Mitogen-activated protein kinase-activated protein kinases (MAPKAPKs) lie immediately downstream of the mitogen-activated protein kinases (MAPKs), extracellular signal-regulated kinase (ERK), and p38 MAPK. Although the family of MAPKAPKs shares sequence similarity, it demonstrates selectivity for the upstream activator. Here we demonstrate that each of the ERK- and p38 MAPK-regulated MAPKAPKs contains a MAPK docking site positioned distally to the residue(s) phosphorylated by MAPKs. The isolated MAPK docking sites show specificity for the upstream activator similar to that reported for the full-length proteins. Moreover, replacement of the ERK docking site of p90 ribosomal S6 kinase with the p38 MAPK docking site of MAPKAPK2 converts p90 ribosomal S6 kinase into a stress-activated kinase in vivo. It is apparent that mechanisms controlling events downstream of the proline-directed MAPKs involve specific MAPK docking sites within the carboxyl termini of the MAPKAPKs that determine the cascade in which the MAPKAPK functions.

The growing family of mitogen-activated protein kinase-activated protein kinases (MAPKAPKs) includes a subfamily of enzymes that contain two distinct kinase catalytic domains in one polypeptide chain and another that contains a single kinase domain (see Fig. 1). The three isoforms of the classic RSKs (RSK1/2/3) and the two isoforms of mitogen- and stress-activated protein kinase (MSK1/2) are in the former subfamily. The single kinase domain subfamily includes MAPKAPK2/3/5 and the two isoforms of MAPK-interacting kinase (MNK1/2). RSK, the initial MAPKAPK to be identified, has been well studied. The amino-terminal kinase domain of RSK phosphorylates exogenous substrates (1–3), whereas the carboxyl-terminal kinase domain regulates amino-terminal kinase domain activity (1, 2, 4, 5). With regard to primary structure, the catalytic core of the single kinase domain MAPKAPKs is related to the carboxyl-terminal kinase domain of RSK (Fig. 1).

In vivo, the MAPKAPKs demonstrate specificity for the upstream activator. RSK1/2/3 are specifically activated by ERK, whereas MAPKAPK2 is activated by p38 MAPK. MNK1 and MSK1/2 are activated by both ERK and p38 MAPK (6–9) although MSK2 (also known as RSKb) was reported to be predominantly regulated by p38 MAPK (9). In vivo activation studies with MNK2 have not been published; however, in vitro studies and yeast two-hybrid assays indicate that full-length MNK2 interacts with both ERK and p38 MAPK (6).

Examination of the amino acid sequence of the MAPKAPK family members revealed a putative MAPK docking site in each MAPKAPK similar to the ERK docking site identified in RSK (10, 11). The following study demonstrates that each of the MAPKAPKs contains a short sequence of amino acids that confer specificity for the upstream activator. The docking site determines the signaling cascade in which the MAPKAPK functions.

**EXPERIMENTAL PROCEDURES**

**Materials**

Antibodies were obtained as follows: polyclonal anti-ERK antibody (6B-129), Upstate Biotechnology; monoclonal FLAG M2 antibody (IBI3025), Kodak; polyclonal anti-p38 MAPK antibody (SC-535-G), monoclonal anti-HA antibody (12CA5), and monoclonal anti-MYC antibody (9E10), the University of Virginia Lymphocyte Culture Center; monoclonal phospho-Ser-380 RSK1 antibody (06-826) and phospho-Ser-167 estrogen receptor (ER) polyclonal antibody, Upstate Biotechnology; ER (EYV a78) polyclonal antibody (12); ER (EYV F9) monoclonal antibody (13); anti-mouse IgG/horseradish peroxidase antibody (NA931) and anti-rabbit IgG/horseradish peroxidase antibody (NA934), Amsberham Pharmacia Biotech.

**Plasmid Construction**

pGEX2T bacterial expression constructs encoding the carboxyl-terminal tail residues of the wild type MAPKAPKs were created either by annealing complementary oligonucleotides corresponding to the 3′ end of the MAPKAPKs or by polymerase chain reaction (PCR) amplification of the 3′ end of each MAPKAPK. The annealed oligonucleotides and PCR products were subsequently subcloned into pGEX2T at the BamHI and EcoRI sites. pGEX2T bacterial expression constructs encoding GST-MAPKAPK2 ("QGIK") and GST-RSK2 ("Q/K-G/K") were also prepared by PCR. pK3H-RSK2 was generated as described previously (10).

