Structure of Mitogen-activated Protein Kinase-activated Protein (MAPKAP) Kinase 2 Suggests a Bifunctional Switch That Couples Kinase Activation with Nuclear Export*

Received for publication, July 22, 2002, and in revised form, August 6, 2002
Published, JBC Papers in Press, August 8, 2002, DOI 10.1074/jbc.C200418200

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MAPK-activated protein kinase 2 (MAPKAPK2), one of several kinases directly phosphorylated and activated by p38 MAPK, plays a central role in the inflammatory response. The activated MAPKAPK2 phosphorylates its nuclear targets CREB/ATF1, serum response factor, and E2A protein E47 and its cytoplasmic targets HSP25/27, LSP-1, 5-lipoxygenase, glycogen synthase, and tyrosine hydroxylase. The crystal structure of unphosphorylated MAPKAPK2, determined at 2.8 Å resolution, includes the kinase domain and the C-terminal regulatory domain. Although the protein is inactive, the kinase domain adopts an active conformation with aspartate 366 mimicking the missing phosphorylated threonine 222 in the activation loop. The C-terminal regulatory domain forms a helix-turn-helix plus a long strand. Phosphorylation of threonine 334, which is located between the kinase domain and the C-terminal regulatory domain, may serve as a switch for MAPKAPK2 nuclear import and export. Phosphorylated MAPKAPK2 masks the nuclear localization signal at its C terminus by binding to p38. It unmasksthe nuclear export signal, which is part of the second C-terminal helix packed along the surface of kinase domain C-lobe, and thereby carries p38 to the cytoplasm.

Mitogen-activated protein kinases, including ERK1/ERK2, JNK/SAPK, and p38/RK, are important signal transducing molecules for control of gene expression, cell proliferation, and apoptosis (1). In response to cellular stresses, such as heat or osmotic shock, bacterial lipopolysaccharide, proinflammatory cytokines, and tumor necrosis factor α, p38/RK is activated by its upstream kinases MKK3 and MKK6. Activated p38 phosphorylates MAPKAPK2, MAPKAPK3/3pk, PRAK, MNK1/2, MSK1, and transcription factors ATF2, CHOP/GADD153, Elk-1, and MEF2C (2). MAPKAPK2 was originally identified as a kinase that is phosphorylated and activated in vitro by the p42/p44 (ERK1/ERK2) MAP kinase isoforms and inactivated by protein phosphatase 2A (3). Later studies showed that MAPKAPK2 is activated in vivo only by p38/p40/RK (4). Mice that lack MAPKAPK2 show increased stress resistance and survive bacterial lipopolysaccharide-induced endotoxic shock due to a 90% reduction in the production of tumor necrosis factor α (5). MAPKAPK2 is in the nucleus of unstimulated cells and moves rapidly to the cytoplasm after stimulation (6, 7). In the nucleus, MAPKAPK2 contributes to the phosphorylation of CREB at Ser133 and may regulate its ability to activate transcription in response to cAMP, Ca2+, and nerve growth factor (8). MAPKAPK2 phosphorylates serum response factor at Ser103 both in vivo and in vitro in response to tumor-promoting and stress-inducing stimuli (9). Both MAPKAPK2 and MAPKAPK3/3pk interact with basic helix-loop-helix transcription factor E47 in vitro and phosphorylate E47 in vivo, suggesting that they are regulators of E47 activity and E47-dependent gene expression (10). In the cytoplasm, MAPKAPK2 phosphorylates small heat shock protein HSP25/HSP27 (11) and lymphocyte-specific protein LSP-1 (12), both F-actin-binding proteins. Other substrates of MAPKAPK2 include glycogen synthase (3), tyrosine hydroxylase (11), and 5-lipoxygenase (13).

