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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[1] 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 chemoattractants, chemokines, bacterial phagocytosis, Fcγ 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-lipooxygenase (29–32). The functional significance of MAPKAPK2 phosphorylation of Hsp27 and 5-lipooxygenase 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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[1] The abbreviations used are: MAPK, mitogen-activated protein kinase; MAPKAPK2, MAPK-activated protein kinase 2; GST, glutathione *S*-transferase; IPG, immobilized pH gradient; Hsp27, heat shock protein 27; p16-Arc, 16-kDa subunit of the Arp2/3 complex; MALDI-MS, matrix-assisted laser desorption and ionization mass spectrometry; LSP1, leukocyte-specific protein-1; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid.

of the Arp2/3 complex. The Arp2/3 complex regulates actin polymerization and cross-linking, and this complex is necessary for neutrophil chemotaxis and phagocytosis (34–36). Two isoforms of p16-Arc have recently been described, both of which are expressed in humans neutrophils (37). To confirm that p16-Arc is a true substrate of MAPKAPK2, we characterized the ability of MAPKAPK2 to interact with and phosphorylate each of the two p16-Arc isoforms, p16-A and p16-B. Our results indicate that MAPKAPK2 interacts with and phosphorylates p16-A, whereas p16-B is relatively resistant to MAPKAPK2 phosphorylation.

EXPERIMENTAL PROCEDURES

Materials—The constructs for pGEX-5X-2-p16A and B as well as pGEX-5X-2-WA and W (SCAR) were described previously (38). Expression of glutathione *S*-transferase (GST)-MAPKAPK2 fusion protein was accomplished by constructing a pGEX-5X-2 (Amersham Biosciences) expression plasmid with the human cDNA for MAPKAPK2 (obtained from Dr. Matthias Gaestel, Martin Luther University, Halle-Wittenberg, Germany) downstream of GST. Recombinant His-p16A and His-p16B were expressed by cloning a *Bam*HI/*Eco*RI insert from pGEX-5X-2p16A and B into pRSETA (Invitrogen). [S^{35}]Methionine-labeled p16-A and p16-B were expressed with the TNT Quick Master expression kit (Promega, Madison, WI).

Ser-77 on pRSET-p16A was mutated to alanine with Clontech transformer site-directed mutagenesis kit using 5′-CCGGGCAGGCGCCATTGTCTTG-3′ as the mutation primer and 5′-GGAATTCGAACCTTGATCCGG-3′ as the selection primer. The appropriate mutation was verified by DNA sequencing. His-tagged p16-A S77A was bacterially expressed and isolated by nickel chromatography.

Neutrophil Isolation—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.

Neutrophil Lysate Preparation for MAPKAPK2 Substrate Identification—Neutrophils (1×10^8) were lysed in 400 μl of lysis buffer containing 2 M thiourea, 7 M urea, 65 mM CHAPS, 58 mM dithiothreitol, and 4.5% ampholytes (pI 3–10) (final pH = 6.4). Lysates were centrifuged at 14,000 rpm for 20 min at 15 °C. Before the addition of exogenous MAPKAPK2, urea concentration of lysates was reduced to 1 M by size exclusion chromatography. One ml spin-out columns (Chemicon International, Inc., Temecula, CA) were equilibrated in kinase buffer (25 mM HEPES, 25 mM β-glycerophosphate, 25 mM $MgCl_2$, 2, mM dithiothreitol, 0.1 mM $NaVO_3$, 65 mM CHAPS) containing 4 M or 1 M urea. Neutrophil lysate (400 μl) was loaded on the column equilibrated with the buffer containing 4 M urea and centrifuged at $500 \times g$ for 1 min. The procedure was repeated with buffer containing 1 M urea. The lysate was stored at −20 °C.

