Identification of the p16-Arc Subunit of the Arp 2/3 Complex as a Substrate of MAPK-activated Protein Kinase 2 by Proteomic Analysis*

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The p38 MAPK pathway regulates multiple neutrophil functional responses via activation of the serine-threonine kinase MAPK-activated protein kinase 2 (MAPKAPK2). To identify substrates of MAPKAPK2 that mediate these responses, a proteomic approach was used in which in vitro phosphorylation of neutrophil lysates by exogenously added active recombinant MAPKAPK2 was followed by protein separation using two-dimensional electrophoresis. Peptide mass fingerprinting of peptides defined by MALDI-MS was then utilized to identify phosphorylated proteins detected by autoradiography. Six candidate substrates were identified, including the p16 subunit of the seven-member Arp2/3 complex (p16-Arc). In vitro studies confirmed that MAPKAPK2 interacts with and phosphorylates the A isoform, but not the B isoform, of p16-Arc with a stoichiometry of 0.6 to 0.7. MAPKAPK2 also phosphorylated p16-Arc in intact Arp2/3 complexes precipitated from neutrophil lysates. Mutation of serine-77 to alanine on the A isoform prevented phosphorylation by MAPKAPK2. The ability of MAPKAPK2 to phosphorylate one isoform of p16-Arc suggests a possible mechanism by which the p38 MAPK cascade regulates remodeling of the actin cytoskeleton.

MAPK cascades are modules containing three kinases, including a MAPK, which is activated by a dual specificity serine-threonine/tyrosine kinase called MAPK/extracellular signal-regulated kinase kinase (MEK), which in turn is activated by a serine-threonine kinase termed MEK kinase (MEKK) (1, 2). One of the cascades, p38 MAPK, is activated in human neutrophils by numerous pro-inflammatory stimuli, including chemotactants, chemokines, bacterial phagocytosis, Fc receptor cross-linking, lipopolysaccharide, tumor necrosis factor-α, and granulocyte-macrophage colony-stimulating factor (3–10). Activation of p38 MAPK is required for a number of neutrophil functions, including chemotaxis, chemokine expression, respiratory burst activity, exocytosis, and priming (8, 11–18). Studies using animal models confirm a role for p38 MAPK in inflammatory diseases (19–25).

The molecular pathways leading from p38 MAPK to various functional responses in neutrophils have not been fully defined. Of the possible targets for p38 MAPK phosphorylation, only the serine-threonine kinase, MAPK-activated protein kinase 2 (MAPKAPK2), has been identified in neutrophils (26). Zu et al. (27) report that a peptide inhibitor of MAPKAPK2 attenuated neutrophil respiratory burst activity stimulated by formylmethionylleucylphenylalanine. Using the same peptide, we showed MAPKAPK2 also participated in exocytosis and chemotaxis (28). Hannigan et al. (26) recently reported that neutrophils from MAPKAPK2−/− mice demonstrated impaired directional migration, whereas adherence was normal. Thus, MAPKAPK2 appears to be a critical downstream kinase for a number of p38 MAPK-dependent neutrophil functions. Previously identified targets of MAPKAPK2 phosphorylation in neutrophils include two actin-binding proteins, heat shock protein (Hsp) 27 and leukocyte-specific protein 1 (LSP1), Akt, and 5-lipoxygenase (29–32). The functional significance of MAPKAPK2 phosphorylation of Hsp27 and 5-lipoxygenase has not been determined, whereas LSP1 participates in neutrophil chemotaxis (33). To define the signal transduction pathways that control p38 MAPK-mediated functional responses in neutrophils, a more complete list of MAPKAPK2 substrates is required.