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§ The abbreviations used are: MAPKAPK, mitogen-activated protein kinase-activated protein kinase; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; RSK, p90 ribosomal S6 kinase; MSK, mitogen- and stress-activated protein kinase; MNK, MAPK-interacting kinase; MEK, MAPK-ERK; RSK2/MK2, RSK2/MAPKAPK2; ER, estrogen receptor; PCR, polymerase chain reaction; WT, wild type; GST, glutathione S-transferase; BHK, baby hamster kidney; EGF, epidermal growth factor; HA, hemagglutinin.
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of pK3H-RSK2, pK3H-RSK2/MK2, pK3H, or pEFMYC-MAPKPK2 as described previously (5). 45 h post-transfection, cells were serum-deprived in the presence of 50 µM PD 98059, 10 µM SB 203580, or Me2SO (vehicle) for 3 h. The cells were treated with EGF (100 ng/ml) for 30 min and NaCl (300 mM) for 15 min (or the appropriate vehicle) and were harvested as described. HA-MAPKAPK2 (3 µl) was incubated in kinase mix (47 µl; 25 mM Heps, pH 7.4, 5 mM β-glycerophosphate, pH 7.4, 3.75 mM MgTA, 1.5 mM dithiothreitol, 30 mM MgCl6, 6 µM protein kinase A inhibitor peptide, 6 µM protein kinase C inhibitor peptide, 150 µM NaVO4, 1 µM microcin R, 300 µM S6 peptide (RRRLSSLRLRA) for WT-RSK2 and RSK2/MK2 chimera or 75 mM glycan synthesize peptide (KKLNLTLSTVA for WT-MAPKAP2, 150 µM ATP, and (γ-32P)ATP (~2000 cpm/pmol)) at 30 °C for 10 min. Each assay was performed in triplicate. Phosphate incorporation into peptide substrate was determined using phosphocellulose P-81 paper as described previously (10). Duplicates of eluted proteins were processed for Western analysis with anti-HA or anti-MYC antibody. The intensity of each immunoblott band from numerous exposures of film was quantitated in the linear range of development. The relative amount of tagged protein was determined using NIH Image 1.61. Specific activity was normalized for the amount of immunoprecipitated protein (pmol/min/unit HA-tagged or MYC-tagged enzyme) and was plotted as percent increase in activation relative to basal kinase activity.

**Phosphorylation of the Estrogen Receptor—BH2 (C-13) cells were co-transfected with 13 µg of HEGO DNA (estrogen receptor) and 13 µg of either pK3H-RSK2, pK3H-RSK2/MK2, pK3H-RSK2/MK2-K100A, or pK3H DNA as described. 45 h post-transfection, all cells were serum-deprived in the presence of 50 µM PD 98059, and a subset of cells was also treated with 10 µM SB 203580 or Me2SO (vehicle) for 3 h. Cells were then treated with NaCl (300 mM) or vehicle for 15 min. Two 150-mm dishes per condition were scraped into 700 µl of lysis buffer C (50 mM Heps, 5 mM β-mercaptoethanol, 1% Nonidet P-40, 0.5% Triton X-100, 100 µg/ml leupeptin and aprotinin, 10 mM Na2MoO4, 2 mM Na3VO4, 10 mM MgCl2, 20 mM β-glycerophosphate, 10% glycerol, 2 µg/ml each leupeptin and aprotinin, 1 mM phenylmethylsulfonyl fluoride, 1 CompleteTM protease inhibitor mixture tablet/50 ml (Roche Molecular Biochemicals) for 1.5 h at 4 °C withgent mixing. The beads were washed once with 0.2 ml of the buffer A and twice with 1 ml of phosphate-buffered saline. SDS sample buffer was added to the washed beads and boiled for 5 min and processed for Western analysis.

**Results and Discussion**

To determine whether the putative docking sites interact with MAPK, GST fusion proteins containing the identified regions of MSK1/2, MK2/1, and MAPKPK2 (Fig. 2A) were incubated with kinase-defective ERK2 (K52R) or inactive p38 MAPK. The isolated carboxyl-terminal tails of the MAPKAPKs formed complexes with MAPK (Fig. 2B), whereas a GST fusion protein containing a randomized RSK1 ERK docking site, RSK1-RANDOM, and a GST fusion protein containing the carboxyl-terminal tail of RSK1 lacking the ERK docking site, RSK1-MUT, did not interact with either ERK or p38 MAPK. The data indicate that these regions of MAPKAPKs are indeed interaction sites for the upstream activators.

**RSK1** (716–735) specifically interacts with ERK (10). Here we demonstrate that MAPKPK2-377–400 and MSK1 (734–750) specifically interacted with p38 MAPK, whereas MK2 (721–737), MK1 (385–415), and MK2 (385–412) interacted with both ERK and p38 MAPK. Thus, the isolated MAPK docking sites demonstrate a similar specificity for ERK or p38 dockin sites.
MAPK as reported for the full-length proteins. MSK1 is activated by both ERK and p38 MAPK (14). However, under these conditions the isolated MSK1 MAPK docking site, MSK1-(734–750), did not interact with ERK in vitro.

