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CHARACTERIZATION OF THE PROLINE-RICH REGION OF MOUSE MAPKAP KINASE 2: INFLUENCE ON CATALYTIC PROPERTIES AND BINDING TO THE C-ABL SH3 DOMAIN *IN VITRO* *

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The primary structure of mouse MAP kinase-activated protein (MAPKAP) kinase 2 contains a proline-rich N-terminal region which might function as a src-homology 3 (SH3) domain-binding motif *in vivo*. To demonstrate the ability of this region to bind SH3 domains, we analyzed the interaction of the SH3 domain of the protein tyrosine kinase c-abl with MAPKAP kinase 2. It is demonstrated, that the proline-rich region specifically binds c-abl-SH3 domain *in vitro*. Furthermore, it is shown, that deletion of this proline-rich region does not significantly influence the substrate binding properties of the enzyme when analyzed with the substrate small heat shock protein Hsp25. The data suggest that the proline-rich region of MAPKAP kinase 2 could interact with proteins containing SH3-domains also *in vivo* regulating its cellular localization and/or modulating its enzymatic properties.

Downstream to MAP kinases, cellular signal transduction proceeds via two serine/threonine kinases, which can be activated by MAP kinases and hence are designated MAP kinase-activated protein (MAPKAP) kinases 1 and 2. These enzymes transform the signal of activated MAP kinases to further cellular targets, which are not directly phosphorylated by MAP kinases at the same sites. MAPKAP kinase 1, also designated p90 ribosomal S6 kinase II (p90 rsk), is known to phosphorylate the serum response transcription factor SRF (1), regulating its contribution to transcriptional activity. For MAPKAP kinase 2, glycogen synthase N-terminal peptide (2), the small heat shock proteins Hsp25 and Hsp27 (3) and tyrosine hydroxylase (4)

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Abbreviations: 3BP1, SH3 domain-binding protein 1; GST, glutathione S-transferase; Hsp, heat shock protein, MAP kinase, mitogen-activated protein kinase; MAPKAP kinase, MAP kinase-activated protein kinase; SH3, src-homology 3.

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have been described as substrates *in vitro*. The physiological role of the phosphorylation of these substrates is still unclear.

The primary sequences of MAPKAP kinase 2 deduced from mouse (5) and human (6,7) cDNA-clones show a proline-rich region N-terminal to the catalytic domain (5-7) and a nuclear translocation signal C-terminal to both the catalytic domain and the MAP kinase phosphorylation site (5,6), which could be related to the intracellular function of this enzyme. Interestingly, a cDNA for a second isoform of human MAPKAP kinase 2, which does not contain the potential nuclear translocation signal and probably results from alternative splicing, has been identified (7). This finding indicates the intriguing possibility of having distinct isoforms of this enzyme in the nuclear and cytosolic compartment as known for p70 and p85 S6 kinases, which differ in an N-terminal nuclear localization signal (8). Possibly, these different isoforms correspond to the two molecular masses (53 kDa and 60 kDa) described for rabbit MAPKAP kinase 2 (2). However, in both isoforms of MAPKAP kinase 2 the proline-rich region N-terminal to the catalytic region could be detected. This region matches the different consensus sequences postulated for SH3 domain-binding protein motifs (9,10). The functional importance of the potential nuclear translocation signal and the ability of the proline-rich region of MAPKAP kinase 2 to specifically bind SH3 domains and, hence, act as a SH3-binding element, have not been demonstrated so far.

In this paper, we used recombinant glutathione S-transferase (GST)-MAPKAP kinase 2 expressed in *E.coli* to analyze the ability of this kinase to specifically interact with SH3 domains. Using a GST-MAPKAP kinase 2 deletion mutant lacking the proline-rich region, it is demonstrated that deletion of this region does not directly influence the catalytic properties of the enzyme analysed with the substrate Hsp25, but that this region is responsible for the binding to the SH3 domain of the tyrosine kinase c-abl.

