p38 KINASE-DEPENDENT MAPKAPK-2 ACTIVATION FUNCTIONS AS PDK2 FOR AKT IN HUMAN NEUTROPHILS

Running Title: p38 Kinase Regulates Akt activation in Human Neutrophils

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SUMMARY

Akt activation requires phosphorylation of T308 and S473 by PDK1 and PDK2, respectively. While PDK1 has been cloned and sequenced, PDK2 has yet to be identified. The present study shows that PI-3K-dependent p38 kinase activation regulates Akt phosphorylation and activity in human neutrophils. Inhibition of p38 kinase activity with SB203580 inhibited Akt S473 phosphorylation following neutrophil stimulation with fMLP, FcγR crosslinking, or PIP3. Concentration inhibition studies showed S473 phosphorylation was inhibited by 0.3 µM SB203580, while inhibition of T308 phosphorylation required 10 µM SB203580. Transient transfection of HEK293 cells with adenoviruses containing constitutively active MKK3 or MKK6 resulted in activation of both p38 kinase and Akt. Immunoprecipitation and GST pull-down with GST-Akt or GST-MK2 showed that Akt was associated with p38 kinase, MK2, and Hsp27 in neutrophils, and Hsp27 dissociated from the complex upon fMLP stimulation. Active recombinant MK2 phosphorylated recombinant Akt and Akt in anti-Akt, anti-MK2, anti-p38 and anti-Hsp27 immunoprecipitates and this was inhibited by an MK2 inhibitory peptide. We conclude that Akt exists in a signaling complex containing p38 kinase, MK2, and Hsp27, and that p38-dependent MK2 activation functions as PDK2 in human neutrophils.
INTRODUCTION

The serine/threonine kinase protein kinase B, also called Akt, is the cellular homologue of a viral oncogene v-Akt (1,2,3). Akt contains a pleckstrin homology (PH) domain at its N-terminus, a catalytic domain, a short C-terminal tail and is closely related to PKA and PKC in its amino- and carboxyl-terminal regions. Akt plays a critical role in mediating cell proliferation, differentiation and survival signals propagated from certain growth factors (4,5). Akt activation is dependent on phosphatidylinositol 3-kinase (PI-3K), as wortmannin and dominant negative mutants of PI-3K block Akt activation (6) and constitutively active mutants of PI-3K activate Akt (7,8). Activation of Akt requires that the products of PI-3K, phosphatidylinositol-3,4,5-trisphosphate (PIP₃) and phosphatidylinositol-3,4-bisphosphate (PIP₂) interact with the PH domain of Akt and recruit it to the plasma membrane (4,9,10,11). Subsequently, Akt undergoes phosphorylation at two sites, T308 in the kinase domain and S473 in the carboxyl terminal domain. PIP₂ and PIP₃ activate 3'-phosphoinositide-dependent kinase-1 (PDK1), which phosphorylates T308 (4,12). Phosphorylation of S473 is also dependent on products of PI-3K; however, the identity of this kinase, termed PDK2, is unknown (11,12).

Cellular stresses, such as heat shock and hyperosmolarity, stimulate both p38 kinase and Akt activity (13). p38 kinase, a homologue of the yeast HOG1, is activated by dual phosphorylation on Thr and Tyr within a Thr-Gly-Tyr motif by MAP kinase kinases MKK3 and MKK6 (14,15). The activation of MKK3 and MKK6 is regulated by phosphorylation on Ser and Thr residues by one of several
MAP kinase kinase kinases (MKKKs). Chemoattractant stimulation and crosslinking of Fcγ receptors stimulate PI-3K-dependent transient activation of Akt and p38 kinase in human neutrophils (16,17,18). MAPK-activated protein kinase-2 (MK2), a direct target of p38, has been reported to phosphorylate S473 of Akt in vitro (19), and activated Akt associates with a substrate of MK2, heat shock protein 27 (Hsp27) (20). Direct regulation of Akt activity by p38 kinase, however, has not been demonstrated previously. Additionally, Alessi et al. (19) suggested that a role for the p38 kinase module in Akt activation was unlikely in intact cells, as IGF-1 activates Akt, but not MK2, in HEK 293 cells. The present study examined the hypothesis that p38 kinase regulates Akt activation in human neutrophils. We show for the first time that Akt activation is regulated by PI-3K-mediated p38 kinase activity in intact cells. We also report that Akt forms a stable complex with p38 kinase, MK2, and Hsp27; and, upon stimulation with fMLP, Hsp27 dissociates from this complex.
MATERIALS AND METHODS

Materials used.