To identify MAPKAPK2 substrates, we developed a proteomic approach using a combination of in vitro phosphorylation of neutrophil lysate by exogenous active recombinant MAPKAPK2, protein separation by two-dimensional electrophoresis, and phosphoprotein identification by matrix-assisted laser desorption and ionization-mass spectrometry (MALDI-MS). Approximately 30 proteins were phosphorylated by MAPKAPK2, as determined by autoradiography of two-dimensional gels. Initial studies identified six proteins as potential substrates, including the known substrate LSP1. One of the previously unknown substrates identified was p16-Arc, a subunit...
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EXPERIMENTAL PROCEDURES

Materials—The constructs for pGEX-5X-2-p16A and B as well as pGEX-5X-2-Neutrophils (108) were lysed in 400 l of lysis buffer containing 400 l of 1 M urea. The lysate was stored at −20°C, whereas p16-B is relatively resistant to MAPKAPK2 phosphorylation.

Neutralization—Neutrophils were isolated from healthy donors using plasma-Percoll gradients as described by Haslett et al. (39). After isolation, neutrophils were suspended in Krebs-Ringer phosphate buffer, pH 7.2, at the desired concentration. The study was approved by the University of Louisville Human Studies Committee.

Neutralization—Preparation for MAPKAPK2 Substrate Identification—Neutrophils (1 × 108) were lysed in 400 l of lysis buffer containing 2 M thiourea, 7 M urea, 65 mM CHAPS, 58 mM dithiothreitol, and 10% glycerol (40). Mass spectral data were obtained using a Tof-Spec 2E (Micromass) and a 35-mm Na2O 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 calibrations were accomplished using peaks from tryptic autohydrolysis. Peptide masses obtained by MALDI-MS analysis were used to search the National Center for Biotechnology Information data base (NCBI, www.matrixscience.com) to identify the intact protein. MS/MS-simulated peptide sequences from tryptic digests were obtained by MALDI-MS analysis were used to search the National Center for Biotechnology Information data base (NCBI, www.matrixscience.com) to identify the intact protein. MS/MS-simulated peptide sequences

Mass spectrometry analysis—Phosphorylation of recombinant p16-A and B by MAPKAPK2 was examined by incubation of active recombinant MAPKAPK2 (40 ng) with 10 l of [γ-32P]ATP and 0.5 l of recombinant protein in 30 l of kinase buffer containing 25 mM HEPES, 25 mM β-glycerophosphate, 25 mM MgCl2, 2 mM dithiothreitol, and 0.1 mM Na3VO4, pH 7.2. Reactions were incubated at 30°C for 1 h. After the incubation, reactions were terminated with Laemmli SDS sample dilution buffer, proteins were separated by 15% SDS-PAGE, and phosphorylation was visualized by autoradiography.

To determine the time course of phosphorylation, 400 ng of recombinant active MAPKAPK2 were incubated with 0.5 l of recombinant Hsp27 or p16-A and 10 l of [γ-32P]ATP at 30°C for times ranging from 30 min to 4 h. Optimal phosphorylation of both substrates was seen between 2 and 3 h. To determine the stoichiometry of phosphorylation, 400 ng of recombinant active MAPKAPK2 were incubated with 0.05, 0.1, and 0.3 l of Hsp27 or 0.5, 1, and 2 l of p16-A, 2 pmol of [γ-32P]ATP, and 200 pmol of ATP in 200 l of kinase buffer at 30°C for 3 h. To separate free ATP from radiolabeled proteins, the reaction mixture was applied to a prewashed Spin-out 6000 Micro column (Chemicon International, Inc.) and collected into 200 l of kinase buffer. Separate reaction mixtures containing 200 pmol of ATP and 0.5 pmol of [γ-32P]ATP in 200 l of kinase buffer were used to determine the specific activity of ATP in each reaction. Fifty l of each sample was counted by scintillation spectrometry, and the molar ratio of phosphorylation was calculated.