MATERIALS AND METHODS

Expression of GST-MAPKAP kinase 2 and the SH3 domain-binding mutant GST-MAPKAP kinase 2 Δ3B. Following PCR mutagenesis to introduce the appropriate restriction sites the mouse cDNA of MAPKAP kinase 2 (5) was cloned into the *Xma I/Xho* I site of the *E. coli* expression vector pGEX-5X-3 (Pharmacia), which was used to express the enzyme as a GST-fusion protein in *E. coli* SURE (Stratagene) cultivated in 2 x YT-G medium containing 16 g/l tryptone, 10 g/l yeast extract and 5 g/l NaCl. Expression was induced at an optical density OD_{600} = 1 by adding IPTG to a final concentration of 1 mM. After additional 90 min cells were harvested, lysed by sonication and GST fusion protein was purified using glutathione-Sepharose 4B (Pharmacia) as described by the manufacturers. The expression of the deletion mutant GST-MAPKAP kinase 2 Δ3B was achieved in a similar manner using an expression vector lacking the coding region for the first 27 amino acids of mouse MAPKAP kinase 2 (cf. Fig. 1).

Activation of GST-MAPKAP kinase 2 and GST-MAPKAP kinase 2 Δ3B by pp44 MAP kinase. 0.5 μM of the purified recombinant fusion proteins GST-MAPKAP kinase 2 and GST-MAPKAP kinase 2 Δ3B were incubated in a kinase reaction mix containing 50 mM β-glycerophosphate, 0.1 mM EDTA, 4 mM $(CH_3COO)_2Mg$, 0.1 mM ATP, 1.5 μCi [γ-^{33}P]ATP

(optional) and 5 ng pp44 MAP kinase (Biomol, purified from sea star) for 30 min at 30°C. Proteins were separated by SDS-PAGE. ^{33}P-labeled GST-MAPKAP kinase 2 and GST-MAPKAP kinase 2 Δ3B were detected using a Bio Imaging Analyzer BAS 2000 (Fuji).

Assay for MAPKAP kinase 2 activity. 5 µl aliquots from the MAP kinase activation mixture were incubated in a kinase reaction mix of a final volume of 25 µl, containing 50 mM β-glycerophosphate, 0.1 mM EDTA, 4 mM $(CH_3COO)_2Mg$, 0.1 mM ATP, 1.5 µCi [γ-^{33}P]ATP and 10 µg recombinant Hsp25 purified from *E. coli* (11). After 10 min at 30°C reactions were terminated by adding 8 µl 4 x SDS sample buffer. Proteins were separated by SDS-PAGE. ^{33}P-labeled proteins were detected by the Bio Imaging Analyzer BAS 2000 (Fuji) and Hsp25-labeling was quantified by photo-stimulated luminescence (PSL).

Abl-SH3 binding assay. The cDNA for the c-abl-SH3 domain (amino acids 80-138 of mouse c-abl, type IV, (12)) was amplified after reverse transcription of mouse testis mRNA by PCR, cloned into the vector pGEX-5X-3 (Pharmacia) and expressed as described for the GST-kinase above. Purified GST-c-abl-SH3 (3 mg/ml) was biotinylated using sulfo-NHS-biotin (Pierce) at a concentration of 50 µg/mg protein in 50 mM Na_2CO_3/$NaHCO_3$-buffer, pH 8.5, for 30 min at 25°C. As a negative control, purified GST was labeled as described above. GST-MAPKAP kinase 2 and GST-MAPKAP kinase 2 Δ3B as well as the control proteins GST and GST-3BP1 were separated by SDS-PAGE and blotted onto nitrocellulose. After a pre-incubation of 2 h in blocking solution (20 mM Tris/HCl, pH 7.4, 150 mM NaCl (TBS), 5 % (w/v) milk powder, 0.1 % (v/v) Tween 20) nitrocellulose was incubated with the biotinylated GST-c-abl-SH3 protein at a probe concentration of 1.5 µg/ml for 15 h at 4°C. After several washings with TBS, biotinylated protein was detected using streptavidin conjugated with alkaline phosphatase (Promega) at a 1:2500 dilution in blocking buffer incubated for 2h at 25°C and a substrate solution containing nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl-1-phosphate (Promega). Western blot analysis was carried out using a mouse monoclonal anti-GST antibody (Santa Cruz Biotechnology) and a secondary anti-mouse antibody conjugated with alkaline phosphatase (Promega).