MAPKAPK2 Substrate Identification—Neutrophil lysates (400 μg of total protein) were incubated with 10 μCi of [γ-^{32}P]ATP (ICN Biomedicals, Inc.) in the presence and absence of 400 ng of active recombinant MAPKAPK2 (Upstate Biotechnology, Lake Placid, NY) at 30 °C for 3 h. Kinase reactions were stopped by adding rehydration buffer (8 M urea, 2% CHAPS, 0.01 M dithiothreitol, 2% ampholytes 3–10, and 0.01% bromphenol blue) to give a total volume of 450 μl. Proteins were separated by isoelectric focusing with pH 3–10 immobilized pH gradient (IPG) strips and by size with 10% Duracyl™ (Genomic Solutions, Ann Arbor, MI). IPG strips were rehydrated in buffer containing 400 μg of protein and focused isoelectrically at 5000 V_{max} and 80 mA/gel for 100,000 V-h. After isoelectric focusing, IPG strips were sequentially incubated for 10 min in equilibration buffers I (6 M urea, 2% dithiothreitol, 30% glycerol) and II (6 M urea, 2.5% iodoacetamide, 30% glycerol). IPG strips were then applied to 10% Duracryl gels, and proteins were separated using a Tricine buffer system (upper reservoir buffer: 0.1 M Tricine, 0.1 M Tris, 0.1% SDS, pH 8.5; lower buffer: 0.2 M Tris-acetate, pH 8.9) at 500 V for 4–5 h. Gels were silver-stained, and phosphorylation was visualized by autoradiography.

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 NH_4HCO_3 and 50% acetonitrile and dried by vacuum centrifugation. Proteins were then reduced by incubation with 20 mM dithiothreitol at 56 °C for 45 min followed by alkylation with 65 mM iodoacetamide in the dark at room temperature for 30 min. Post-alkylation gel pieces were incubated for 15 min in 100 mM NH_4HCO_3 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-cyanohydroxycinnamic 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 337-nm N_2 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 auto-hydrolysis. 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 proteins. A MOWSE score >71 indicated a significant match, ensuring the probability of the match not being a random event.

In Vitro Kinase Assays—Phosphorylation of recombinant p16-A and p16-B by MAPKAPK2 was examined by incubation of active recombinant MAPKAPK2 (40 ng) with 10 μCi of [γ-^{32}P]ATP and 0.5 μg of recombinant protein in 30 μl of kinase buffer containing 25 mM HEPES, 25 mM β-glycerophosphate, 25 mM $MgCl_2$, 2 mM dithiothreitol, and 0.1 mM $NaVO_3$, 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 Hsp27 and p16-A phosphorylation, 400 ng of recombinant active MAPKAPK2 were incubated with 0.5 μg of recombinant Hsp27 or p16-A and 10 μCi of [γ-^{32}P]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 μg of Hsp27 or 0.5, 1, and 2 μg of p16-A, 2 pmol of [γ-^{32}P]ATP, and 200 pmol of ATP in 200 μl 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 μl of kinase buffer. Separate reaction mixtures containing 200 pmol of ATP and 2 pmol of [γ-^{32}P]ATP in 200 μl of kinase buffer were used to determine the specific activity of ATP in each reaction. Fifty μl 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 5×10^7 cells in 200 μl of lysis buffer containing 20 mM Tris, pH 7.4, 1% Triton X-100, 0.5% Nonidet P-40, 150 mM NaCl, 25 mM $MgCl_2$, 20 mM NaF, 0.2 mM $NaVO_3$, 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 μg of protein) overnight at 4 °C. After incubation, beads were washed 4 times with lysis buffer. Proteins were eluted with 6× Laemmli SDS sample dilution buffer, separated by 10% 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 ^{35}S-labeled p16-A or ^{35}S-labeled p16-B in 50 μl of kinase buffer containing 25 mM HEPES, 25 mM β-glycerophosphate, 25 mM $MgCl_2$, 2 mM dithiothreitol, 0.1 mM $NaVO_3$, 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 [S^{35}]methionine-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 precipitate was eluted with Laemmli buffer and separated by 12% SDS-PAGE, 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 $MgCl_2$, 2 mM dithiothreitol, and 0.1 mM $NaVO_3$) in the presence and absence of 40 ng of active recombinant MAPKAPK2 and [γ-^{32}P]ATP. Reactions were incubated at 30 °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 phosphorylation was visualized by autoradiography.