To examine whether the p38 MAPK docking site of MAPKAPK2 acts as a docking site in the context of a full-length protein, the carboxyl-terminal 23 amino acids of RSK2 were replaced with the carboxyl-terminal 35 amino acids of MAPKAPK2 containing the putative bipartite nuclear localization signal of MAPKAPK2 (RSK2/MK2 chimera) (Fig. 3A). HA-tagged RSK2/MK2 chimera and WT-RSK2 were immunoprecipitated from BHK cells in which either ERK or p38 MAPK was co-expressed. The RSK2/MK2 chimera co-immunoprecipitated with p38 MAPK but not with ERK, whereas WT-RSK2 co-immunoprecipitated only with ERK (Fig. 3B). One explanation for interaction of the chimera with p38 MAPK in vitro is that replacement of the RSK2 tail with that of MAPKAPK2 may result in co-localization of the chimera with p38 MAPK, whereas WT-RSK2 is co-localized with ERK. To address this question, purified His-tagged ERK and His-tagged p38 MAPK were combined and incubated with immunoprecipitated WT-RSK2 or RSK2/MK2 chimera. The chimera formed a complex only with p38 MAPK, whereas WT-RSK2 preferentially bound to ERK above levels observed with empty vector immunoprecipitates (Fig. 3C). Thus, replacing the carboxyl-terminal tail of RSK2 with that of MAPKAPK2 is sufficient to switch specificity for the upstream activator.

Alignment of the MAPK docking sites of RSK2 and MAPKAPK2, which bind exclusively to ERK and p38, respectively, reveals a distinction in the number of contiguous basic amino acids. The RSK2 MAPK docking site contains two adjacent basic residues, whereas that of MAPKAPK2 contains five. Specificity for the upstream activator may be dictated by the number of contiguous basic amino acids within the MAPK docking sites such that the number of basic residues is inversely related to the affinity for ERK and directly related to...
the affinity for p38 MAPK. To address this question, the number of basic residues in the isolated MAPK docking site of MAPKAPK2 and RSK2 was decreased and increased, respectively, to determine whether specificity for the upstream activator could be altered (Fig. 4A). Reducing the number of basic residues in the p38 MAPK docking site of MAPKAPK2 to mimic the ERK docking site of RSK2 attenuated p38 MAPK binding (Fig. 4B). These data indicate the necessity of basic residues for interaction with p38 MAPK as has been reported for interaction of RSK with ERK (10, 11). However, the mutant p38 MAPK docking site of MAPKAPK2 did not interact with ERK (not shown). Therefore, whereas the basic residues are essential for interaction with the upstream activator, additional specificity determinants must exist within the isolated MAPK docking sites. However, the number of basic residues, in combination with the additional specificity determinants, may indeed be inversely proportional to ERK affinity because increasing the number of basic residues in the ERK docking site of RSK2 decreased the affinity for ERK (Fig. 4B); as in the case of the mutant p38 MAPK docking site, mutation of the ERK docking site did not increase the affinity for p38 MAPK (not shown), supporting the role for additional determinants of specificity.

To examine whether interaction between the RSK2/MK2 chimera and p38 MAPK resulted in activation of the chimera in vivo, ectopically expressed WT-RSK2, RSK2/MK2 chimera and MAPKAPK2 were immunoprecipitated from BHK cells treated with inhibitors or stimulants of the MAPK pathways. The enzymes were eluted from the immune complex, and kinase activity toward S6 or MAPKAPK2 substrate peptides was measured. NaCl stimulation of the p38 pathway resulted in a ~130% increase in RSK2/MK2 chimera activity compared with the activity observed when cells were treated with SB 203580, whereas WT-RSK2 activity was unchanged by NaCl treatment (Fig. 5A). SB 203580 is an ATP analog that specifically inhibits catalytic activity of p38 MAPK (15) and eliminates activation of components downstream of p38 MAPK. SB 203580 pretreatment abrogated NaCl-stimulated activity of the RSK2/MK2 chimera (Fig. 5A). The wild type MAPKAPK2 (WT-MAPKAPK2) control was also activated by NaCl, and this stimulation was inhibited by pretreatment with SB 203580 (not shown). Thus, a stress-activated kinase was created by replacing the carboxyl-terminal 23 amino acids of RSK2 with the carboxyl-terminal 35 amino acids of MAPKAPK2.