GST Pull Down—Neutrophil lysates were prepared by suspending 5 × 107 cells in 200 l of lysis buffer containing 20 mM Tris, pH 7.4, 1.4 mM Trion X-100, 0.5% Nonidet P-40, 25 mM MgCl2, 20 mM NaF, 0.2 mM Na3VO4, 1 mM EDTA, 1 mM EGTA, 5 mM phenylmethylsulfonyl fluoride, and 10% glycerol. GST, GST-p16A, or GST-p16B glutathione-coupled Sepharose were incubated with neutrophil lysate (400 l of protein) overnight at 4°C. After incubation, beads were washed 4 times with lysis buffer. Proteins were eluted with 15 l of 10× SDS sample dilution buffer containing 15 l of 337-nm N2 laser at 20°C for 1 h. After incubation, reactions were terminated by the addition of Laemmli SDS sample dilution buffer, proteins were separated by 15% SDS-PAGE, and immunoblotted for MAPKAPK2 using a polyclonal anti MAPKAPK2 antibody (Sigma).

GST pull downs of recombinant proteins were carried out by incubating GST or GST-MAPKAPK2 glutathione-coupled Sepharose with recombinant 35S-labeled p16-A or 35S-labeled p16-B in 50 l of kinase buffer containing 25 mM HEPES, 25 mM β-glycerophosphate, 25 mM MgCl2, 2 mM dithiothreitol, 0.1 mM Na3VO4, and 10% glycerol for 2 h at 4°C. Beads were then washed 4 times with kinase buffer, proteins were eluted with Laemmli SDS sample dilution buffer and separated by 15% SDS-PAGE, and the 35S-labeled proteins were detected by autoradiography.

In separate experiments, the Arp2/3 complex was precipitated from neutrophil extract as previously described (38). Briefly, lysate was subjected to pull-down by GST or GST fused to the C-terminal region (WA) of the Scar1 protein that interacts with the Arp2/3 complex. GST fused to a truncated portion of the WA protein, termed GST-W, was utilized as a negative control. One aliquot of each preparation was incubated with GST and GST-W, transferred to nitrocellulose membrane, and subsequently immunoblotted for Arp3 using a polyclonal anti-Arp3 antibody (Santa Cruz, CA). A second aliquot was suspended in kinase buffer (25 mM HEPES, 25 mM β-glycerophosphate, 25 mM MgCl2, 2 mM dithiothreitol, and 0.1 mM Na3VO4) in the presence and absence of 40 ng of active recombinant MAPKAPK2 and 0.5 pmol of [γ-32P]ATP at 4°C overnight. Tryp- sin-generated peptides were applied by a thin film-spotting procedure for MALDI-MS analysis using α-cyano-4-hydroxycinnamic acid as the matrix on stainless steel targets, as described by Jensen et al. (40).

MAPKAPK2 Substrate Identification—To obtain peptides for mass spectrometry analysis, protein spots were excised and digested with trypsin by modification of the method of Jensen et al. (40). The excised gel pieces were incubated for 15 min in 100 mM NH4HCO3, and 50% acetonitrile and dried by vacuum centrifugation, and then proteins were hydrolyzed by incubation in 20 ng/ml modified trypsin (Promega) at 37°C overnight. Trypsin-generated peptides were applied by a thin film-spotting procedure for MALDI-MS analysis using α-cyano-4-hydroxycinnamic acid as the matrix on stainless steel targets, as described by Jensen et al. (40). Mass spectral data were obtained using a Tof-Spec 2E (Micromass) and a 35-mm Na2O 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 calibrations were accomplished using peaks from tryptic autohydrolysis. Peptide masses obtained by MALDI-MS analysis were used to search the National Center for Biotechnology Information data base (NCBI, www.matrixscience.com) to identify the intact protein. MS/MS-simulated peptide sequences from tryptic digests were obtained by MALDI-MS analysis were used to search the National Center for Biotechnology Information data base (NCBI, www.matrixscience.com) to identify the intact protein. MS/MS-simulated peptide sequences