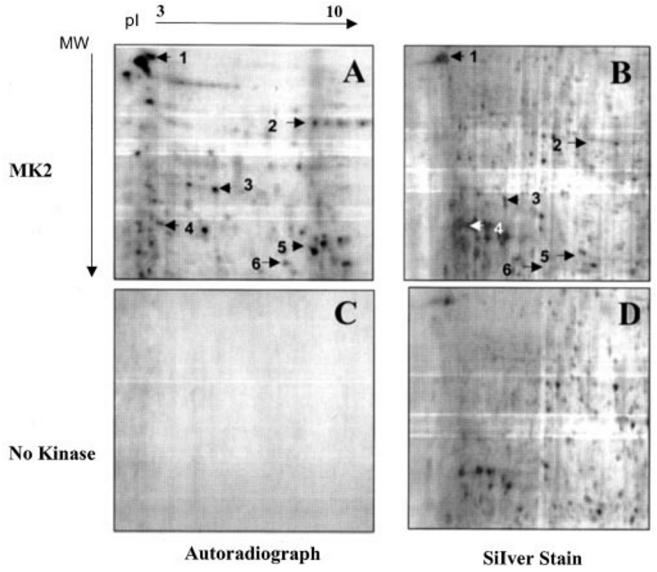

FIG. 1. **Phosphorylation of neutrophil lysate by recombinant active MAPKAPK2.** Candidate MAPKAPK2 substrates were identified by phosphorylation of neutrophil lysates with [γ-^{32}P]ATP in the presence and absence of active recombinant MAPKAPK2. Proteins were separated by two-dimensional gel electrophoresis and phosphoproteins detected by autoradiography. Proteins were identified by comparing autoradiographs (*panels A* and *C*) with their corresponding silver-stained gels (*panels B* and *D*). No endogenous kinase activity was detected in neutrophil lysates incubated without active recombinant MAPKAPK2 (*panel C*). Six candidate substrates of MAPKAPK2 were identified by peptide mass fingerprinting of trypsin-digested phosphoproteins using MALDI-MS and protein data base analysis (*numbered arrows*). These proteins were LSP1 (*1*), integrin-linked kinase (*2*), proteosome activator 1 (*3*), myosin regulatory light chain (*4*), cyclophilin A (*5*), and p16-Arc (*6*).

Structural Modeling—The starting models were based on the p16-Arc protein in the Arp2/3 x-ray crystal structure (41). The A isoform of p16-Arc was generated by homology modeling with p16-Arc in the crystal structure using Modeler (42) and an alignment generated with ClustalW (43). The S77A-mutated p16-Arc was created by replacing the Ser-77 with Ala in a standard conformation. The simulations to determine the effect of the S77A mutation on p16-Arc core structure included the central core of p16-Arc (residues 35–151) but did not include the first 34 residues that interact with Arp2 and p41-Arc, as this region of p16-Arc is dynamic, and the structure is stabilized by interaction partners that were not included in the calculations.

Models were hydrated in a 10+ box of TIP3P waters using standard AMBER (44) (amber.scripps.edu) rules; Cl^- counterions were placed randomly for charge neutrality. Box sizes were adjusted to include 7060 waters for each model. Simulations were performed in the isothermal isobaric ensemble (300 K, 1 atm) with the AMBER 7.0 program (43) and parameters from parm96.dat using periodic boundary conditions and the PME algorithm. Molecular dynamics simulations used the message passing interface version of the Sander routine (1.5-fs time step) with SHAKE to freeze all bonds involving hydrogen. Initial equilibrium for 155 ps following the general protocol (44) were performed with gradual removal of positional restraints on the protein complex. The production runs were 4.0 ns in length, and average structures for each complex (taken from 50 snapshots accumulated in the last 50 ps) were obtained and subsequently minimized. The calculations were run on a 32 processor SGI Origin 2000.

RESULTS

Identification of MAPKAPK2 Substrates—MAPKAPK2 substrate screening was performed by incubating human neutrophil lysates with [γ-^{32}P]ATP in the presence and absence of active recombinant MAPKAPK2. Proteins were separated by two-dimensional gel electrophoresis, gels were stained, and phosphorylated proteins were visualized by autoradiography. Autoradiographs were compared with silver-stained gels to identify the proteins that were MAPKAPK2 substrates (Fig. 1). No phosphorylation was observed after incubation of neutrophil lysates with [γ-^{32}P]ATP in the absence 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 56% 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 [S^{35}]p16-B. Fig. 2*A* shows that GST-MAPKAPK2 coupled, but not GST-coupled, glutathione-Sepharose precipitated p16-A. Neither GST-MAPKAPK2 nor GST-glutathione-Sepharose was able to precipitate p16-B (Fig. 2*B*). 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 [γ-^{32}P]ATP in an *in vitro* kinase assay. The proteins were separated by 15% SDS-PAGE and subjected to autoradiography. Fig. 4*A* 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 ($KDRAGS_{77}$) 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. 4*B* 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