PD98059, SB203580, wortmannin and LY294002 were obtained from Calbiochem (San Diego, CA). Final concentrations used were PD98059 50 µM, SB203580 10 µM, wortmannin 100 nM and LY294002 100 µM, except where otherwise indicated. fMLP and Histone H2B were obtained from Sigma Chemical Co. (St. Louis, MO). GST-Akt agarose beads, active recombinant MK2, anti-Akt2 and anti-Akt3 were obtained from Upstate Biotechnology (Lake placid, NY). Anti-phospho-p38, anti-p38, anti-phoshoS473-Akt, anti-phosphoT308-Akt, and anti-Akt antibodies were obtained from New England Biolabs, Inc. (Beverly, MA). Recombinant Hsp27 and anti-mouse Hsp27 were obtained from Stressgen Biotechnologies Corp. (Victoria BC, Canada). Anti-MK2 was obtained from Research Biochemicals International (Natick, MA). Anti-FcγRIIa Fab mAb (IV.3) and FcγRIIIb F(ab’)2 mAb (3G8) were obtained from Medarex (Annandale, NJ). Goat anti-mouse IgG and goat anti-rabbit IgG were obtained from Vector (Burlingam, CA). Goat anti-mouse IgG (GAM), specific for F(ab’)2, was obtained from Jackson ImmunoResearch Laboratories (West Grove, PA). Phosphatidylinositol-3,4,5-trisphosphate (PIP₃) was obtained from Matreya (Pleasant Gap, PA). Adenovirus vector and adenovirus containing genes for constitutively active MKK3 and MKK6 were obtained from Dr. Wibin Wang (University of Maryland). GST-MK2 was kindly provided by Dr Matthias Gaestel (Martin-Luther-University Halle-Wittenberg, Germany). The synthetic MK2
inhibitory peptide (AFHRAFNRQLANGVAEIR-amine) was obtained from Macromolecular Structure Analysis Facility at the University of Kentucky (Lexington, KY). The synthetic EGFR peptide (NH$_2$-RRELVEPLTPSGEAPNQALLR-COOH) was obtained from Macromolecular Resources, Colorado State University (Fort Collins, CO).

**Neutrophil Isolation.**

Neutrophils were isolated from healthy donors using plasma-Percoll gradients, as described previously (21). After isolation, neutrophils were washed and resuspended with lipopolysaccharide-free Krebs-Ringer phosphate buffer (pH 7.2) containing 0.2% dextrose (Krebs). Microscopic evaluation of isolated cells treated by trypan-blue exclusion indicated that 95% of cells were neutrophils and those were > 98% viable.

**FcγR Cross-linking.**

FcγR cross-linking was performed as described previously (17).

**Adenovirus Transfection of 293 cells.**

HEK 293 cells in DMEM containing 10% fetal Bovine serum (FBS) were plated onto 100 mm tissue culture dishes one day prior to transfection. Immediately prior to transfection, the medium was replaced by 4 ml complete DMEM containing 2% FBS, and the cells were infected with 500 pfu of appropriate adenovirus. Following incubation at 37°C in a 5% CO$_2$ incubator for
one hour, 6 ml DMEM containing 2% FBS was added back to each plate. Following 20 hours of incubation, cells were lysed and assayed for p38 kinase or Akt kinase activity.

**Delivery of synthetic peptide into human neutrophils.**

The synthetic MK2 inhibitory peptide or control peptide representing a portion of EGF receptor (EGFR) was introduced into human neutrophils by incubating cells with peptide for 40 minutes at 37°C in a solution containing 20 mM Hepes, 5 mM KCl, 150 mM NaCl, 1 mM MgCl₂, 1 mM CaCl₂, and 10 mM glucose before exposing the cells to hypotonic shock for 20 sec to stimulate intracellular delivery of the peptide, as described previously by Zu et al. (22).

**Measurement of Akt Kinase Activity.**

Akt kinase activity was measured by the ability of immunoprecipitated enzyme to phosphorylate histone H2B. Briefly, 1 X 10⁷ neutrophils were prewarmed for 5 min at 37°C prior to stimulation with fMLP. The reaction was terminated by centrifugation at 2,500 x g followed immediately by lysis in buffer containing NP-40 1% (v/v), glycerol 10% (v/v), NaCl 137 mM, Tris-HCl 20 mM pH 7.4, aprotinin 1 µg/ml, leupeptin 1 µg/ml, PMSF 5 mM, NaF 20 mM, sodium pyrophosphate 1 mM, sodium orthovanadate 1 mM and Triton X-100 1% (v/v). Lysates were centrifuged at 15,000 x g for 15 min at 4°C and supernatants were incubated with 2 µl of anti-Akt antisera rotating continuously for 1 hr at 4°C and protein A sepharose beads for an additional hr. Beads were washed once each
in lysis buffer and kinase buffer (HEPES 20 mM, MgCl_2 10 mM, MnCl_2 10 mM) and incubated in a 30 \mu l reaction mixture containing [\gamma^{32}P]ATP 5 \mu Ci, DTT 1 mM, histone H2B 85.7 \mu g/ml and kinase buffer. Reactions were incubated at 25°C for 30 min, and terminated by the addition of 6 \mu l of 6X Laemmli buffer. The samples were boiled for 3 min and the products were resolved by 10% SDS-PAGE and products were visualized by autoradiography.

