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The MAP kinase-activated protein kinase 2 contains a proline-rich SH3-binding domain

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The protein sequence of MAP kinase-activated protein kinase 2 (MAPKAP kinase 2) deduced from mouse cDNA sequence reveals structural features of the enzyme, which could be of importance for its function: a proline-rich SH3-binding domain N-terminal to the catalytic region, a MAP kinase phosphorylation site and a bipartite nuclear targeting sequence located C-terminal to the catalytic region. The catalytic domain itself has the strongest homology to calcium/calmodulin-dependent protein kinase II. Northern blot analysis demonstrates a 3.5 kb MAPKAP kinase 2 transcript which is ubiquitously expressed and, hence, co-expressed with the mRNA of the recently identified substrate Hsp25 in all tissues analysed. However, the functional consequences of the nuclear targeting sequence present in MAPKAP kinase 2 suggest the existence of further substrates for the enzyme in the nucleus.

Protein kinase; MAPKAP kinase 2; Nnuclear targeting sequence; Heat shock protein; Proline-rich SH3-binding domain; cDNA

1. INTRODUCTION

Protein phosphorylation represents a key mechanism of transduction in mitogenic signalling, e.g. by activating MAP kinase via the MAP kinase cascade. MAP kinase-activated protein kinase 2 (MAPKAP kinase 2) has been described as an enzyme which is activated by phosphorylation via MAP kinases [1]. This property of MAPKAP kinase 2 is shared with the 90 kDa ribosomal S6 kinases (RSKs; members of the RSK family are also referred as S6 kinase I and II, ISPK1 or MAPKAP kinase 1) which are also phosphorylated and activated by MAP kinase as a result of mitogenic stimulation [2]. The substrate used for initial purification of MAPKAP kinase 2 was a peptide derived from the N-terminal part of glycogen synthase [1]. The small heat shock proteins (sHsps) have been identified as further substrates. Both, the small heat shock protein from mouse, Hsp25, and from human, Hsp27, have been demonstrated to be phosphorylated very efficiently by MAPKAP kinase 2 in vitro [3] at the same sites which are phosphorylated in vivo [4,5]. It is not clear whether the sHsps are the only physiological substrates of this enzyme and whether phosphorylation of sHsps is the main process relevant for the biological function of this enzyme.

In this paper we describe cloning and sequencing of mouse MAPKAP kinase 2 cDNA resulting in identification of primary structure motifs which may prove to be of important influence on regulation of function and subcellular localisation of MAPKAP kinase 2.

2. EXPERIMENTAL

2.1. *Polymerase chain reaction*

Degenerate oligonucleotide 5′-GA(TC)GTNAA(AG)CCNGA-(AG)AA(TC)(TC)T-3′ and 5′-TCCANGG(AG)TG(AG)TTCAT(AG)-AA-3′ derived from the sequence of the tryptic peptides of domains VIb (DVKPENL) and XI (FMNHPWI) [1,6] were used as primers in a PCR reaction with mouse cDNA as template. The fragment amplified was cloned into pUC18 (Sure Clone Ligation Kit, Pharmacia). DNA sequencing was carried out in both directions using double-strand plasmid and Sequenase 2.0 (US Biochemical Corp.). The deduced amino acid sequence of the fragment was verified to be a part of the MAPKAP kinase 2 sequence by showing identity to the peptides known from rabbit MAPKAP kinase 2 [1]. A hybridisation probe for screening of the cDNA library and northern hybridisation was generated from the cloned PCR fragments by random priming and labelling with [α-^{32}P]ATP (3000 Ci/ mmol) (Megaprime labelling kit, Amersham).

2.2. *cDNA-screening and rapid amplification of cDNA ends (RACE)*

A mouse lung cDNA library in gt10 was screened using the PCR fragment as probe. Four different clones were identified and sequenced as described above. Rapid amplification of cDNA ends [7] was carried out using the 5′-AmpliFINDER RACE Kit (Clontech, Palo Alto, CA) and the primers P1 (5′-ATGTGTGGGCACTGGG-AGG-3′) and P2 (5′-CGAAGATCCGCAGCACC-3′) complementary to the cloned 5′ region of the cDNA.

2.3. *Northern blot experiments*

A mouse Multi Tissue Northern Blot containing 2 g of electro-

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Abbreviations: bp, base pair(s); Hsp25, small mouse heat shock protein; Hsp27, small human heat shock protein; ISPK1, insulin-stimulated protein kinase 1; MAP, mitogen activated protein; MAPKAP kinase, MAP kinase-activated protein kinase; NTS, nuclear targeting sequence; sHsp, small heat shock protein; RACE, rapid amplification of cDNA ends; RSKs, ribosomal S6 kinases, members of this kinase family are also referred as S6 kinase I, S6 kinase II, ISPK1 or MAPKAP kinase 1; SH3, src homology 3.