TABLE I
Identification of p16-Arc as an MAPKAPK2 substrate

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	VLISFK
1069.62	1070.71	132	143	ALAAGGVGSIVR
1315.69	1316.71	48	60	QGNMTAALQAALK
1352.61	1353.64	13	23	KVDVDEYDENK
2161.97	2162.99	113	131	GFESPSDNSSAMLLQWHEK
2552.04	2553.05	24	47	FVDEEDGGDGQAGPDEGEVDSCLR
3758.54	3759.24	14	47	VDVDEYDENKFVDEEDGGDGQAGPDEGEVDSCLR

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  1 MSKNTVSSAR FRKVDVDEYD ENKFVDEEDG GDGQUAGPDEG EVDSCLRQGN
 51 MTAALQAALK NPPINTKSQA VKDRAGSIVL KVLISFKAND IEKAVQSDLDK
121 NGVDLLMKYI YKGFESPSDNSSAMLLQWHE KALAAGGVGS IVRVLTARKT
151 V
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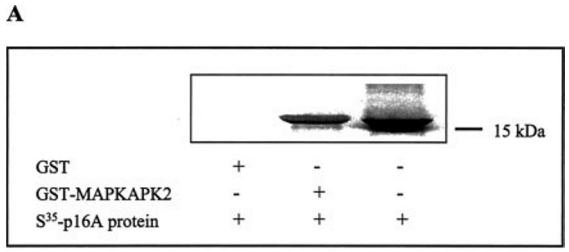

FIG. 2. **MAPKAPK2 interacts with p16-Arc isoforms.** To determine whether MAPKAPK2 interacts with p16-A and p16-B, recombinant [^{35}S]methionine-labeled p16-A (*panel A*) or p16-B (*panel B*) was incubated with GST (*first lane*, *panels A* and *B*) or GST-MAPKAPK2 glutathione coupled Sepharose (*second 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.

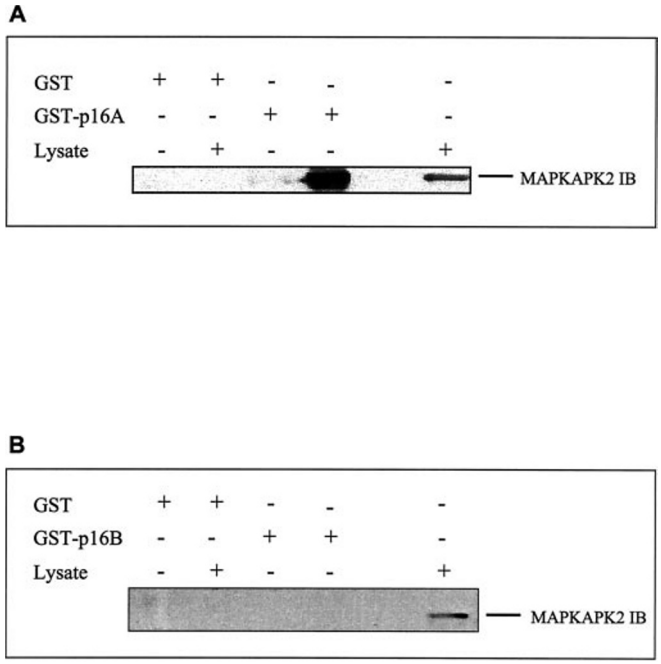

FIG. 3. **Interaction of p16-Arc isoforms with MAPKAPK2 in neutrophil lysate.** To determine whether endogenous MAPKAPK2 interacts with p16-A and p16-B, neutrophil lysates (400 μg of protein) were incubated with GST-p16-A (*panel A*, *first lane*) or GST-p16-B (*panel B*, *fourth lane*) and subjected 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.