Trypsin Digestion and Mass Spectrometry Analysis—To obtain peptides for mass spectrometry analysis, protein spots were excised and digested with trypsin by modification of the method of Jensen et al. (40). The excised gel pieces were incubated for 15 min in 100 mM NH4HCO3, and 50% acetonitrile and dried by vacuum centrifugation, and then proteins were hydrolyzed by incubation in 20 ng/ml modified trypsin (Promega) at 37°C overnight. Trypsin-generated peptides were applied by a thin film-spotting procedure for MALDI-MS analysis using α-cyano-4-hydroxycinnamic acid as the matrix on stainless steel targets, as described by Jensen et al. (40). Mass spectral data were obtained using a Tof-Spec 2E (Micromass) and a 35-mm Na2O 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 calibrations were accomplished using peaks from tryptic autohydrolysis. Peptide masses obtained by MALDI-MS analysis were used to search the National Center for Biotechnology Information data base (NCBI, www.matrixscience.com) to identify the intact protein. MS/MS-simulated peptide sequences from tryptic digests were obtained by MALDI-MS analysis were used to search the National Center for Biotechnology Information data base (NCBI, www.matrixscience.com) to identify the intact protein. MS/MS-simulated peptide sequences.
RESULTS

Identification of MAPKAPK2 Substrates—MAPKAPK2 substrate screening was performed by incubating human neutrophil lysates with \[^{32}P\]ATP in the presence of active recombinant MAPKAPK2, indicating the absence of endogenous kinase activity under the conditions of neutrophil lysate preparation. In the presence of active recombinant MAPKAPK2 more than 30 proteins demonstrated phosphorylation. Six proteins identified as candidate MAPKAPK2 substrates by this method are marked on the autoradiograph in Fig. 1. LSP1 and myosin regulatory light chain were previously shown to be substrates for MAPKAPK2 (30, 46). A phosphorylated protein with a molecular mass below 25 kDa was identified as p16-Arc with 50% protein coverage (Table I). The possibility that p16-Arc was a false positive was considered for several reasons. First, cell lysis may permit access of exogenous kinases to proteins with restricted localization in intact cells. Second, urea denaturation may expose phosphorylation sites that are inaccessible in properly folded proteins. Third, disruption of protein-protein interactions may result in dissociation of signaling modules that direct kinase activity. Finally, protein spots may contain more than one protein, leading to identification of false substrates. Therefore, additional studies were performed to confirm the ability of MAPKAPK2 to interact with and phosphorylate p16-Arc.

MAPKAPK2 Interacts With and Phosphorylates p16-Arc—p16-Arc was recently shown to consist of two different isoforms, p16-A and p16-B, which are differentially expressed in various tissues and may determine which proteins bind to the intact Arp2/3 complex (40). To determine whether MAPKAPK2 interacts with p16-A and p16-B, GST-MAPKAPK2 pull-down of recombinant \[^{35}S\]labeled p16-Arc isoforms was performed by incubating GST or GST-MAPKAPK2 glutathione-coupled Sepharose with recombinant \[^{35}S\]p16-A or \[^{35}S\]p16-B. Fig. 2A shows that GST-MAPKAP2 coupled, but not GST-coupled, glutathione-Sepharose precipitated p16-A. Neither GST-MAPKAP2 nor GST-glutathione-Sepharose was able to precipitate p16-B (Fig. 2B). To determine whether endogenous MAPKAPK2 in neutrophil lysates interacts with p16-A or p16-B, neutrophil lysates were incubated with recombinant GST, GST-p16-A, or GST-p16-B coupled to glutathione-Sepharose. Precipitated proteins were separated by SDS-PAGE followed by immunoblotting for MAPKAPK2. Figs. 3, A and B, show that endogenous MAPKAPK2 was precipitated by GST-p16-A but not by GST-p16-B or GST-glutathione-Sepharose. These data suggest that MAPKAPK2 physically associates with p16-A but not p16-B.