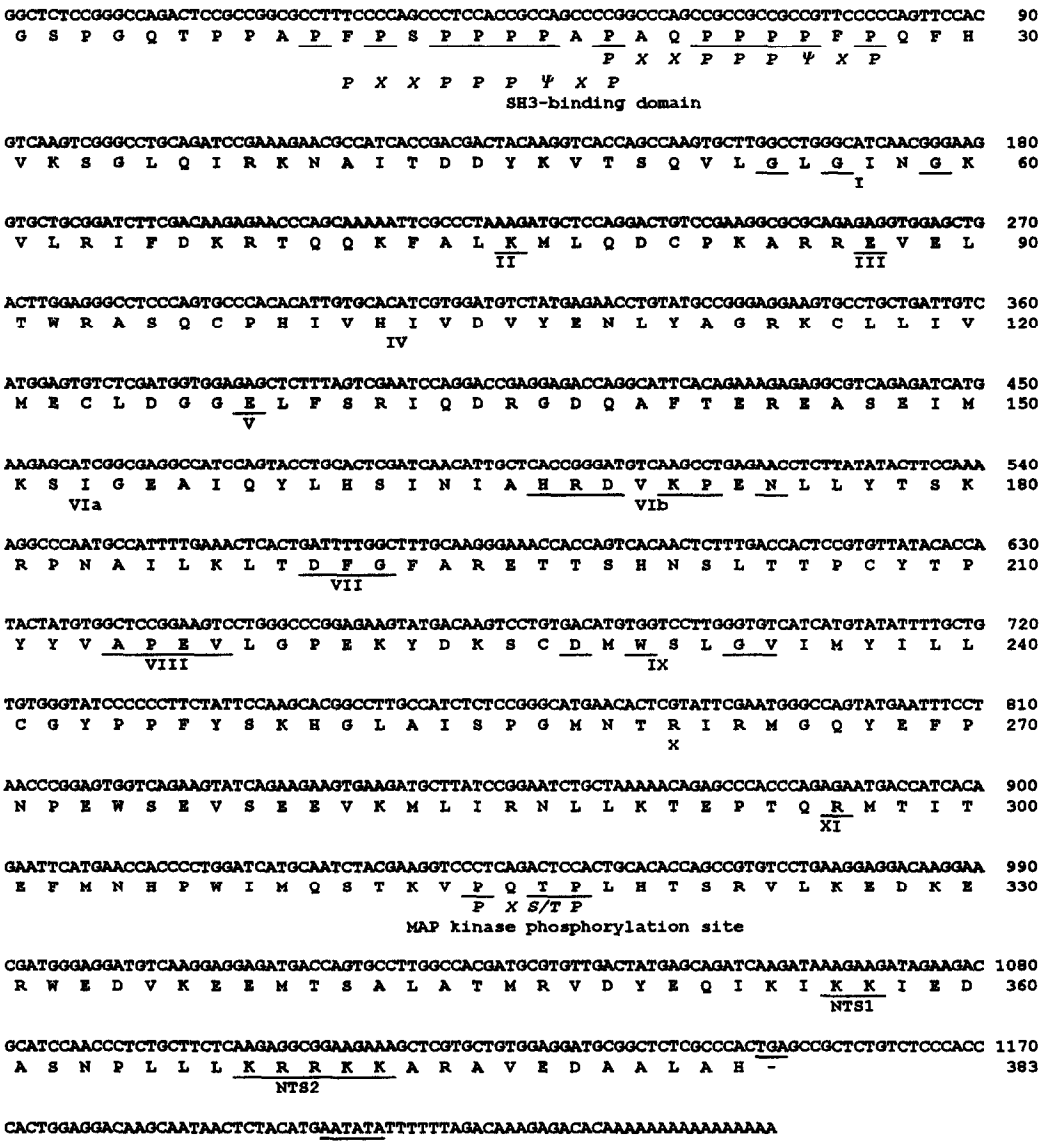

Fig. 1. cDNA sequence and deduced protein structure of MAPKAP kinase 2. The stop codon and polyadenylation signal are underlined. The protein sequence of MAPKAP kinase 2 deduced from the open reading frame is shown in one letter code. The proline-rich SH3-binding domain, the MAP kinase phosphorylation site and the bipartite nuclear targeting sequence are underlined, the consensus sequence for the SH3-binding domain and the MAP kinase recognition motif are given in italics and aligned to the sequence. The bipartite nuclear targeting sequence is indicated by NTS1 and NTS2. The protein kinase catalytic domain sequence motifs (I–XI) are marked and the highly conserved amino acid residues of these motifs are underlined.