and 3 h for both proteins (Fig. 4*C*). 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 phosphorylated p16-Arc in the intact Arp2/3 complex, we precipitated the entire complex from the neutrophil lysate. The Scar-1 protein directly binds to and activates the Arp2/3 complex (34, 50). The C-terminal region of Scar-1, termed Scar-WA, is responsible for this interaction, and GST-Scar-WA precipitates the intact Arp2/3 complex from cell lysates (38). A truncated form of the WA protein, termed Scar-W, fails to bind to Arp2/3 complex and was used as a negative control. Neutrophil lysates were incubated with GST, GST-WA, or GST-W coupled to glutathione-Sepharose. Aliquots of each assay were used to confirm the precipitation of the Arp2/3 complex by immunoblotting for Arp3. Fig. 5*A* shows that GST-WA, but not GST-W or GST, precipitated the Arp2/3 complex from lysates. An *in vitro* kinase assay was performed by the addition of active recombinant MAPKAPK2 to an aliquot of each precipitate. Fig. 5*B* demonstrates that MAPKAPK2 phosphorylated a 16-kDa protein precipitated by GST-WA but not GST-W or GST alone. These data suggest that MAPKAPK2 phosphorylates p16-Arc when it is a component of the intact Arp2/3 complex.

The p16-Arc subunit is bound to Arp2 and p41-Arc at the base of the Arp2/3 complex (Fig. 6*A*). The Ser-77 residue of p16-Arc identified as the MAPKAPK2 phosphorylation site is located on the fifth α helix, as defined by the crystal structure,

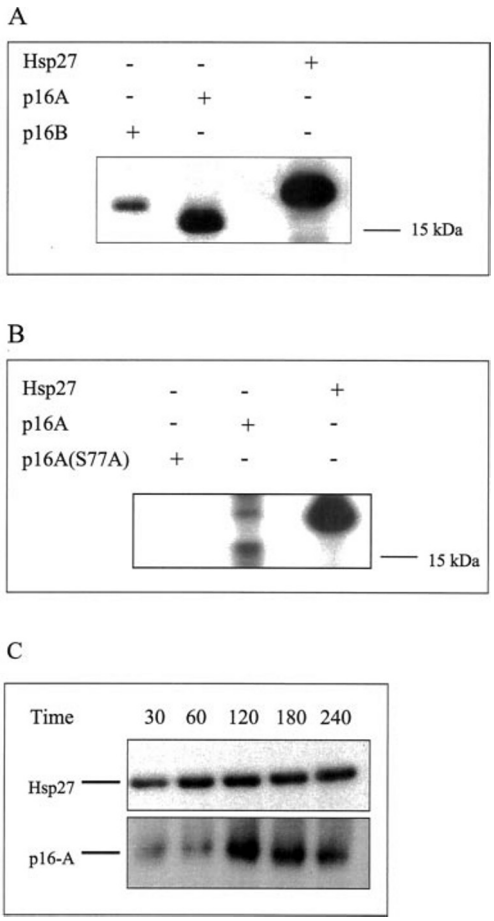

FIG. 4. ***In vitro* MAPKAPK2 phosphorylation of p16-Arc isoforms.** *Panel A*, equal amounts of recombinant p16-A (*second lane*) and p16-B (*first lane*) were subjected to an *in vitro* kinase assay with [γ-^{32}P]ATP and active recombinant MAPKAPK2. Proteins were separated by 15% SDS-PAGE, and results 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-^{32}P]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-^{32}P]ATP at 30 °C for 30 min to 4 h. Optimal phosphorylation was visualized between 2 and 3 h for both proteins.