*Measurement of p38 Kinase Activity.*

p38 MAPK activity was measured by assaying the ability of immunoprecipitated enzyme to phosphorylate ATF2, as described previously (23).

*Measurement of MK2 Activity.*

MK2 activity was measured by assaying the ability of immunoprecipitated enzyme to phosphorylate recombinant Hsp27, as described previously by Krump et al (24).

*Preparation of GST and GST-MK2 Sepharose Beads.*

GST-pGEX-2T and MK2-GST-pGEX2T cDNAs were transformed into *E.coli* BL21PlysS and the expression and purification of GST and GST-MK2 fusion protein was performed as described previously (25).

*Western blot analysis of phospho-p38 and phospho-Akt.*
Tyrosine phosphorylation of ERK and p38 kinase and phosphorylation of S473 or T308 on Akt was determined by western blotting with phosho-specific antisera. Following lysis, proteins were separated with 10% SDS-PAGE, transferred onto nitrocellulose membrane, and blocked with 5% milk in Tween-20 Tris Buffered Saline (TTBS) (w/v) for one hour. Blots were probed with appropriate phospho-specific antibody in 5% BSA/TTBS overnight, and antibodies were detected using peroxidase-conjugated, secondary antibody in 5% milk/TTBS for an hour. Products were visualized by chemiluminescence. To verify equal loading of protein in each lane, the blots were stripped and reprobed for total p38, ERK, or Akt.

*Immunoprecipitation of p38, MK2, Hsp27 and Akt.*

Neutrophils (2 x 10^7) were prewarmed at 37°C for 5 min. prior to stimulation with or without 0.3 μM fMLP. The reactions were stopped by centrifugation followed immediately by addition of 200 μl immunoprecipitation (IP) lysis buffer containing Tris-HCl 20 mM pH 7.4, NaCl 150 mM, Triton-X-100 1% (v/v), NP-40 0.5% (v/v), EDTA 1 mM, EGTA 1 mM, sodium orthovanadate 20 mM, p-nitrophenol phosphate 10 μM, NaF 20 mM, PMSF 5 mM, aprotinin 21 μg/ml, and leupeptin 5 μg/ml. Following centrifugation at 15,000 x g for 15 min at 4°C, cleared lysates were incubated with 5 μl of anti-Akt antisera, 3 μl anti-p38, 2 μl anti-Hsp27, or 2 μl anti-MK2 overnight with continuous rotation at 4°C. Protein A Sepharose beads (15 μl) were then added and samples were rotated for an additional 2 hours at 4°C. Beads were washed once by centrifugation in Krebs
buffer, then resuspended in 50 µl 2X Laemmli buffer and boiled for 3 min. Proteins were separated by 10% SDS-PAGE, transferred onto nitrocellulose membrane, and blocked with 5% milk /TTBS for an hour. Blots were probed with anti-p38 (1:1000), anti-Hsp27 (1:1000), anti-MK2 (1:2000), or anti-Akt (1:1000) antiserum in 5% BSA/TTBS (w/v) and peroxidase-conjugated, secondary antibody in 5% milk/TTBS (w/v). Products were visualized by chemiluminescence.

GST Pull-Down Assay.

Neutrophils (2 x 10^7) were lysed with 200 µl IP lysis buffer. GST-Akt agarose, GST-MK2 sepharose, Protein A agarose, Protein A sepharose or GST sepharose beads were added to the lysates and incubated at 4°C for one hour with shaking. The beads were washed three times with Krebs buffer and 15 µl of 2x Laemmli buffer was added to each tube. The samples were boiled for 3 minutes and then subjected to 10% SDS-PAGE. Proteins were transferred onto nitrocellulose and immunoblotted for p38, MK2, Hsp27 and Akt as described above.

Phosphorylation with Active Recombinant MK2:

Neutrophils (2 x 10^7) were lysed with 200 µl IP lysis buffer. Lysates were precleared with 15 µl protein A sepharose beads for one hour at 4°C with shaking. Anti-Akt (3 µl), anti-MK2 (3 µl), anti-Hsp27 (3 µl), or anti-p38 (3 µl) antisera was added to the precleared neutrophil lysate and incubated overnight.
at 4°C with shaking. Protein A sepharose beads (15 µl) were then added to lysates and incubated for one hour at 4°C with shaking. Beads were washed once each in lysis buffer and kinase buffer (HEPES 20 mM, MgCl₂ 10 mM, MnCl₂ 10 mM) and incubated in a 30 µl reaction mixture containing 3 µl [γ⁻³²P]ATP (1 mCi/100 µl), or 1 µM ATP, 1 µl active recombinant MK2 (25 ng/µl) and 26 µl kinase buffer. Reactions were incubated at 30°C for 2 hours, and the reaction terminated by the addition of 30 µl of 2x Laemmli buffer. The samples were boiled for 3 min and products were resolved by 10% SDS-PAGE. Phosphorylation was visualized by autoradiography.