phoretically separated poly(A)$^+$ mRNA from each tissue was obtained from Clontech (Palo Alto, CA). Probes used were the MAPKAP kinase 2 PCR fragment, an about 600 bp *Hin*dIII cDNA fragment of Hsp25 [8] and a 2 kb probe for human β-actin. Hybridisation was carried out in 5 × SSPE, 10 × Denhardt's reagent, 2% SDS, 50 μg/ml fragmented salmon sperm DNA, 50% formamide at 42°C overnight. Filters were washed three times for 10 min in 2 × SSC, 0.05% SDS at room temperature, and twice for 15 min in 2 × SSC, 0.1% SDS at 50°C. Northern blot analysis and quantification was performed using the Bio Imaging analyser BAS 2000 (Fuji).

3. RESULTS

3.1. *cDNA and deduced protein sequence of MAPKAP kinase 2*

Two degenerate oligonucleotides were derived from the sequence of tryptic peptides of the catalytic domain motifs VIb and XI of rabbit MAPKAP kinase 2 [1]. The oligonucleotides were used to amplify a 418 bp mouse cDNA fragment of MAPKAP kinase 2, which was further taken as probe for screening of a mouse lung cDNA library in λgt10. Four different types of overlap-

ping cDNA clones were isolated and analysed. All clones obtained lack the part corresponding to the extreme 5′-end of the MAPKAP kinase 2-mRNA and the translation start site. Further screening of the cDNA library and RACE did not provide cDNA clones which code for the translation start of MAPKAP kinase 2. This is probably a result of the extremly GC-rich region at the 5′-end of the mRNA sequence, which may produce stable secondary mRNA structure and thereby inhibits full-length first strand cDNA synthesis [7]. Similar problems caused by 5′ GC-rich regions have been described in cloning for the MAP kinase erk-1 cDNA [9].The cDNA sequence and translated protein sequence of MAPKAP kinase 2 is shown in Fig. 1. The cDNA sequence represents an open reading frame of 1149 bp, coding for a polypeptide of 383 amino acids. It contains a stop codon and a polyadenylation signal AATATA 21 bp upstream of the poly(A) tail in the 3′ non-translated region. The deduced protein sequence of MAPKAP kinase 2 shows two overlapping proline-rich SH3-binding domains with the sequence PFPAPPPPAPAQPPPPFP N-terminal to the catalytic domain of the kinase. At least one of these overlapping motifs perfectly matches the SH3-binding consensus sequence PXXPPPψXP (ψ represents a hydrophobic amino acid residue) [10]. The catalytic domain of MAPKAP kinase 2 is characterised by the various conserved motifs (I–XI, cf. [6]) as well as by the occurrence of the sequences of the tryptic peptides obtained form rabbit MAPKAP kinase 2 [1]. The MAP kinase phosphorylation site in rabbit MAPKAP kinase 2 has already been identified [1]. In the deduced amino acid sequence of mouse MAPKAP kinase 2 this site is located C-terminal to the catalytic domain within the sequence motif PQT*P, which is in agreement with the MAP kinase recognition motif PXS/TP [11]. This finding is in contrast to the position of the phosphorylation sites of other protein kinases as ISPK1 (RSK, MAPKAP kinase 1) [12], cAMP-dependent protein kinase [13], MAP kinase [14] and p34cdc2 kinase [15], which are located within the catalytic domain N-terminal to the motif VIII [6]. Interestingly, the phosphorylation site of MAPKAP kinase 2 is followed by an ideal bipartite nuclear targeting signal KK$(X)_{10}$KRRKK, which has been described to be sufficient for nuclear localisation of a variety of proteins [16].