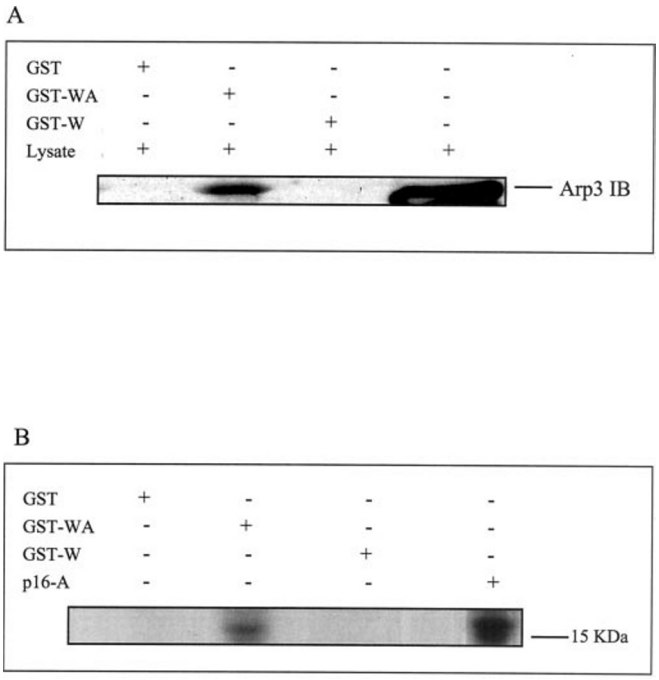

FIG. 5. **GST pull-down of Arp2/3 complex from neutrophil lysate.** *Panel A*, neutrophil lysate was incubated with GST-coupled to glutathione-Sepharose (*first lane*), coupled to GST-WA (*second lane*), or GST-W (*third lane*). Proteins were separated by SDS-PAGE and immunoblotted for Arp3. Immunoblots (*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-^{32}P]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*).

situated between helices 8 and 9 (orthogonal to helix 5) and helices 3 and 4 (parallel to helix 5). The Ser-77 residue is located in the center of helix 5, and the side chain is exposed with no structural interaction with other p16-Arc residues (Fig. 6*B*). Thus, it is accessible for interaction with and phosphorylation by MAPKAPK2. The identification of Ser-77 as the site of phosphorylation by MAPKAPK2 depends on the absence of significant structural alterations in p16-A S77A. Therefore, the structural alterations produced by this mutation were predicted by computer-generated structural modeling. Fig. 6*C* depicts the results of this modeling, which predicted no major local structural perturbations caused by the S77A mutation. Additionally, the mutation site is remote to the binding sites of p16-Arc to Arp2 and p41-Arc. These results suggest that the S77A mutation does not produce structural changes that would interfere with MAPKAPK2 phosphorylation of amino acid residues distinct from Ser-77.

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. 7*A* 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. 7*B*). 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 [^{32}P]ATP labeling of ki-

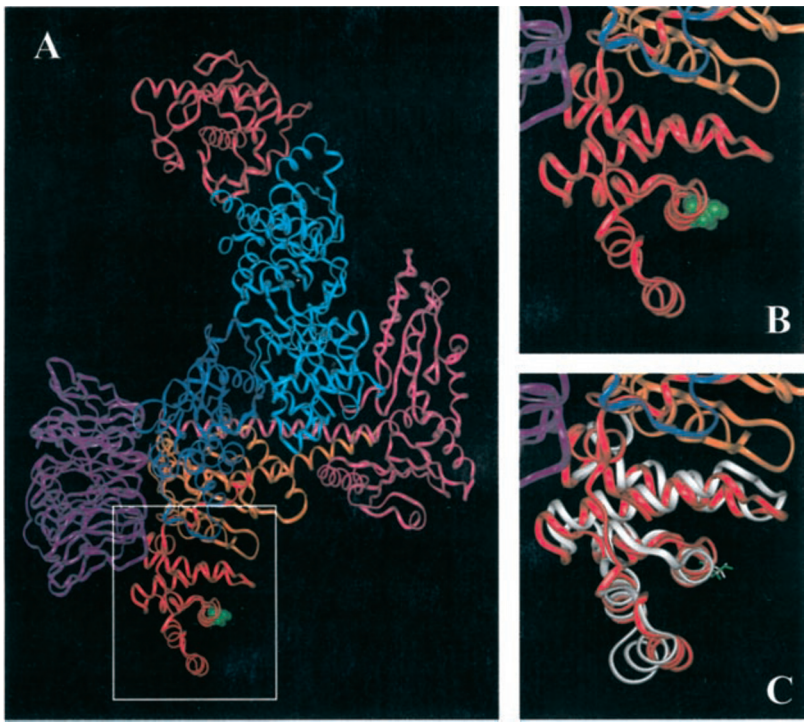

FIG. 6. **Structure of the Arp2/3 complex.** *Panel A* depicts the structure of the intact Arp2/3 complex as *ribbons* based on the x-ray crystal structure, as described under "Experimental Procedures." p16-Arc is shown in *red*, and Ser-77 is in *green* as a Corey-Pauling-Koltun representation. *Panel 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.