Trypsin digestion and Mass Spectroscopic Analysis:

Coomassie blue stained regions from 1D PAGE were cut from the gel in approximately 1 mm³ sections and were taken for tryptic hydrolysis using a modification of the method of Jensen et al. (26). Essentially, the gel was washed using NH₄HCO₃ and CH₃CN, then proteins were reduced using dithiothreitol (DTT) and alkylated using iodoacetamide. After washing, proteins were hydrolyzed using modified trypsin (Promega). Differences from the method of Jensen et al. were the use of higher (20mM) DTT concentration, larger volume (0.1 mL) washes following alkylation and exclusion of calcium from the trypsin mixture.

Peptides were then taken for thin film spotting for MALDI using α-cyanohydroxycinnamic acid as matrix on stainless steel targets with 1-2 µL spots. Mass spectral data were obtained using a Tof-Spec 2E (Micromass) and a 337
nm N₂ laser at 20-35% power in the reflector mode. Spectral data were obtained by averaging 10 spectra, each of which was the composite of 10 laser firings. Mass axis calibration was accomplished using peaks from tryptic autohydrolysis. Data were analyzed using MassLynx ProteinProbe software and the Mascot database.
RESULTS

*P38 Kinase but not ERK, Regulates Akt Phosphorylation.*

Both formyl peptide receptors and Fcγ receptors stimulate Akt phosphorylation in human neutrophils (16). To determine the optimal time of stimulation, a time course of Akt S473 phosphorylation following addition of 3 x 10^{-7} M fMLP or FcγR crosslinking was performed. Both agonists stimulated optimal Akt phosphorylation at 2 minutes (data not shown).

To investigate the involvement of ERK and p38 in PI-3K-dependent Akt activation in neutrophils, we measured fMLP-stimulated Akt S473 phosphorylation in the presence or absence of LY294002, wortmannin, PD98059, or SB203580. Figure 1a shows that wortmannin, LY294002, and SB203580 inhibited fMLP-stimulated Akt S473 phosphorylation, while PD98059 had no effect. To determine whether p38 regulation of Akt S473 phosphorylation was unique to chemoattractant receptors, we examined the effect of SB203580 on FcγR-stimulated Akt S473 phosphorylation (fig. 1b). Pre-treatment with SB203580 blocked Akt S473 phosphorylation stimulated by FcγIIa and FcγIIIb receptor crosslinking. Thus, p38 kinase inhibition attenuates both formyl peptide receptor and FcγR-stimulated Akt phosphorylation.

*S473 is More Sensitive to SB203580 Inhibition than T308.*

A previous study found that SB203580 inhibited PDK1 phosphorylation of Akt T308 at concentrations greater than 3 μM (27). To separate the effects of
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SB203580 on PDK1 and p38 kinase, we performed concentration inhibition experiments on phosphorylation of T308 and S473. Neutrophils were pretreated with varying concentrations of SB203580 for an hour prior to stimulation with fMLP. Concentration inhibition studies showed that SB203580 reduced fMLP-stimulated phosphorylation of S473 at 0.3 µM, while at least 10 µM SB203580 was required to see a diminution of T308 phosphorylation (fig. 2). The concentrations of SB203580 required to inhibit Akt T308 phosphorylation (10 µM) are approximately 20-fold higher than the IC₅₀ for p38 kinase inhibition (0.3-0.5 µM) (27), while Akt S473 phosphorylation is inhibited by SB203580 at the IC₅₀ for p38 kinase. These results suggest that inhibition of p38 kinase attenuates S473 phosphorylation, while T308 phosphorylation is independent of p38 kinase.

*p38 Kinase Mediates PIP₃-Dependent Akt Phosphorylation.*

To determine whether p38 kinase is upstream or downstream of PI-3K in the pathway leading to Akt activation, we examined the ability of PIP₃ to stimulate p38 kinase and Akt S473 phosphorylation in neutrophils. PIP₃ stimulated both p38 kinase (fig 3a) and Akt S473 phosphorylation (fig. 3b) in a time dependent manner with optimal stimulation at 1 min. To determine if PIP₃-stimulated Akt phosphorylation was mediated by p38 kinase, neutrophils were pretreated with 10 µM SB203580 prior to addition of PIP₃. Fig 3b shows that SB203580 inhibited PIP₃-mediated Akt S473 phosphorylation, indicating that p38 kinase activation is necessary for PI-3K mediated activation of Akt in human neutrophils. PIP₃ also
stimulated ERK activation with a time course similar to p38 activation (data not shown).