To determine whether the interactions with MAPKAPK2 result in p16-Arc phosphorylation, active recombinant MAPKAPK2 was incubated with equivalent amounts of recombinant p16-A or p16-B and \[^{32}P\]ATP in an \it{in vitro} kinase assay. The proteins were separated by 15% SDS-PAGE and subjected to autoradiography. Fig. 4A shows that, despite equal loading of p16-A and p16-B, MAPKAPK2-phosphorylated p16-A more extensively than p16-B. A search of the amino acid sequences of p16-A and p16-B determined that p16-A contains a motif similar to the consensus MAPKAPK2 phosphorylation motif (KDRAGSS\(_{77}\)) that is absent in the B isoform. To determine whether this consensus site was the primary phosphorylation site, serine 77 was mutated to alanine (p16-A S77A). Fig. 4B shows that substitution of alanine for Ser-77 completely inhibited MAPKAPK2 phosphorylation of p16-A. These data suggest that MAPKAPK2 directly interacts with and phosphorylates the A isoform of p16-Arc at Ser-77, whereas interaction with and phosphorylation of the B isoform is minimal.

The time course of Hsp27 and p16-A phosphorylation by MAPKAPK2 were similar under identical incubation conditions. At 30 °C maximal phosphorylation occurred between 2
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Peptide mass fingerprint analysis of a 16-kDa neutrophil protein phosphorylated by active recombinant MAPKAPK2 (Fig. 1) matches seven peptide masses with p16-Arc. The total coverage is 56% of the protein. Peptides covered in p16-Arc are shown in bold letters.

| Masses submitted | Masses matched | Start | End | Peptide |
|------------------|----------------|-------|-----|---------|
| 705.44           | 706.35         | 82    | 87  | VLI5FK  |
| 1069.62          | 1070.71        | 132   | 143 | ALAAGGVSIVR |
| 1315.69          | 1316.71        | 48    | 60  | QGNMTALQALK |
| 1352.61          | 1353.64        | 13    | 23  | KVDVDEYDRENK |
| 2161.97          | 2162.99        | 113   | 131 | GFESPDSNNSAMLIQHEK |
| 2552.04          | 2553.05        | 24    | 47  | FVDEEDGSGAQPGDEGEVDSCLR |
| 3758.54          | 3759.24        | 14    | 47  | VVDVDEYDRENKPVDEEDGOOGQAGGEGEVDVDSCLR |

1 MSKNTVSSAR FRKVDVDEYD ENKVFDDEEG GDQQUAAPDEG EVDSLRQSN
51 MTIALQALKE NPIINTESQA VKDASSIVL KVLISFKAMD ERKQSDLDK
121 NVDLMKVT1 YKGFESPDSNNSAMLIQHEK TALLAAGGVS IVKVTARKT
151 V

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and 3 h for both proteins (Fig. 4C). Stoichiometry of phosphorylation was 0.6 and 0.7 mol of phosphate/mol of protein for p16-A in two separate experiments. The stoichiometry of phosphorylation for Hsp27 was calculated at 4 mol of phosphate/mol of Hsp27. Previous studies identified up to 4 sites of phosphorylation on Hsp27, Ser-15, Ser-78, Ser-82, and Ser-90 (47–49).

Phosphorylation of p16-Arc in the Arp2/3 Complex—To determine whether MAPKAPK2 interacts with p16-A and p16-B, recombinant [35S]methionine-labeled p16-A (panel A) or p16-B (panel B) was incubated with GST (first lane, panels A and B) or GST-MAPKAPK2 glutathione coupled Sepharose (second lane, panels A and B) and subjected to a pull-down assay. Proteins associated with the Sepharose beads were separated by SDS-PAGE and visualized by autoradiography. p16-A, but not p16-B, precipitated with MAPKAPK2. The third lane in both panels represents radiolabeled recombinant proteins run as a positive control.

A

![GST-MAPKAPK2 interacts with p16-Arc isoforms.](image1.png)

B

![GST-MAPKAPK2 interacts with p16-Arc isoforms.](image2.png)

Fig. 2. MAPKAPK2 interacts with p16-Arc isoforms. To determine whether MAPKAPK2 interacts with p16-A and p16-B, recombinant [35S]methionine-labeled p16-A (panel A) or p16-B (panel B) was incubated with GST (first lane, panels A and B) or GST-MAPKAPK2 glutathione coupled Sepharose (second lane, panels A and B) and subjected to a pull-down assay. Proteins associated with the Sepharose beads were separated by SDS-PAGE and visualized by autoradiography. p16-A, but not p16-B, precipitated with MAPKAPK2. The third lane in both panels represents radiolabeled recombinant proteins run as a positive control.