Human MAPKAPK2 (14, 15), a 400-residue enzyme, has two proline-rich segments at its N terminus followed by the kinase domain and the C-terminal regulatory domain. The N-terminal proline-rich segments have been shown to bind to the c-ABL Src homology 3 domain in vitro (16). The kinase domain has low homology with other serine/threonine kinases except MAPKAP3/4 (Fig. 2). The N-terminal proline-rich domain and the C-terminal regulatory domain have no significant homology with other non-MAPKAPK proteins. The C-terminal regulatory domain contains a bipartite nuclear localization signal and a nuclear export signal (6, 7). We report here the crystal structure of unphosphorylated MAPKAPK2 and suggest a possible mechanism for its nuclear export with p38.

EXPERIMENTAL PROCEDURES

Protein Expression—A construct encoding human MAPKAPK2 residues 47–400 was cloned into pBEV1, a T7 polymerase-based Escherichia coli expression vector. BL21 (DE3) competent cells were transformed with pBEV1/Hisg×5-tagged MAPKAPK2(47–400) using a standard transformation protocol. Freshly transformed cells were grown at 37 °C in a complex medium supplemented with 100 μg/ml carbenicillin for 16 h at 37 °C. This culture was used to inoculate additional flasks of M9/carbenicillin (1:10). These cultures were grown to A590 0.7–0.9, and the amino acids lysine, phenylalanine, and threonine were added to final concentrations of 100 mg/liter, while the amino acids selenomethionine, isoleucine, and valine were added to final con-
 concentrations of 50 mg/liter. The growth temperature was reduced to 30 °C, and after 30 min induction was initiated by the addition of 1 mM isopropyl-1-thio-β-D-galactopyranoside. Cells were harvested by centrifugation 14 h postinduction and flash frozen at −80 °C prior to purification.

**Protein Purification**—Frozen cell pellets were thawed in 10 volumes of Buffer A (50 mM HEPES, pH 7.8, 10% glycerol, 2 mM β-mercaptoethanol, 200 mM NaCl, 0.02% Tween 20) + 0.5 mM Pefabloc, 2 μg/ml pepstatin, 1 μg/ml E64, 1 μg/ml leupeptin and were lysed in a microfluidizer. The lysate was centrifuged at 54,000 × g for 1 h, and the supernatant was collected and incubated batchwise with Talon metal affinity resin. After extensive washing with Buffer A, the resin was eluted with Buffer A + 150 mM imidazole. 1 unit of thrombin/mg of

| Table 1: Statistics of data collection, phasing, and refinement |
|---------------------------------------------------------------|
| **Space group** | R3 |
| **Unit cell** | a = b = 143.994 Å, c = 90.273 Å, α = β = 90°, γ = 120° |
| **Resolution (Å)** | 32.6–2.8 |
| **Molecules/a.u.** | 2 |
| **Roverall (%)** | 10.6 |
| **Reflections (total/unique)** | 386,514/17,189 |
| **Completeness (%)** | 100.00 |
| **Rmerge (%)** | 0.066 |
| **Phasing power** | 1.833 |
| **Rfree (%)** | 0.605 |
| **FOM** | 0.397 |
| **Number of mercury sites** | 14 |
| **Refinement** | |
| **Rcryst/Rfree (%)** | 0.233/0.245 |
| **r.m.s.d.' (bond/angle)** | 0.014 Å/3.2° |

*a.u., asymmetric unit.
†FOM, figure of merit.
‡r.m.s.d., root mean square deviation.

![FIG.1. Ribbon diagram of the MAPKAPK2 structure.](image1)

The N-lobe of the kinase domain is colored light blue. The C-lobe of the kinase domain is colored dark blue. The regulatory domain is colored red. The key regulatory residue threonine 334 is labeled. The dotted line indicates the missing part of activation loop.