*Constitutively Active MKK3/6 Stimulates Akt Activation in HEK 293 Cells.*

As neutrophil half-life is not long enough to allow genetic manipulation, HEK 293 cells were transiently transfected with adenoviruses containing empty vector, MKK3bE (constitutively active MKK3), MKK3A (dominant negative MKK3), MKK6bE (constitutively active MKK6) or MKK6A (dominant negative MKK6) to directly examine the ability of p38 kinase to stimulate Akt activation. Figure 4a shows that MKK3bE and MKK6bE stimulated increased Akt activity, while the dominant negative mutants had no effect. Both MKK3bE and MKK6bE stimulated increased p38 kinase activity (fig. 4b), as measured by an *in vitro* kinase assay using ATF2 as substrate.

*Akt is Physically Associated with Components of the p38 Kinase Pathway.*

Previous studies reported that MK2 phosphorylates Akt S473 *in vitro* and that Hsp27 associates only with active Akt (19,20). Coupled with our data that phosphorylation of S473 is dependent on p38 kinase, we postulated that Akt exists in a signaling complex with MK2 and p38 kinase. Therefore, the association of Akt with p38 kinase, MK2 and Hsp27 was examined in unstimulated and stimulated neutrophils. Lysates prepared from unstimulated and fMLP-stimulated cells were immunoprecipitated with anti-Akt antibody. Proteins in the immunoprecipitate were separated by SDS-PAGE and
immunoblotted with anti-Akt, anti-p38, anti-MK2, and anti-Hsp27. Figure 5a shows that p38 kinase, MK2 and Hsp27 were all present in Akt immunoprecipitates from unstimulated cells. Stimulation with fMLP resulted in a time-dependent dissociation of Hsp27 from the complex. Additionally, neutrophil lysates immunoprecipitated with anti-Hsp27, anti-MK2, or anti-p38 were immunoblotted for Akt. Akt was detected in all three immunoprecipitates (data not shown).

Another method for detecting of protein-protein interactions is a GST pull-down assay. GST fused Akt or MK2 proteins were expressed in E.coli and were immobilized on glutathione-agarose or glutathione-sepharose beads. Neutrophil lysates were incubated with the protein-immobilized beads or GST sepharose beads. The proteins attached to the beads were separated by SDS-PAGE and immunoblotted for Akt, p38 kinase, MK2 and Hsp27. Figure 5b shows that GST-Akt was associated with p38 kinase, MK2 and Hsp27 and GST-MK2 associated with Akt, p38 kinase and Hsp27. GST sepharose beads alone did not bind to Akt, p38 kinase, MK2 or Hsp27 from neutrophil lysates (data not shown).

**MK2 Phosphorylation of Akt.**

A direct target of p38 kinase, MK2, phosphorylates Akt in vitro (19). A previous report suggested, however, that MK2 is unlikely to mediate Akt activation because agonists which activate Akt in HEK 293 cells failed to activate MK2. We examined the ability of recombinant active MK2 to phosphorylate recombinant Akt (fig. 6a) and Akt present in anti-Akt, anti-MK2, anti-p38 and anti-
Hsp27 immunoprecipitates from human neutrophils. Figures 6a and 6b show that MK2 stimulated phosphorylation of a 66-kDa protein in all conditions. The phosphorylated protein was trypsin digested and resulting peptides were subjected to matrix assisted laser desorption mass spectroscopic analysis, and identified by peptide mass fingerprinting to be Akt. Recombinant MK2 also stimulated phosphorylation of S473 Akt in anti-Akt, anti-MK2, and anti-Hsp27 immunoprecipitates from neutrophils (figure 6c). To determine if the differences in the role of MK2 as PDK2 could be due to cell-specific differences in the Akt isoforms, we immunoblotted neutrophil lysates with anti-Akt1, anti-Akt2 and anti-Akt3 antibodies. All 3 isoforms of Akt were present in human neutrophils (data not shown).

Inhibition of Akt Phosphorylation and Activation by MK2 Inhibitory Peptide.

Zu et al. reported that a peptide representing the phosphorylation site of Hsp27 inhibited MK2 phosphorylation of substrates (22). A concentration inhibition study showed that 160 μM of the MK2 inhibitory peptide was required to reduce fMLP-stimulated MK2 phosphorylation of Hsp27 (figure 7a). A non-related peptide at the same concentration did not reduce MK2-mediated Hsp27 phosphorylation. The concentration of the MK2 peptide required was significantly greater than the 30 μM concentration reported by Zu et al (22). A similar concentration of the MK2 peptide was required to inhibit the ability of active recombinant MK2 to phosphorylate Akt from neutrophil anti-Akt immunoprecipitates (figure 7b). To determine the role of MK2 in Akt
phosphorylation and activation, we preincubated neutrophils with the MK2 inhibitory peptide prior to stimulation with fMLP. Intracellular delivery of the inhibitory peptide reduced both fMLP-stimulated Akt S473 phosphorylation (fig 7c) and Akt activity (fig 7d), while a non-related peptide had no effect. Taken together, our results indicate that MK2 phosphorylates S473, which leads to activation of Akt in human neutrophils.