Identification of p16-Arc with MAPKAPK2 in neutrophil lysate.

To determine whether endogenous MAPKAPK2 interacts with p16-A and p16-B, neutrophil lysates (400 µg of protein) were incubated with GST-p16-A (panel A, first lane) or GST-p16-B (panel B, fourth lane) and subjected to a pull-down assay. GST glutathione-Sepharose (first lane, panels A and B), GST-p16-A coupled to glutathione-Sepharose (third lane, panel A), GST-p16-B coupled to glutathione-Sepharose (third lane, panel B), and neutrophil lysate (fifth lane, panels A and B) were used as controls. Proteins were separated by SDS-PAGE and immunoblotted (IB) for MAPKAPK2. Immunoblots show that MAPKAPK2 precipitated with p16-A but not with p16-B.

A

![GST-MAPKAPK2 interacts with p16-Arc isoforms.](image3.png)

B

![GST-MAPKAPK2 interacts with p16-Arc isoforms.](image4.png)

Fig. 3. Interaction of p16-Arc isoforms with MAPKAPK2 in neutrophil lysate. To determine whether endogenous MAPKAPK2 interacts with p16-A and p16-B, neutrophil lysates (400 µg of protein) were incubated with GST-p16-A (panel A, first lane) or GST-p16-B (panel B, fourth lane) and subjected to a pull-down assay. GST glutathione-Sepharose (first lane, panels A and B), GST-p16-A coupled to glutathione-Sepharose (third lane, panel A), GST-p16-B coupled to glutathione-Sepharose (third lane, panel B), and neutrophil lysate (fifth lane, panels A and B) were used as controls. Proteins were separated by SDS-PAGE and immunoblotted (IB) for MAPKAPK2. Immunoblots show that MAPKAPK2 precipitated with p16-A but not with p16-B.
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**Fig. 4. In vitro MAPKAPK2 phosphorylation of p16-Arc isoforms.** Panel A, equal amounts of recombinant p16-A (second lane) and p16-B (first lane) were subjected to an in vitro kinase assay with [γ-32P]ATP and active recombinant MAPKAPK2. Proteins were separated by 15% SDS-PAGE, and results were visualized by autoradiography. Autoradiographs show that p16-A was extensively phosphorylated compared with p16-B. Phosphorylation of recombinant Hsp27 by MAPKAPK2 was used as the control (third lane). Panel B, recombinant active MAPKAPK2 was incubated with [γ-32P]ATP and recombinant p16-A wild type (WT) (second lane) or p16-A S77A (first lane). Proteins were separated by SDS-PAGE, and results were visualized by autoradiography. Autoradiographs show that MAPKAPK2 phosphorylated p16-A WT, but not p16-A (S77A). The third lane represents MAPKAPK2-phosphorylated recombinant Hsp27 run as a positive control. Panel C, the time course of phosphorylation of p16-A and Hsp27 by MAPKAPK2 is shown. Hsp27 (2 μg) or p16-A (1 μg) were incubated with 400 ng of recombinant active MAPKAPK2 in the presence of [γ-32P]ATP at 30 °C for 30 min to 4 h. Optimal phosphorylation was visualized between 2 and 3 h for both proteins.