S6 peptide kinase activity of WT-RSK2 from cells treated with EGF, which activates the ERK pathway, was ~290% greater than that from cells treated with the MAPK-ERK kinase (MEK) inhibitor PD 98059 (Fig. 5B). PD 98059 specifically inhibits activation of MEK1/2 (16), the upstream activator of ERK, thereby reducing phosphorylation and activation of the downstream components. EGF treatment increased kinase activity of the immunoprecipitated RSK2/MK2 chimera by only 76% compared with that of RSK2/MK2 chimera immunoprecipitated from cells pretreated with PD 98059. However, this activity is likely due to cross-talk between the mitogen- and stress-activated pathways because EGF treatment also resulted in a 70% increase in WT-MAPKAPK2 activity (Fig. 5B). The RSK2/MK2 chimera and WT-MAPKAPK2 EGF-stimulated activities were inhibited by pretreatment with PD 98059 (Fig. 5B) but not by pretreatment with SB 203580 (not shown). Thus, replacing the carboxyl-terminal tail of RSK2 with that of MAPKAPK2 removes the enzyme from the ERK pathway.
To examine whether expression of the RSK2/MK2 chimera resulted in p38 MAPK-regulated phosphorylation of a physiological RSK substrate, the α isoform of the human estrogen receptor (ERα) was co-expressed in BHK cells with either WT-RSK2 or RSK2/MK2 chimera. RSK phosphorylates Ser-167 of the ERα (17). A phospho-specific Ser-167 antibody revealed that overexpression of either RSK2/MK2 chimera or WT-RSK2 resulted in phosphorylation of ERα (Fig. 6A). ERα immunoprecipitated from NaCl-treated cells co-expressing the RSK2/MK2 chimera was phosphorylated 2.8-fold more on Ser-167 than ERα immunoprecipitated from SB 203580-treated cells co-expressing RSK2/MK2 chimera, whereas NaCl did not increase Ser-167 phosphorylation of ERα in cells co-expressing WT-RSK2 (Fig. 6A). Pretreatment of cells co-expressing the RSK2/MK2 chimera and ERα with SB 203580 eliminated the NaCl-induced phosphorylation of Ser-167 (Fig. 6A), indicating that in vivo phosphorylation of the RSK substrate by the RSK2/MK2 chimera is a p38 MAPK-regulated event. As stated above, the amino-terminal catalytic domain of RSK phosphorylates exogenous substrates. Mutation of the critical lysine (Lys-100) in the catalytic core to an alanine eliminates kinase activity. ERα immunoprecipitated from cells co-expressing the kinase-defective RSK2/MK2 chimera (K100A chimera) exhibited no Ser-167 phosphorylation (Fig. 6B). Therefore, the p38-MAPK-regulated phosphorylation of ERα requires catalytic activity of the RSK2/MK2 chimera. These data indicate that the signaling pathway in which a MAPKAPK functions in vivo is dictated by the MAPK docking site in the carboxyl-terminal tail of the MAPKAPK.

Herein we demonstrate that specific MAPK docking sites are located in the carboxyl-terminal tails of the MAPKAPKs. Determinants for specificity of interaction with the upstream MAPK are contained within the isolated MAPK docking sites. Replacing the ERK docking site of RSK with the p38 MAPK docking site of MAPKAPK2 results in an enzyme that complexes with p38 MAPK instead of ERK in vitro, relocating the enzyme from the ERK pathway to the p38 MAPK pathway and
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creating a stress-activated kinase that phosphorylates a RSK substrate in vivo. Thus, complex formation of MAPKs with the MAPK docking site of the MAPKAPKs is a general feature important for specific regulation of these MAPK-targeted kinases in vivo and dictates the signal transduction pathway in which a MAPKAPK is involved.

The MAPK docking site is not limited to MAPKAPKs. The transcription factor Elk-1 has overlapping but distinct interaction sites for ERK and c-Jun NH2-terminal kinase (18), and recently the tyrosine phosphatase PTP-SL was shown to contain a MAPK docking site (19). In addition, specific docking sites are not limited to MAPKs. The retinoblastoma protein has a sequence similar to the MAPK docking sites that targets it as a substrate for the proline-directed cyclin-dependent kinase (20).

Each of these interaction motifs is required for efficient phosphorylation of the substrate by the proline-directed kinases. Thus, it is clear that substrate specificity of the proline-directed kinases involves docking sites in the downstream targets that are distinct from the consensus phosphorylation sequence. In addition, docking sites may enable co-localization of a specific proline-directed kinase with a specific target in signaling complexes where they collaborate to elicit physiological responses of pathway activation. Upon the elucidation of the determinants for specificity of the docking sites, the amino acid sequence would be a useful tool for identifying novel substrates.

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