**ERK Activation is Independent of Akt.**

We examined the effect of pretreatment with 50 µM PD98059 on PIP$_3$-stimulated Akt 473 phosphorylation and found that PD98059 did not alter PIP$_3$-stimulated Akt phosphorylation (data not shown), suggesting ERK is not upstream of Akt. To determine if ERK is downstream of Akt, we pretreated neutrophils with SB203580 prior to stimulation with fMLP and measured ERK activity by an *in vitro* kinase assay and by immunoblot for phospho-ERK. Inhibition of p38 kinase did not alter fMLP-stimulated ERK activity by either of the two methods (data not shown). These data suggest that ERK does not participate in the Akt signaling pathway, despite the requirement of PI-3K for ERK activation.
DISCUSSION

Both Akt and p38 kinase are rapidly activated in neutrophils by a number of inflammatory mediators, and one or both kinases participate in respiratory burst activity, chemotaxis, priming, and apoptosis (17,18,28,29,31,32). Previous studies indicated that activation of both kinases is mediated by products of PI-3K (18,30). Our study provides evidence for the first time that p38 kinase participates in the signal transduction pathway leading to Akt activation. Akt activation requires its translocation from a cytosolic location to the plasma membrane, phosphorylation of T308 by PDK1, and phosphorylation of S473 by an unknown kinase heretofore called PDK2 (12,33). All three of these activation steps are dependent on products of PI-3K (30,33). Previous reports suggested that phosphorylation of S473 was the result of auto-phosphorylation following PDK1-dependent phosphorylation of Akt T308 or was due to sequential phosphorylation of T308 and then S473 by PDK1 (34). Our results indicate that p38 kinase is required for PIP3 stimulated activation of p38 kinase and Akt in human neutrophils, and PIP3-dependent phosphorylation of S473 on Akt is inhibited by SB203580. The pyridinyl imidazole SB203580 is a relatively specific inhibitor of the α and β isoforms of p38 kinase (35). Recently, Lali et al. (27) reported that SB203580 inhibited PDK1 at concentrations significantly greater than the IC50 for p38 kinase. We excluded this explanation for our results by demonstrating different SB203580 concentration-inhibition curves for T308 and S473 phosphorylation. SB203580 at the IC50 for p38 kinase (0.3 μM) inhibited S473 phosphorylation, while concentrations of SB203580 below 10 μM failed to
inhibit T308 phosphorylation. Further evidence that p38 kinase participates in Akt activation was provided by the ability of constitutively active MKK3 and MKK6 to stimulate Akt activation following transfection into HEK 293 cells. These studies also suggest that p38 kinase-mediated S473 phosphorylation is sufficient to induce Akt activation independent of PI-3K. Neutrophils were inadequate for these genetic studies, as they undergo constitutive apoptosis in culture resulting in survival of less than 40% of cells at 48 hr (36).

Alessi et al. (19) previously showed that MK2 phosphorylates recombinant Akt on S473; however, they discounted a role for MK2 in intact cells, as fibroblasts showed IGF-1-dependent Akt activation in the absence of MK2 activation. The present study demonstrates that p38 kinase-stimulated Akt S473 phosphorylation is mediated by MK2. Not only did active recombinant MK2 phosphorylate recombinant Akt in vitro, but Akt immunoprecipitated from neutrophil lysates was phosphorylated by active recombinant MK2, as well. Direct evidence for MK2-mediated phosphorylation of Akt S473 in intact neutrophils was obtained using an MK2 inhibitory peptide described by Zu et al. (22). Introduction of the inhibitory peptide into freshly isolated neutrophils inhibited phosphorylation of Hsp27 following immunoprecipitation of MK2 from fMLP-stimulated cells. Similarly, the MK2 inhibitory peptide reduced fMLP-stimulated Akt activation and S473 phosphorylation, while the control EGFR peptide had no affect. Taken together, these data indicate that MK2 acts as PDK2 in human neutrophils.