**Fig. 5. GST pull-down of Arp2/3 complex from neutrophil lysate.** Panel A, neutrophil lysate was incubated with GST-coupled to glutathione-Sepharose (first lane), coupled to GST-WA (second lane), or GST-W (third lane). Proteins were separated by SDS-PAGE and immunoblotted for Arp3. Immunoblots (IB) confirm that GST-WA, but not GST-W or GST, precipitated the Arp2/3 complex from neutrophil lysate. The fourth lane represents neutrophil lysate run as a positive control. Panel B, neutrophil lysate was incubated with GST coupled to glutathione-Sepharose (first lane), GST-WA-coupled glutathione-Sepharose (second lane), or GST-W-coupled glutathione-Sepharose (third lane). Aliquots from the pull-down assay were incubated with active recombinant MAPKAPK2 in presence of [γ-32P]ATP. Proteins were separated by 10% SDS-PAGE, and results were visualized by autoradiography. Autoradiographs show that MAPKAPK2 phosphorylated a 16-kDa protein only in the assay containing the intact Arp2/3 complex. Phosphorylation of recombinant p16-A by MAPKAPK2 was used as a positive control (fourth lane).

**MAPKAPK2 Interacts with the Arp2/3 Complex in Neutrophils—**To determine whether MAPKAPK2 interacts with the Arp2/3 complex in human neutrophils, this complex was precipitated from neutrophil lysates, and MAPKAPK2 was detected by immunoblot analysis. GST-WA coupled to glutathione-Sepharose was used to precipitate the Arp2/3 complex, whereas GST and GST-W coupled to glutathione-Sepharose were used as controls. Fig. 7A demonstrates that MAPKAPK2 was present in the complex precipitated by GST-WA but not GST-W or GST. To confirm that the Arp2/3 complex was only precipitated by GST-WA, the blots were stripped and reprobed for Arp3 (Fig. 7B). These data indicate that MAPKAPK2 interacts with the Arp2/3 complex in intact neutrophils.

**DISCUSSION**

Transient phosphorylation of serine, threonine, or tyrosine is a common mechanism of signal transduction. Phosphorylation can alter the catalytic activity or conformation of a protein or create binding sites for protein-protein interactions. A variety of approaches have been developed to identify proteins that interact with or are phosphorylated by kinases. The limitations associated by each approach prevent any one method from being universally applicable. For example, whereas the transcriptional-based yeast two-hybrid system is an extremely sensitive method to study protein-protein interactions, proteins must be expressed in the nucleus and cannot possess transcriptional activity. Approaches utilizing [32P]ATP labeling of ki-
nase substrates in intact cells have proven effective; however, identification of specific substrates is complicated by activation of multiple kinases under basal conditions and after physiological stimuli. Genetic introduction of constitutively active and dominant negative kinases reduces interference from endogenous kinases, but this approach requires long-lived isolated cells, usually in the form of transformed cell lines. Additionally, the ability to simultaneously identify a large number of protein substrates has until recently been limited by available technology.

We report here a proteomic approach that allows the identification of multiple substrates of a single kinase. This approach involves the in vitro phosphorylation of cellular lysate by recombinant kinase followed by two-dimensional gel electrophoresis. The phosphoproteins are subsequently identified by MALDI-MS. This methodology eliminates the need for pharmacological kinase inhibitors or the genetic introduction of mutant kinases into intact cells. The use of urea based lysis buffer improves protein solubility, effectively separates proteins from interfering lipids, salts, and nucleic acids, and eliminates endogenous kinase activity. Additionally, urea denatures and inactivates proteases that degrade cellular proteins. The preparatory methods described herein may also be applicable to methods for phosphopeptide enrichment by metal chelation columns, chemical modification for affinity chromatography, and tandem mass spectrometry.

The proteomic approach described, however, also possesses a number of limitations. For some phosphorylated proteins, insufficient mass spectra for identification were observed. Possible explanations included phosphorylation of low abundance proteins, incomplete trypsin digestion, interference of the silver stain with extraction of peptides from the gel, or incomplete protein transfer from the IPG strips to the second dimension gel. Because of the problems created by silver staining, we are now using a fluorescent-based dye (Sypro Ruby®), which has a greater dynamic range for protein expression and does not impair extraction of peptides from the gel (51). False positives may be generated for several reasons. Cell lysis may permit access of an exogenously added kinase to proteins with restricted localization in intact cells. Urea denaturation may expose phosphorylation sites that are inaccessible in properly folded proteins. Disruption of protein-protein interactions results in dissociation of signaling modules that direct kinase activity. Finally, protein spots may contain more than one protein, leading to identification of false substrates. On the other hand, some substrates may be missed due to protein unfolding leading to disruption of docking sites.