![FIG.2. Alignment of the amino acid sequences of MAPKAPK2 (mk2), MAPKAPK3 (mk3), CaMKI, and cAPK.](image2)

The secondary structure of MAPKAPK2 (blue) and cAPK (green) are shown above the sequences. Residues that are identical in all four sequences are shaded red. Residues that are similar in all four sequences are colored red. For convenience, the MAPKAPK2 secondary structure elements are numbered starting from aB and β2. The secondary structure nomenclature of cAPK is taken from Knighton (21).
 His-tagged protein was added to the Talon eluate and allowed to incubate at room temperature for 1 h. The thrombin activity was quenched by addition of 0.5 mM Pefabloc. The protein was diluted 1:4 to lower the NaCl to 50 mM and loaded onto a Q-Sepharose column pre-equilibrated with Buffer A. The flow-through fractions, containing MAPKAPK2, were collected and directly loaded to an SP-Sepharose column pre-equilibrated with Buffer B (25 mM HEPES, pH 7.2, 5% glycerol, 2 mM dithiothreitol, 0.5 mM Pefabloc). The protein eluted from the SP-Sepharose column was concentrated in a Centriprep-30 for size exclusion chromatography on a Sephacryl S-200 column pre-equilibrated with Buffer C (25 mM Tris, pH 7.8, 200 mM NaCl, 2 mM dithiothreitol). The peak fractions were collected and concentrated to 5–10 mg/ml for crystallization.

Crystallization and X-ray Data Collection—Crystals grown in 2 mM sodium/potassium phosphate at pH 5.15 appeared as small multiple crystals. A several-step seeding helped to produce single large crystals. Most of the diffraction data sets we collected from these crystals could only be processed in space group P2₁ with six molecules in asymmetric unit. One crystal, which had been soaked overnight in a solution containing a potential mercury derivative and flash frozen in liquid nitrogen, belonged to space group R3. Data were collected from this crystal at The Advanced Light Source (ALS) beamline 5.0.2 using an ADSC Quantum-4 detector. Data were integrated using MOSFLM (17) and scaled using SCALA in the CCP4 package (18).

Structure Determination and Refinement—Single-wavelength anomalous dispersion data from these mercury derivative data were used to calculate an anomalous difference Patterson map. Fourteen sites were located by difference Patterson and difference Fourier maps (19) (CNX). Phases calculated using these 14 sites were improved by a combination of solvent flattening, histogram matching, phase extension, and non-crystallographic symmetry averaging. Several cycles of model building (QUANTA2000, MSI) and phase combined refinement (CNX) led to the crystallographic symmetry averaging. Several cycles of model building of solvent flattening, histogram matching, phase extension, and non-crystallographic symmetry averaging. Several cycles of model building (QUANTA2000, MSI) and phase combined refinement (CNX) led to the initial model. The model was extended by many cycles of rebuilding and refinement. The final model includes two protein molecules plus 14 mercury atoms and 133 water molecules positioned by ARP/WARP-REFMAC (20). Detailed information is presented in Table I.

RESULTS AND DISCUSSION

Overview of the Structure—The MAPKAPK2 structure (Fig. 1) has the standard two-lobe kinase architecture plus an extra C-terminal regulatory domain (red). Although there are two molecules in asymmetric unit (denoted molecules A and B), the structure shows no evidence for a dimer, nor does MAPKAPK2 form dimers in solution. The two molecules are essentially identical except at the bottom of the C-lobe of the kinase domain residues 260–290, see Fig. 3A). Several residues in this region are missing due to poor electron density in both molecules. Ser272 in this region is one of the three major regulatory phosphorylation sites (4). The activation loop (residues 217–235) including Thr222, a common regulatory phosphorylation site in most serine/threonine kinases, is disordered in our structure (Fig. 1, dotted line). The C-terminal regulatory domain of MAPKAPK2 has a quite different conformation from the C-terminal elements in cyclic AMP-dependent kinase (21) (cAPK, Protein Data Bank code 1FMO, Fig. 3C), calcium/calmodulin-dependent protein kinase I (22) (CaMKI, Protein Data Bank code 1A06, Fig. 3B), or twitchin kinase (23) (Protein Data Bank 1KOB). Electron density of molecule A ends at residue 377, and the electron density of molecule B ends at residue 374. The structure of MAPKAPK2 analyzed here is limited to the coordinates from molecule B except as otherwise indicated.