3.2. *Homology to other protein kinases*

The primary structure of the catalytic domain of MAPKAP kinase 2 was compared to the other protein kinases contained in the Protein Kinase Catalytic Domain Database (cf. [6]) using the method of Needleman and Wunsch [17]. The sequence of highest homology is the a α subunit of rat calcium/calmodulin-dependent protein kinase II (score 50.8) followed by myosin light chain kinase from chicken smooth muscle (score 30.8), the C-terminal catalytic domain of mouse RSK kinase

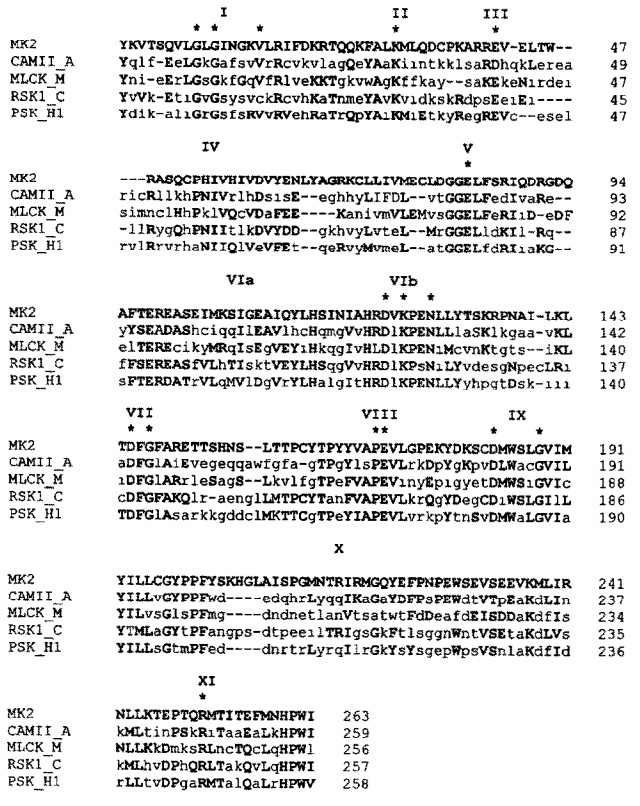

Fig. 2. Comparison of MAPKAP kinase 2 catalytic domain with catalytic domains of similar proteins kinases. MK2 = mouse MAPKAP kinase 2; CAMII_A = α subunit of rat calcium/calmodulin-dependent protein kinase II; MLCK_M = myosin light chain kinase from chicken smooth muscle; RSK1_C = C-terminal catalytic domain of mouse RSK kinase and PSK_H1 = putative protein-serine kinase PSK H1 from HeLa cells. Identities and strong similarities (Y = F, Q = E, V = I, M = L, R = K, D = E, S = T, N = E, H = Q, N = H) to MAPKAP kinase 2 are shown by uppercase letters. The subdomains I to XI (cf. [6]) are indicated and the conserved amino acid residues of these domains are marked with an asterisk.

(score 27.8) and the putative protein-serine kinase cells PSK H1 from HeLa (score 27.8). The alignment of the catalytic domains of these kinases to MAPKAP kinase 2 is shown in Fig. 2. Interestingly, the homologous kinases are exclusively located in a cluster of the phylogenetic kinase tree known to contain calcium/calmodulin-dependent protein kinases. However, until now no calcium/calmodulin dependence of the MAPKAP kinase 2 [1] and other members of this cluster as RSK C1, RSK C2 and PSK H1 has been observed [6].

3.3. *Co-expression of MAPKAP kinase 2 and Hsp25*

Northern blot experiments revealed an about 3.5 kb mRNA for mouse MAPKAP kinase 2 (Fig. 3). The large size of the MAPKAP kinase 2-mRNA cannot be explained by the coding and 3′ non-translated region analysed and is probably the result of the size of the 5′ non-translated region. In all mouse tissues under investigation expression of MAPKAP kinase 2 could be detected. Using specific DNA probes we have also monitored expression of the MAPKAP kinase 2 substrate

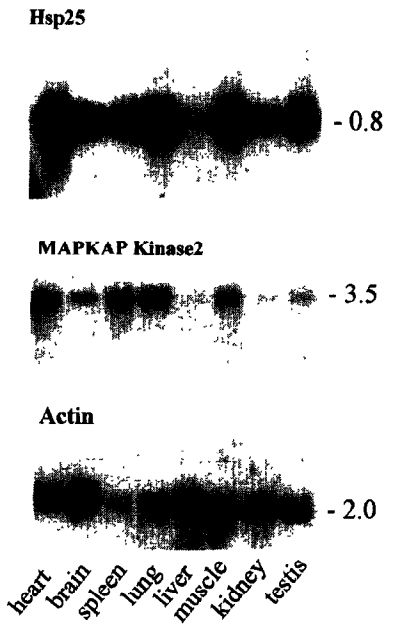

Fig. 3. Northern blot analysis of expression of MAPKAP kinase 2 and Hsp25. Expression of Hsp25-mRNA (a), MAPKAP kinase 2-mRNA (b) and β-actin-mRNA (c) in different mouse tissues.