The concept that signal transduction pathway components form multimeric
complexes held together by scaffolding proteins, rather than existing free in the cytosol, has received significant experimental support recently. Scaffolding proteins have been described for two other MAPK modules, ERK and JNK (37,38). Therefore, the possibility that p38 kinase, MK2 and Akt form a signaling complex was examined. Using two separate methods, immunoprecipitation and GST pull-down, the present study shows for the first time that p38 kinase, MK2 and Akt exist as a complex that does not dissociate upon activation. Hsp27 was previously reported to associate with activated Akt (20). Therefore, we examined the association of Hsp27 with Akt, MK2, and p38 kinase following Akt, p38 or MK2 immunoprecipitation and following GST pull-down with MK2 or Akt. Hsp27 was present in these complexes in unstimulated neutrophils. As opposed to MK2 and p38 kinase, Hsp27 dissociated from Akt immunoprecipitates following neutrophil stimulation with fMLP. Taken together, our data indicate that three components of the p38 kinase module, p38 kinase, MK2, and Hsp27, form a signaling complex with Akt. MK2, which has been shown to phosphorylate Hsp27 (39,40), directly phosphorylates S473 on Akt. Hsp27 dissociates from the complex during stimulation, suggesting that Hsp27 performs a regulatory function. Our data do not indicate whether Hsp27 dissociates from the complex before or after translocation or phosphorylation of Akt; and, therefore, no conclusion as to whether Hsp27 acts as a positive or negative regulator is possible. The presence of other components in the signaling complex and the scaffolding protein which binds the complex together remain to be determined.
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The abbreviations used are:

Akt, protein kinase B; PDK1/PDK2, 3'-phosphoinositide-dependent kinase-1/2; PIP₂, phosphatidylinositol-3,4-bisphosphate; PIP₃, phosphatidylinositol-3,4,5-trisphosphate; PI-3K, phosphatidylinositol 3-kinase; fMLP, fMet-Leu-Phe; MAP, mitogen activated protein; MK2, MAP kinase activated protein kinase-2; MKK3bE/6bE, constitutively active MAP kinase kinase 3/6; MKK3A/6A, dominant negative MAP kinase kinase 3/6; Hsp27, heat shock protein 27; GST, glutathione S-transferase.
FIGURE LEGENDS

Figure 1. Effect of PI-3K, p38 kinase and ERK Inhibition on fMLP-Stimulated Akt Phosphorylation.  
a, Immunoblot of neutrophil lysates probed with phospho-specific Akt S473 (pS473-Akt) antisera. Pretreatment with the PI-3K inhibitors wortmannin and LY294002 and the p38 kinase inhibitor SB203580 inhibited S473 phosphorylation following fMLP stimulation, whereas the MEK/ERK inhibitor PD98059 did not block fMLP-stimulated Akt phosphorylation, (n=4).  
b, Immunoblot of neutrophil lysates probed with phospho-specific Akt S473 (pS473-Akt) and Akt antisera. Pretreatment with the p38 kinase inhibitor SB203580 blocked FcγRIIa- and FcγRIIIb-stimulated Akt phosphorylation, (n=3).

Figure 2. Effect of p38 Inhibition on Phosphorylation of T308/S473. 
Immunoblot of neutrophil lysates probed with phospho-specific Akt S473 (pS473-Akt), T308 (pT308-Akt), or Akt antisera. Pretreatment with SB203580 at concentrations of 0.3 µM or higher inhibited fMLP-stimulated Akt S473 phosphorylation, while inhibition of fMLP-stimulated Akt T308 phosphorylation was not observed at concentrations below 10 µM, (n=3).

Figure 3. PIP3-Stimulated Time Course of p38 and Akt Phosphorylation and Effect of p38 Inhibition on Akt phosphorylation.  
a, Immunoblot of neutrophil lysates probed with phosphotyrosine p38 (pp38) and p38 antisera. PIP3-stimulated optimal p38 phosphorylation by 1 min, (n=3).  
b, Immunoblot using phospho-specific Akt S473 (pS473-Akt) and Akt antisera. PIP3-stimulated optimal Akt S473 phosphorylation at 1 min. This phosphorylation was inhibited by pre-treatment with 10 µM SB203580, (n=3).
Figure 4. Expression of Constitutively Active MKK3 or MKK6 Adenoviruses Stimulated p38 and Akt Activation in HEK 293 Cells. Twenty hours following adenoviral transfection, HEK 293 cells were lysed, and lysates were subjected to p38 and Akt in vitro kinase assay. a, Akt kinase activity was measured in an in vitro kinase assay by $^{32}$P-ATP phosphorylation of histone H2B. Constitutively active MKK3/6, but not dominant negative MKK3/6, activated Akt in HEK 293 cells. b, p38 kinase activity was measured in an in vitro kinase assay by $^{32}$P-ATP phosphorylation of ATF2. These results indicate that constitutively active MKK3/6 activated p38 kinase in HEK 293 cells, (n=2).

Figure 5. Association of Akt with Components of the p38 Signal Module. a, Immunoblot for Akt, p38, MK2, and Hsp27 in immunoprecipitates with Akt antibody. All three components of the p38 kinase module were present. Only Hsp27 dissociated from Akt upon fMLP stimulation, (n=3). b, Immunoblot for MK2, p38, and Hsp27 indicate they associate with GST-Akt agarose beads while Akt, p38, Hsp27 associate with GST-MK2 sepharose beads. These results confirm the association of Akt, p38, MK2 and Hsp27.