Fig. 6. **Structure of the Arp2/3 complex.** Panel A depicts the structure of the intact Arp2/3 complex as ribbons based on the x-ray crystal structure, as described under “Experimental Procedures.” p16-Arc is shown in red, and Ser-77 is in green as a Corey-Pauling-Koltun representation. Panel B is a magnification of the box in panel A showing p16-Arc (red) and the Corey-Pauling-Koltun representation of Ser-77 (green) in the fifth α helix. The figure demonstrates that side chain of Ser-77 is exposed, potentially allowing access to kinases. Panel C compares the ribbon representation of wild type p16-Arc (red) with that of p16-A S77A (white). This figure demonstrates that substitution of Ala for Ser-77 does cause significant alterations in secondary structure.

Fig. 7. **MAPKAPK2 interacts with the Arp2/3 complex in neutrophils.** Panel A, the Arp2/3 complex was precipitated from neutrophil lysates by incubating with GST-coupled glutathione-Sepharose, GST-W-coupled glutathione-Sepharose, or GST-WA-coupled glutathione-Sepharose. Proteins were separated by SDS-PAGE and immunoblotted for MAPKAPK2. Immunoblots confirm that MAPKAPK2 co-precipitated with GST-WA but not GST-W or GST. Neutrophil lysate served as a positive control. Panel B, nitrocellulose membrane from the MAPKAPK2 immunoblot was stripped and reprobed with Arp3. The immunoblot confirms that GST-WA, but not GST-W or GST, precipitate the Arp2/3 complex.

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From the ~30 phosphorylation events observed by autoradiography, we identified six potential MAPKAPK2 substrates; LSP1, integrin-linked kinase, proteasome activator-1, myosin regulatory light chain, cyclinophilin-A, and p16-Arc. The identification of LSP1 and myosin regulatory light chain, both previously identified as MAPKAPK2 substrates (30, 46), supports our proteomic approach. Because of the possibility of false positive substrate identification, however, we consider this approach to be a screening method requiring further confirmation.

It has been postulated that the Arp2/3 complex acts as the final common pathway for a variety of signaling inputs leading to actin polymerization (38). The actin cytoskeleton is a dynamic filament network involved in multiple functions, including cell locomotion, chemotaxis, phagocytosis, and vesicle exocytosis. The Arp2/3 complex enhances actin nucleation and cell locomotion, chemotaxis, phagocytosis, and vesicle exocytosis. The Arp2/3 complex regulates cellular functions dependent on the Ser-77 residue is located in the center of the activation loop (39). Phosphorylation of p16-Arc by MAPKAPK2 has been shown to be a screening method requiring further confirmation.

We identified six potential MAPKAPK2 substrates; cytosis. The Arp2/3 complex enhances actin nucleation and cell locomotion, chemotaxis, phagocytosis, and vesicle exocytosis. The Arp2/3 complex regulates cellular functions dependent on the Ser-77 residue is located in the center of the five a helix of p16-A, and the side chain is exposed, potentially allowing interaction with kinases. Additionally, the secondary structure of p16-Arc was not significantly altered in the S77A mutant. Thus, the failure of p16-A S77A to undergo phosphorylation is unlikely to be due to an altered structure of the S77A mutant. Thus, the failure of p16-A S77A to undergo phosphorylation is unlikely to be due to an altered structure of the S77A mutant. Thus, the failure of p16-A S77A to undergo phosphorylation is unlikely to be due to an altered structure of the S77A mutant. Thus, the failure of p16-A S77A to undergo phosphorylation is unlikely to be due to an altered structure of the S77A mutant. Thus, the failure of p16-A S77A to undergo phosphorylation is unlikely to be due to an altered structure of the S77A mutant.
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