RESULTS AND DISCUSSION

From the analysis of the cDNA sequence from mouse and human MAPKAP kinase 2, a proline-rich region has been identified (5-7) which could act as an SH3-binding element. As seen from the alignment of the protein primary structure of this region from mouse and human MAPKAP kinase 2 (Fig. 1), this region is not extremely conserved, but does contain several core

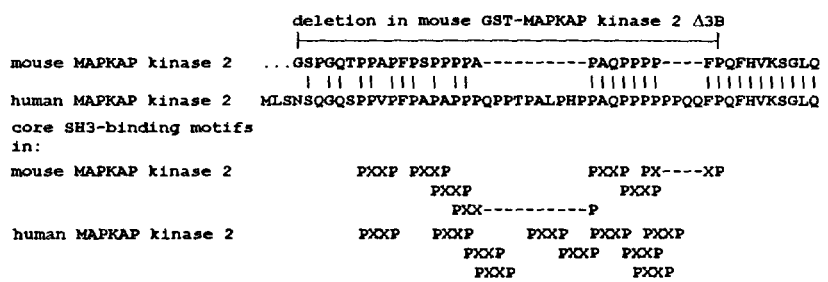

Figure 1. Alignment of the amino acid sequence of the N-terminal part of mouse and human MAPKAP kinase 2. Identities between both sequences are indicated by vertical bars. The incomplete mouse sequence is shown from the first amino acid known, the first amino acid of the human sequence shown is the methionine at the translation start site. The core of the SH3 domain-binding motif, the sequence P X X P, is aligned to the mouse and human sequence separately. The deletion in the mutant GST-MAPKAP kinase 2 Δ3B is indicated.