Hsp25 and as a control the expression of β-actin. The relative amount of specific MAPKAP kinase 2-mRNA and Hsp25-mRNA compared to the β-actin signal was quantitatively analysed by phospho-imaging and is shown in Table I. Interestingly, the lowest expression of both MAPKAP kinase 2-mRNA and Hsp25-mRNA compared to β-actin-mRNA is detected in liver, kidney and brain. The highest relative expression of enzyme and substrate-mRNA is calculated for spleen, caused by relatively low abundant β-actin-mRNA. A high relative expression of MAPKAP kinase 2- and Hsp25-mRNA is also detected in lung and testis, followed by heart and muscle.

4. DISCUSSION

To our knowledge, MAPKAP kinase 2 is the first protein kinase which contains a proline-rich SH3 domain-binding sequence motif. This motif has been identified in different proteins, e.g. formins, muscarinic acetylcholine receptor [10] and *Drosophila* Sos [18]. The src homology 3 (SH3) domain binding to the proline-rich sequence is present in a variety of molecules involved in signal transduction, as the family of SH3-SH2-adaptors including *vav* [19] and *Drosophila drk* [20], the tyrosine kinase oncogenes c-*abl* and c-*src* [21] as well as phospholipase Cγ, phosphatidyl inositol-3-kinase and GTPase activating protein GAP (for review see [22]). Furthermore, SH3 domains are also found in proteins involved in cytoskeletal architecture as spectrin and actin-binding proteins (see [22]). The presence of a proline-rich SH3 domain-binding motif in MAPKAP kinase 2, which is located at a relatively late position in the mitogenic signal transduction pathway, opens up new possibilities in feedback control of signalling or in further signal transduction influencing cytoskeletal organisation. The identification and characterisation of SH3 domain containing target proteins which bind to MAPKAP kinase 2 will probably provide better understanding of these processes.

The MAPKAP kinase 2-mRNA is demonstrated to be ubiquitously expressed in the various mouse tissues used for the Northern blot analysis. Since Hsp25-mRNA is also constitutively expressed in these tissues, a co-expression of MAPKAP kinase 2 and the substrate Hsp25 can be demonstrated (cf. Table I). However, this result does not exclude that both proteins are localised in different cellular compartments. The ideal bipartite NTS motif in the C-terminal region of MAPKAP kinase 2 raises the question of functional significance of this motif. Taking into account the ideal fit of the MAPKAP kinase 2 sequence motif with the NTS consensus sequence and that degenerated NTS motifs are present only in 4.2% of the non-nuclear protein sequences contained in the SwissProt Database [16], it is most likely that this motif is responsible for nuclear localisation of MAPKAP kinase 2. Furthermore, the close proximity of the MAP kinase phosphorylation site, threonine-317, to the bipartite NTS (lysine-356 to lysine-372) raise the possibility of modulation of nuclear targeting activity of MAPKAP kinase 2 by phosphorylation [23]. As demonstrated for the simian virus 40 large T-antigen, nuclear transport kinetics is increased after phosphorylation at sequences flanking the NTS [24]. Accordingly, it could be speculated that activation of MAPKAP kinase 2 via phosphorylation by MAP kinase also modulates its cellular localisation by increasing translocation to the nu-

Table I

Relative level of expression of MAPKAP kinase 2 (MK2) and Hsp25 mRNA in different mouse tissues

	Heart	Brain	Spleen	Lung	Liver	Muscle	Kidney	Testis
Hsp25	3.1	0.4	5.5	5.2	0.3	3.7	0.5	5.2
MK2	0.5	0.15	3.6	1.0	0.2	0.8	0.2	1.0

Hybridisation signal was quantified by phospho-imaging and normalised by the signal obtained with the β-actin probe.

cleus. This hypothesis would be parallel to the observation that a fraction of MAP kinases and RSKs enters the nucleus after phosphorylation [25], indicating a general mechanism for transduction of mitogenic signals to the nucleus.

In any case, nuclear localisation of MAPKAP kinase 2 would favour also nuclear substrates, e.g. transcription factors which are phosphorylated in response to mitogenic signals. In contrast, the substrates identified so far, glycogen synthase [1] and the small heat shock proteins [3] are localised in the cytosol. A translocation of the sHsps to the nuclear region has been described as a result of heat shock [26] but not as the result of mitogenic stimulation. Furthermore, after heat shock no significant differences in the degree of phosphorylation of sHsps in the cytosol and in the nuclear fraction could be demonstrated [27,28]. These data could be explained by a cytosolic activity of MAPKAP kinase 2. The analysis of the intracellular localisation of MAPKAP kinase 2 in dependence on its degree of phosphorylation should provide further inside into these questions.

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