Kinase Domain—Here we describe the kinase domain in relation to the structure of cAPK (21). The two lobes of the MAPKAPK2 kinase domain have a “closed” conformation, usually characteristic of the active state of a kinase, although our protein is unphosphorylated (Figs. 2 and 3C). The N-lobe of the MAPKAPK2 kinase domain starts with a long strand in place of the long helix (aA) in cAPK. The N-terminal part of the glycine-rich loop (nucleotide binding loop) flips up by 120° and moves −11 Å to form a short helix (aB, corresponding to b1 of cAPK), and a three-residue turn replaces the aB of cAPK. The helix aC and the central catalytic cleft, up to the DFG of the activation loop, superimpose very well on the active cAPK structure. Moreover, helices of the C-lobe superimpose nicely on cAPK except for residues 260–290, which are poorly ordered

![Fig. 3. A, superimposition of molecule A (yellow) and molecule B (blue) in the asymmetric unit of the MAPKAPK2 crystal. B, superimposition of molecule B (blue) with CaMKI (yellow); the regulatory domain of MAPKAPK2 is colored red, and the regulatory domain of CaMKI is colored green. C, superimposition of molecule B (blue) with cAPK (yellow); the regulatory domain of MAPKAPK2 is colored red, and PKI peptide bound to cAPK is colored green.](image-url)
and have different conformations in the two molecules in the asymmetric unit. All catalytically important residues, including Lys93 (corresponding to Lys47 of cAPK), Glu104 (corresponding to Glu62 of cAPK), Arg185 (corresponding to Arg140 of cAPK), Asp186 (corresponding to Asp141 of cAPK, a conserved residue in all kinases), and Asp207 (corresponding to Asp159 of cAPK), align very well with the active form of cAPK (root mean square deviation, 0.44 Å; Fig. 4). Asp366 of the C-terminal regulatory domain of MAPKAPK2 occupies the position of phosphothreonine Thr(p)195 in cAPK. The salt bridge between Arg185 and phosphothreonine (or phosphoserine) in the activation loop is critical for promoting the correct conformation of Asp186, the catalytic base, and for stabilizing positively charged residues Arg185 and Lys212 in the active form.

C-terminal Regulatory Domain of MAPKAPK2—The C-terminal regulatory domain of MAPKAPK2 contains residues 328–400. Deletion of this domain results in a marked increase in catalytic activity with or without pretreatment by MAP kinase (24). There are two phosphorylation sites in this domain, Thr334 and Thr338. Thr334 is a major regulatory phosphorylation site, and Thr338 is likely an autophosphorylation site (4). Both Thr334 and Thr338 are in a very acidic environment. Thus phosphorylation of these residues would be expected to weaken or interrupt binding of the C-terminal regulatory domain to the catalytic domain.

In the MAPKAPK2 structure, the N-terminal part of this regulatory domain including the first helix (αJ) and the three-residue turn (residues 328–345) occupies very similar positions to those taken by αR1 and adjacent residues in CaMKI (22) (Fig. 3B). CaMKI does not have a phosphorylation site in this region, but Thr195 of CaMKI (corresponding to Val306 of CaMKI), which is autophosphorylated when the enzyme is activated (25), occupies the same position as MAPKAPK2 Thr338. Interaction between conserved Glu145 (corresponding to Glu127 of cAPK and Glu102 of CaMKI) and Lys353, which mimics the P-3 arginine of the cAPK substrate analog PKI (Lys18, corresponding to Lys300 of CaMKI), supports the assumption that the C-terminal regulatory segment occupies the substrate binding pocket and may act like a pseudosubstrate. A surface representation of this substrate binding pocket is shown in Fig. 7. The tail of the second helix overlaps with the activation loop of cAPK (Figs. 3C and 5), and the position of the cAPK phosphorylation site Thr(p)195 is replaced by Asp366 as indicated above (Figs. 4 and 5). The long C-terminal strand, which appears to adopt its conformation solely for crystal packing, is probably flexible in solution.