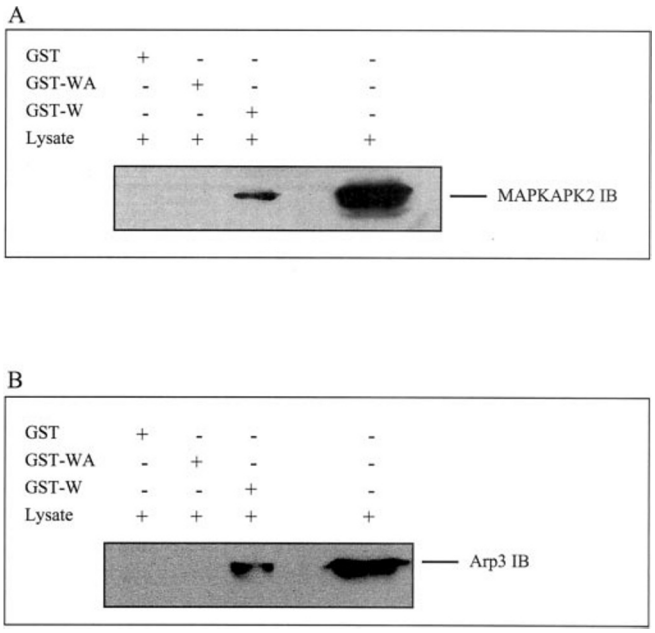

FIG. 7. **MAPKAPK2 interacts with the Arp2/3 complex in neutrophils.** *Panel A*, the Arp2/3 complex was precipitated from neutrophil lysates by incubating with GST-coupled glutathione-Sepharose, GST-W-coupled glutathione-Sepharose, or GST-WA-coupled glutathione-Sepharose. Proteins were separated by 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.

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.

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, cyclophilin-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 causes branching and cross-linking *in vitro*. In intact cells the Arp2/3 complex controls actin-based motility by driving the formation of lamellipodia (34, 52–55). It has been shown that neutrophils from MAPKAPK2-deficient mice have impaired directional migration (26), and MAPKAPK2 is involved in respiratory burst activity, exocytosis, and chemotaxis (27, 28). Our results indicate that MAPKAPK2 co-precipitated with the Arp2/3 complex and MAPKAPK2 phosphorylated p16-Arc in the intact Arp2/3 complex. The intensity of interaction and phosphorylation differed significantly between the two p16-Arc isoforms. The interaction of MAPKAPK2 with p16-A was clear-cut, whereas the physical interaction with p16-B was negligible. Additionally, p16-A was phosphorylated by active recombinant MAPKAPK2 to a much greater extent than p16-B. By mutational analysis, serine 77 was identified as the phosphorylation site on the p16-A isoform. This residue is contained in a consensus MAPKAPK2 phosphorylation domain (KDRAGS) (56), which is not present in the p16-B isoform. Our structural analysis found that the Ser-77 residue is located in the center of the fifth α 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 mutant protein. Additionally, this MAPKAPK2 phosphorylation site is exposed when p16-A is contained in intact Arp2/3 complexes. No MAPKAPK2 consensus sequence was located on p16-B, and the site(s) of the minimal MAPKAPK2 phosphorylation is unknown.

The functional consequences of p16-Arc phosphorylation by MAPKAPK2 and the role of differences in phosphorylation between p16-A and p16-B remain to be determined. We speculate that MAPKAPK2 plays a regulatory role in Arp2/3 functions only in cells expressing greater quantities of p16-A than p16-B. The ability of MAPKAPK2 to phosphorylate p16-Arc in the Arp2/3 complex from neutrophils and the preponderance of p16-A in hematopoietic cells (37) supports the hypothesis that MAPKAPK2 regulates Arp2/3 functions in human neutrophils through phosphorylation of p16-Arc. MAPKAPK2 has been recently implicated in hypoxia-related actin cytoskeleton changes in endothelial cells via phosphorylation of its downstream substrate Hsp27 (57). Phosphorylation of p16-Arc by MAPKAPK2 may provide an additional mechanism by which the p38 MAPK cascade regulates cellular functions dependent on the actin cytoskeleton.

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