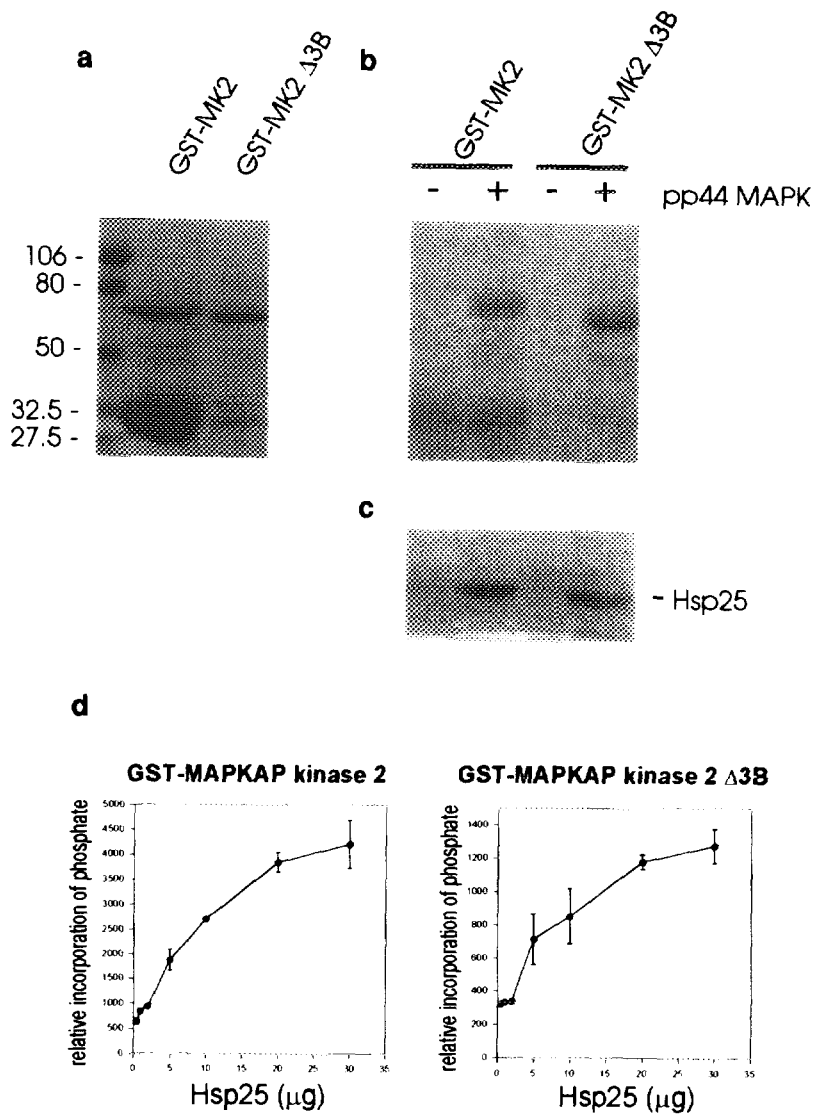

Figure 2. Analysis of the enzymatic activity of GST-MAPKAP kinase 2 and GST-MAPKAP kinase 2 Δ3B after activation with MAP kinase. a) SDS-PAGE gel of the purified GST-fusion proteins GST-MAPKAP kinase 2 (GST-MK2) and GST-MAPKAP kinase 2 Δ3B (GST-MK2 Δ3B). b) Incorporation of ^{33}P from into the recombinant proteins after treatment with pp44 MAP kinase. c) Detection of MAPKAP kinase 2 activity in the different preparations of b), activated in parallel with MAP kinase omitting [γ-^{33}P]ATP. Preparations phosphorylated with pp44 MAP kinase were incubated with the substrate Hsp25 and [γ-^{33}P]ATP. MAPKAP kinase 2 activity is demonstrated by incorporation of ^{33}P into Hsp25. d) Dependence of the incorporation of ^{33}P into Hsp25 on the amount of substrate used. K_M values of 16 μM for GST-MAPKAP kinase 2 and 13 μM for GST-MAPKAP kinase 2 Δ3B could be calculated from Lineweaver-Burk-analysis.

motifs P X X P which are characteristic of the SH3-binding consensus sequence (10,11) in both the human and the mouse protein, raising the question about the functional relevance of these primary structure motifs. To answer this question, we expressed mouse MAPKAP kinase 2 and a

mutant lacking this potential SH3 domain-binding region (cf. Fig. 1) as a GST-fusion protein (Fig. 2a).

To verify that the structures of the recombinant fusion proteins are close to the native structure of the enzyme, we first analyzed the enzymatic activity of the recombinant fusion proteins. Recombinant GST-MAPKAP kinase 2 and its mutant Δ3B were incubated in a phosphorylation reaction with pp44 MAP kinase and the phosphorylation, as well as the enzymatic activity of the phosphorylated and nonphosphorylated fusion proteins, were determined. Both GST-MAPKAP kinase 2 and the deletion mutant GST-MAPKAP kinase 2 Δ3B could be phosphorylated and were enzymatically activated by phosphorylation as judged from incorporation of ^{33}P from [γ-^{33}P]ATP into the recombinant fusion protein (Fig. 2b) and into the MAPKAP kinase 2 substrate small heat shock protein Hsp25 (3) (Fig. 2c). As known for the wild type MAPKAP kinase 2 from rabbit muscle, which could be activated by pp42 MAP kinase (2), only the phosphorylated recombinant fusion protein shows enzymatic activity. To analyze the possible influence of the deletion in GST-MAPKAP kinase 2 Δ3B on the catalytic activity of the enzyme, we determined the K_M values of both GST-MAPKAP kinase 2 and GST-MAPKAP kinase 2 Δ3B. Using a constant ATP concentration of 100 μM with varying Hsp25 concentrations (Fig. 2d), Lineweaver-Burk-analysis indicated K_M values of about 16 μM and 13 μM for the fusion proteins, which correlate with the K_M value determined for the wild type protein (19 μM, (3)). This clearly indicates that the SH3-binding motif does not significantly influence the intrinsic substrate binding properties of the catalytic domain of MAPKAP kinase 2 for Hsp25. Hence, it is more probable that the potential SH3 domain-binding proline-rich region interacts with SH3 domain-containing proteins. This interaction could be involved in regulation of the cellular localization of MAPKAP kinase 2 and/or in recognition of unknown substrate proteins, which, in contrast to Hsp25, may contain a SH3 domain in an appropriate spatial arrangement to the phosphorylated site.

