2 is involved in human neutrophil activation. In response to PMA or fMLP, neutrophils are rapidly activated and produce a respiratory burst response. To investigate the role of MAPKAP kinase 2 in the generation of superoxide anions, the competitive inhibitory peptide was loaded into neutrophils and the resulting effects were analyzed as described in Materials and Methods after simulation with PMA or fMLP. As shown in
Fig 6, loading of the competitive inhibitory peptide partially reduced the respiratory burst response to PMA (Fig 6B and E) and almost completely repressed that induced by fMLP (Fig 6C and F). These results indicate that MAPKAP kinase 2 may be involved in the respiratory burst response of neutrophils.

DISCUSSION

This study provides the first evidence that MAPKAP kinase 2 is involved in the activation of human neutrophils. Stimulation of neutrophils by PMA or fMLP induced a rapid increase in MAPKAP kinase 2 enzymatic activity. Repression of endogenous MAPKAP kinase 2 activity by loading a competitive inhibitory peptide into neutrophils reduced the respiratory burst response initiated by PMA or fMLP. Additionally, by using a highly active kinase (mutant T334A), proteins of approximately 60 kDa (p60) and 27 kDa (p27) were identified as potential substrate molecules for MAPKAP kinase 2 in human neutrophils.

In response to extracellular stimuli, a number of neutrophil proteins have been shown to be rapidly phosphorylated, and are linked to functional responses. Several studies have shown that tyrosine as well as serine/threonine phosphorylation mediates the oxidative burst response of neutrophils. Phorbol esters such as PMA activate neutrophils in a receptor-independent manner. PMA induces PKC translocation to the membrane and subsequent activation, which leads to the phosphorylation and regulation of its substrate molecules. Recent studies showed that phorbol ester stimulates MAP kinase signal pathway by regulating MAP kinase kinase kinases. In contrast to PMA, fMLP, a chemotactic factor, activates neutrophils through heptahelical membrane receptors. Binding of fMLP to its receptor results in the activation of phospholipases and two pertussis toxin-sensitive G proteins, G12 and G13. After stimulation with fMLP, MAP kinases have been shown to be activated in human neutrophils through a Ras-mediated pathway. These observations suggest that stimulation of neutrophils by PMA or fMLP may activate signal transduction pathways that regulate a universal downstream signaling pathway. The findings in this report strongly support this hypothesis, because activation either through the PKC pathway stimulated by PMA or the G-protein-coupled receptor pathway induced by fMLP stimulates the activation of MAPKAP kinase 2 in neutrophils.

The introduction of a competitive inhibitory peptide for MAPKAP kinase 2 into neutrophils inhibited the fMLP-induced oxidative burst almost completely, whereas it only partially inhibited that induced by PMA (Fig 6). Such a result suggests that PMA and fMLP may activate a common MAPKAP kinase 2-mediated pathway as well as a signaling pathway(s) unique to the initiating stimulant. Several supporting observations have been obtained. First, analysis of in vivo protein phosphorylation experiments indicate that PMA stimulates not only the phosphorylation of p60 and p27, but also that of p47 phox, a major substrate for PKC in human neutrophils, which fMLP did not (Fig 5, lanes 1 through 3). Inhibition of cellular MAPKAP kinase 2 by the competitive inhibitory peptide markedly repressed both PMA and fMLP-stimulated protein phosphorylation of p60, but had little effect on the PMA-induced p47 phox phosphorylation (Fig 5A, lanes 4 through 6). Moreover, downregulation of p38 MAP kinase, an enzyme that phosphorylates and thus activates MAPKAP kinase 2 in vivo, by
a specific inhibitor, SKF86002, inhibited both PMA or fMLP-stimulated MAPKAP kinase 2 activation to a similar extent. However, the effect of SKF86002 on neutrophil function indicated that inhibition of cellular p38 MAP kinase remarkably repressed fMLP-stimulated oxidative burst, while having little effect on that stimulated by PMA in human neutrophils (Huang et al, manuscript in preparation). Thus, it is likely that, to induce the respiratory burst of neutrophils, PMA stimulates both MAPKAP kinase 2-dependent and -independent signaling pathways, whereas fMLP, in contrast, activates only the MAPKAP kinase 2-dependent signaling pathway. Whether FMA-stimulated p47 phox phosphorylation and cellular MAPKAP kinase 2 activation result from the same signaling pathway at different stages or from two separate signaling pathways has not yet been determined.

Identification of potential substrates should help in the understanding of MAPKAP kinase 2 cellular function. To date, only a few proteins, such as glycogena synthase, tyrosine hydroxylase, and low molecular weight heat shock proteins, have been reported to act as substrates for the kinase. Studies of human neutrophils showed that interleukin-3 and granulocyte-macrophage colony-stimulating factor induce MAPKAP kinase 2 activation, which stimulates in vitro phosphorylation of a small heat shock protein. Using a highly active recombinant kinase and in vitro protein phosphorylation analysis, we show that p60 is a major substrate for MAPKAP kinase 2 in human neutrophils. However, in other cells, such as undifferentiated human HL-60 cells or rat cardiac myocytes, Hsp27 or Hsp25 served as the major substrate for MAPKAP kinase 2 (Zu et al, unpublished observation). Thus, it seems likely that p60 is a neutrophil-specific substrate for MAPKAP kinase 2 and may be involved in neutrophil function, including the respiratory burst phenomenon. Further characterization of p60 may more clearly define the role of the MAPKAP kinase 2-mediated signal transduction pathway in human neutrophils.

Protein phosphorylation analysis showed that the ERK group (p42/44 MAP kinases) and p38 MAP kinases phosphorylate recombinant MAPKAP kinase 2 on threonine 334. Interestingly, the ERK group seemed to be more effective in phosphorylating recombinant MAPKAP kinase 2 in vitro than p38 MAP kinase (unpublished observation). In contrast, the SAPK/JNK group of MAP kinases did not phosphorylate MAPKAP kinase 2 in vitro (unpublished results). These findings suggest that p42/44 MAP kinases, as well as p38 MAP kinase, may play a role in regulating MAPKAP kinase 2 in vivo. The mechanism by which the ERK group and p38 MAP kinases govern the MAPKAP kinase 2 activity in response to various stimuli needs to be clarified. In addition to fMLP and PMA, an enhanced calcium flux stimulated by a calcium ionophore A23187 also activates MAPKAP kinase 2 in human neutrophils (unpublished observation), suggesting that MAPKAP kinase 2 may be activated in response to various stimuli and serve as a convergent molecule for distinct signaling pathways in neutrophils. Further investigation of MAPKAP kinase 2 should yield a better understanding of phosphorylation-coupled regulation that lead to neutrophil functional responses.

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Activation of MAP kinase-activated protein kinase 2 in human neutrophils after phorbol ester or fMLP peptide stimulation

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