Figure 6. MK2 Acts as PDK2 in Human PMNs. a, Autoradiograph of SDS-PAGE following addition of active recombinant MK2 to recombinant Akt in the presence of $^{32}$p-ATP. Significant Akt phosphorylation was seen at 2 hours. b, Autoradiograph of SDS-PAGE following addition of active recombinant MK2 to neutrophil lysates immunoprecipitated with anti-Akt, anti-MK2, anti-p38, and anti-Hsp27 (immuno-Akt, immuno-MK2, immunop-38, and immuno-Hsp2) in the presence of $^{32}$p-ATP. Active recombinant MK2 phosphorylated a 66 kDa protein
in all the immunoprecipitates. c, Active recombinant MK2 was added to neutrophil lysates immunoprecipitated with anti-Akt, anti-MK2, and anti-Hsp27 in the presence of 1 µM ATP. The samples were run on SDS-PAGE and immunoblotted with phospho-specific Akt S473 (pS473-Akt). Active recombinant MK2 phosphorylated S473 of Akt in all the immunoprecipitates.

**Figure 7. Effect of MK2 Inhibitory Peptide on MK2 and Akt Activation in Human Neutrophils.** a,. Concentration-inhibition assay of MK2 inhibitor peptide in neutrophils. Preincubation of human neutrophils with 160 µM MK2 inhibitory peptide (MK2 peptide), but not EGFR peptide, inhibited fMLP-stimulated MK2 kinase activity as measured by phosphorylation of recombinant Hsp27 by immunoprecipitated MK2 in an *in vitro* kinase assay. b, Autoradiograph of SDS-PAGE following addition of active recombinant MK2 in the presence of 80 µM or 160 µM MK2 inhibitory peptide or 160 µM EGFR peptide and 32p-ATP to neutrophil lysates immunoprecipitated with anti-Akt. MK2 inhibitory peptide, but not EGFR peptide inhibited the ability of active recombinant MK2 to phosphorylate a 66 kDa protein in a concentration dependent manner. c, Immunoblots using phospho-specific Akt S473 (pS473-Akt). Preincubation of human neutrophils with MK2 inhibitory peptide, but not EGFR peptide, inhibited fMLP-stimulated Akt (S473) phosphorylation. d, Preincubation of human neutrophils with MK2 inhibitory peptide, but not EGFR peptide, inhibited fMLP-stimulated Akt kinase activity measured by phosphorylation of histone H2B in an *in vitro* kinase assay.
Figure 1

**Panel A**

- pS473-Akt
- fMLP
- 100 nM Wortmannin
- 100 µM LY294002
- 10 µM SB203580
- 50 µM PD98059

**Panel B**

- Akt
- pS473-Akt
- FoγRlla + Gam
- FoγRllb + Gam
- 10 µM SB203580
Figure 2

- SB 203580
- Akt
- pS473-Akt
- pT308-Akt
- 0.3 μM fMLP
- 0.1 μM
- 0.3 μM
- 3 μM
- 1 μM
- 10 μM
**Figure 3**

(a) Time (min) vs. p38, pp38, and 5 μM PIP₃

(b) Time (min) vs. Akt, pS473-Akt, 5 μM PIP₃, and 10 μM SB203580
Figure 4

(a) Histone 2HB

(b) ATF2
Figure 5

a

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<th>0</th>
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</tr>
<tr>
<td>MK2</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>p38</td>
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0.3 µM fMLP
- + + +

b

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<tr>
<th></th>
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Akt
p38
Hsp27
Figure 6

Panel a:
- Time (hr)
- 66 kDa
- Active recomb. MK2

Panel b:
- 66 kDa
- Active recomb. MK2
- Immuno- Akt
- Immuno-MK2
- Immuno- p38
- Immuno- Hsp27

Panel c:
- pS473-Akt
- Active Recomb. MK2
- Immuno Akt
- Immuno MK2
- Immuno Hsp27
Figure 7

a

Hsp27

MK2 peptide
EGFR peptide
0.3 μM fMLP

b

66 kDa
Active Recomb. MK2
MK2 Peptide
EGFR peptide

3347-Akt

0.3 μM fMLP
160 μM MK2 peptide
160 μM EGFR peptide

Histone H2B

0.3 μM fMLP
160 μM MK2 peptide
160 μM EGFR peptide
p38 KINASE-DEPENDENT MAPKAPK-2 ACTIVATION FUNCTIONS AS PDK2 FOR AKT IN HUMAN NEUTROPHILS
Madhavi J. Rane, Patricia Y. Coxon, David W. Powell, Rose Webster, Jon B. Klein, Peipei Ping, William Pierce and Kenneth R. McLeish

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