To show the ability of the proline-rich region of MAPKAP kinase 2 to bind SH3 domain proteins, we chose the SH3 domain of the tyrosine kinase c-abl, since it is known that this SH3 domain has the ability to bind to a variety of SH3 domain-binding proteins (9,13). The SH3 domain of mouse c-abl was expressed as a GST fusion protein and labeled by biotinylation. As a negative control, GST alone was expressed and biotinylated. Both proteins were used in an assay which detects interaction with GST-MAPKAP kinase 2 and GST-MAPKAP kinase 2 Δ3B bound to nitrocellulose. As a negative and positive control, GST and the GST-fusion of the SH3-binding protein 3BP1 (14) were bound. Fig. 3a and 3b demonstrate that comparable amounts of the different proteins were applied to the blot and that most of these proteins are full length or truncated GST-fusion proteins as judged by their ability to bind the anti-GST antibody. Fig. 3c demonstrates a clear binding of the biotinylated GST-c-abl-SH3 fusion protein to the GST-

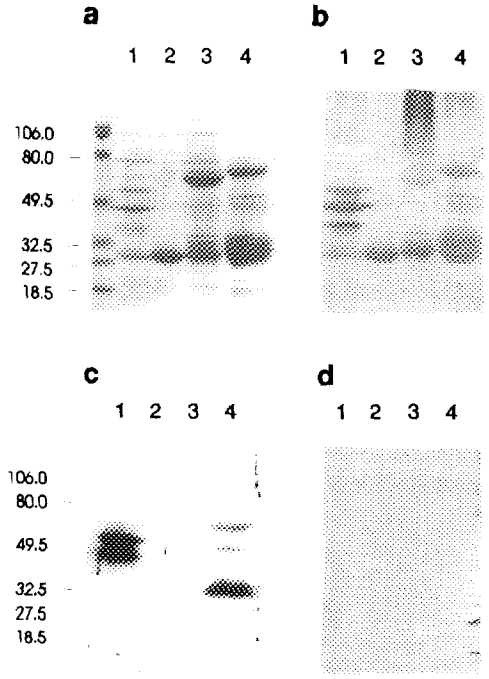

Figure 3. ***In vitro*-binding of GST-c-abl-SH3 to the proline-rich motif of MAPKAP kinase 2.** Lanes 1: GST-3BP1, lanes 2: GST, lanes 3: GST-MAPKAP kinase 2 Δ3B and lanes 4: GST-MAPKAP kinase 2. a) Coomassie stain of the SDS-gel of the total purified recombinant proteins applied to electrophoresis before transfer to nitrocellulose. The molecular mass of standard proteins (in kDa) is indicated. b) Western blot detection of GST-fusion proteins with a specific antibody to GST, c) binding of biotinylated GST-c-abl-SH3 and d) binding of biotinylated GST to the proteins transferred and bound to nitrocellulose as in b).

MAPKAP kinase 2 containing the proline-rich region but not to the deletion mutant GST-MAPKAP kinase 2 Δ3B. In contrast, biotinylated GST itself shows no binding to any of the proteins on the blot (Fig. 3d). This result demonstrates the ability of MAPKAP kinase 2 to bind to SH3 motifs *in vitro* and suggests that this interaction can be of importance also *in vivo*. In addition to the binding of GST-c-abl-SH3 to the full length GST-MAPKAP kinase 2, a binding to smaller fragments having a molecular mass greater than 31 kDa was observed. Taking into account that GST alone has a molecular mass of about 27 kDa, this finding indicates that the first 30 amino acids of the MAPKAP kinase 2 fused to GST are sufficient for SH3-binding. Together with the finding that the deletion mutant GST-MAPKAP kinase 2 Δ3B missing the first 27 amino acids of mouse MAPKAP kinase 2 can not bind GST-c-abl-SH3, it is clearly shown that the N-terminal proline-rich region is necessary and sufficient for SH3 domain-binding.

The biological function of the SH3 domain-binding element in this serine/threonine kinase is still unknown. It can be speculated that the binding of MAPKAP kinase 2 to SH3 domain-containing proteins within the cell can influence the intracellular distribution of this enzyme. Since one known substrate, Hsp25, is assumed to influence the actin polymerization in a

phosphorylation dependent manner (15, 16) and SH3 domains have also been identified in actin-binding proteins (17), a targeting of the enzyme to the cytoskeleton seems possible. Furthermore, since several SH3 domain-containing proteins are relatively upstream in the cellular signal transduction pathways, one may also consider a feedback mechanism in this process by the downstream MAPKAP kinase 2. A final consideration is that the SH3 domain-binding element of MAPKAP kinase 2 may contribute directly to the binding of MAPKAP kinase 2 substrates or inhibitors before interacting with the catalytic domain or to the interaction with MAPKAP kinase 2 kinases.

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