NLS and NES of MAPKAPK2—MAPKAPK2 and its activator p38, both located predominantly in the nucleus before stimulation, quickly translocate to the cytoplasm together after stimulation (6, 7). The C-terminal regulatory domain of MAPKAPK2 (also MAPKAPK3/3pk) contains both a functional nuclear localization signal and a functional nuclear export signal (6, 7). The NLS (residues 373KKKX10KKRRKK389) of MAPKAPK2 is required for its activation by p38 in the nucleus. The NES of MAPKAPK2 (residues 345DKERWEDVKEELMTRDVYE368) is sufficient to trigger nuclear export, which can be inhibited by leptomycin B, an inhibitor of the interaction between CRM1/exportin 1 and Rev-type leucine-rich NES (6, 7). The structure of the MAPKAPK2 NES is very similar to the NES of p53 (26) and the NES of 14-3-3 proteins (27) (Fig. 6). All have three hydrophobic residues (Leu, Ile, or Met) pointing to one side of the helix and another hydrophobic residue (Leu or Val) pointing to the other side (Figs. 6A and 7). Some well-known leucine-rich NES sequences are aligned in Fig. 6B.

Conclusions—MAP kinase cascades mediate signal transduction from the cell surface to the nucleus. At least three
parallel MAP kinase pathways have been identified, known as ERK/MAPK, p38/RK, and JNK/SAPK pathways. Signal transduction through MAP kinases depends not only on the catalytic activity of the kinases but also on the spatial redistribution accompanying the activation. The mechanisms that control redistribution are largely unknown and appear to be different among the three pathways. In the p38 pathway, the activators of p38, MKK3, and MKK6, are present in both the cytoplasm and the nucleus (6). The phosphorylation of p38 and subsequently MAPKAPK2 occurs in the nucleus. The phosphorylation of MAPKAPK2 by p38 involves the interaction between the CD/ED domain of p38 and the NLS of MAPKAPK2 (28). Phosphorylation of MAPKAPK2 by p38 at threonine 334 disrupts the CD/ED domain of p38 and the NLS of MAPKAPK2 (28). Phosphorylation of MAPKAPK2 by p38 involves the interaction between the regulatory domain thus making the NES available for nuclear receptor binding. The complex of p38 and MAPKAPK2 moves to the cytoplasm in a phosphorylation-dependent manner (6). Study of the budding yeast p38 homolog HOG1 (29) showed that a small GTP-binding protein (Ran-GSP1), an importin β homolog (NMD5), and the NES receptor (XPO/CRM1) are involved in the regulation of nuclear transport of HOG1. However, unlike p38, HOG1 (MAPK), PBS2 (MAPKK), and STE11 (MAPKKK) localize to the cytoplasm of unstressed cells. Following osmotic stress, HOG1 translocates into the nucleus. Although yeast homologs of MAPKAPK2 have not been identified, RCK2/CLK1 (30), a HOG1 substrate, is a good candidate for two reasons. First, both RCK2/CLK1 and MAPKAPK2 share high homology with CaMK. Second, RCK2/CLK1 has a C-terminal regulatory domain (residues 569-DEQLEQNMFQLTLDTS584 match the Rev leucine-rich nuclear export sequence, and residues 589-RRKK592 match the nuclear localization signal sequence) similar to that of MAPKAPK2 that binds HOG1.

Acknowledgments—We thank S. C. Harrison, V. L. Sato, J. A. Thompson, K. P. Wilson, U. A. Germann, E. Fox, and S. P. Chambers for comments on the manuscript and G. McDermont for the assistance with data collection at ALS.

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