The substrate specificity and structure of mitogen-activated protein (MAP) kinase-activated protein kinase-2

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The substrate specificity of mitogen-activated protein (MAP) kinase-activated protein kinase-2 (MAPKAP kinase-2) was investigated by using synthetic peptides related to the N-terminus of glycogen synthase. The minimum sequence required for efficient phosphorylation was found to be Xaa-Xaa-Hyd-Xaa-Arg-Xaa-Xaa-Ser-Xaa-Xaa, where Hyd is a bulky hydrophobic residue (Phe > Leu > Val ≫ Ala), and the peptide Lys-Lys-Phe-Asn-Arg-Thr-Leu-Ser-Val-Ala was phosphorylated with a K_m of 9.3 μ M and V_{max} of 10 μ mol/min per mg. MAPKAP kinase-1 (a homologue of ribosomal protein S6 kinase) also requires an arginine three residues N-terminal to the serine (position $n-3$), but not a hydrophobic residue at position $n-5$. Neither MAPKAP kinase-1 nor MAPKAP kinase-2 could tolerate a proline residue at position $n+1$, indicating that their specificities do not overlap with that of MAP kinase. The specificity of calmodulin-dependent protein kinase-II resembled that of MAPKAP kinase-2, except that it could tolerate replacement of

the arginine by a lysine and the phosphorylation-site serine by a threonine residue. Partial cDNAs encoding MAPKAP kinase-2 were isolated from rabbit and human skeletal muscle and human teratocarcinoma libraries, and Northern-blotting experiments revealed a single 3.3 kb mRNA transcript present at similar levels in six human tissues examined. The catalytic domain was most similar (35–40 % identity) to calmodulin-dependent protein kinases II and IV, phosphorylase kinase, putative serine kinase H1 and the C-terminal domain of MAPKAP kinase-1, which form one branch of the protein kinase phylogenetic tree. The sequence N-terminal to the catalytic domain is proline-rich and contains two putative SH3-binding sites. The threonine residue phosphorylated by MAP kinase lies immediately C-terminal to the catalytic domain and is followed by a nuclear localization signal, Lys-Lys-(Xaa)₁₀-Lys-Arg-Arg-Lys-Lys, near the C-terminus.

INTRODUCTION

We have recently identified a protein kinase that is only active after phosphorylation by mitogen-activated protein kinase (MAP kinase) and is distinguished from the family of ribosomal protein S6 kinases (RSKs), which are also activated by MAP kinase, by its substrate specificity, sensitivity to protein kinase inhibitors and peptide sequences (Stokoe et al., 1992a). We have therefore termed this enzyme MAP kinase-activated protein kinase-2 (MAPKAP kinase-2) to distinguish it from RSKs (or MAP kinase-activated protein kinases-1, MAPKAP kinases-1).

MAPKAP kinase-2 was originally identified by its phosphorylation of glycogen synthase at serine-7 and the corresponding serine in a peptide (GS peptide-1) modelled after the N-terminus of glycogen synthase (Stokoe et al., 1992a). It is unclear whether MAPKAP kinase-2 phosphorylates glycogen synthase *in vivo*, but one physiological target appears to be the small heat-shock proteins, termed hsp25 in murine and hsp27 in human cells (Stokoe et al., 1992b). MAPKAP kinase-2 phosphorylates hsp25 *in vitro* at a similar rate to that at which it phosphorylates glycogen synthase, and hsp25 kinase activity co-purifies with MAPKAP kinase-2 throughout the purification of the latter from skeletal muscle (Stokoe et al., 1992b). MAPKAP kinase-2 phosphorylates the same serine residues in hsp25 and hsp27 (Stokoe et al., 1992b) that are phosphorylated *in vivo* during heat shock (Gaestel et al., 1991; Landry et al., 1992) or in response to signals which activate MAP kinase, such as tumour necrosis factor (Hepburn et al., 1988; Kaur and Saklatvala, 1988),

interleukin-1 (Kaur and Saklatvala, 1988; Saklatvala et al., 1991), platelet-derived growth factor (Saklatvala et al., 1991), fibroblast growth factor (Saklatvala et al., 1991; Landry et al., 1992) and phorbol esters (Welch, 1985; Regazzi et al., 1988). Furthermore, MAP kinase is also activated when mammalian cells are heat-shocked (Dubois and Bensaude, 1993). However, the physiological role(s) of hsp25/hsp27 (and hence of hsp25/hsp27 phosphorylation) is (are) uncertain. These small heat-shock proteins are present constitutively in almost all mammalian cells, although their levels increase during heat shock and other stresses. They are reported to have chaperonin-like properties (Jakob et al., 1993), and their over-expression appears to increase the thermotolerance of some cells and to inhibit cell proliferation (Landry et al., 1989; Knauf et al., 1992).

MAPKAP kinase-2 does not phosphorylate two MAPKAP kinase-1 substrates, namely the glycogen-binding subunit of protein phosphatase-1 or peptides related to the C-terminus of ribosomal protein S6 (Dent et al., 1990; Lavoinne et al., 1991; Stokoe et al., 1992a). Conversely MAPKAP kinase-1 does not phosphorylate hsp25/hsp27 (Stokoe et al., 1992b). However, both kinases phosphorylate glycogen synthase (at serine-7) and GS peptide-1 (Dent et al., 1990; Stokoe et al., 1992a) and they both phosphorylate (activate) tyrosine hydroxylase, the rate-limiting enzyme in catecholamine synthesis (Sutherland et al., 1993a). MAPKAP kinase-2 phosphorylates Ser-40 of tyrosine hydroxylase about twice as fast as Ser-19, whereas MAPKAP kinase-1 phosphorylates Ser-40 specifically. In order to understand why MAPKAP kinase-1 and MAPKAP kinase-2 have

Abbreviations used: MAP kinase, mitogen-activated protein kinase; MAPKAP, MAP kinase-activated protein; RSK, ribosomal protein S6 kinase; CaM kinase-II, calmodulin-dependent protein kinase-II; PKI, peptide inhibitor of cyclic AMP-dependent protein kinase; hsp, heat-shock protein.

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The nucleotide sequence data reported will appear in the EMBL, GenBank and DDBJ Nucleotide Sequence Databases under the accession numbers X75345 (rabbit) and X75346 (human).

Figure 1 Oligonucleotides used for the amplification of a MAPKAP kinase-2 PCR fragment from a rabbit skeletal-muscle cDNA library

The amino acid sequences were from tryptic peptides 5 and 6 (Stokoe et al., 1992a). Abbreviations: N is A, U, G or C; I is inosine.

distinct, yet overlapping, specificities, we have therefore examined the phosphorylation of GS peptide-1 analogues by these two protein kinases.

MAPKAP kinase-2 purified from rabbit skeletal muscle extracts shows three protein staining bands when examined by SDS/PAGE, a closely spaced 60 kDa doublet and a 53 kDa component, whose proportions are relatively constant from preparation to preparation. After separation by gel-filtration on Superose 12, the 60 kDa doublet and the 53 kDa species have similar specific activities, and six tryptic peptides from each species (about 75 residues) were found to have identical sequences (Stokoe et al., 1992a). This suggests that the three forms of MAPKAP kinase-2 are either closely related isoenzymes or derived from the same gene. In this paper we also present the isolation and sequence analysis of cDNAs encoding MAPKAP kinase-2 from mammalian libraries, which reveal an interesting structure in which the catalytic domain is followed by the phosphorylation site and a putative nuclear localization signal.

EXPERIMENTAL

Proteins and peptides

MAPKAP kinase-1 (Sutherland et al., 1993b) and MAPKAP kinase-2 (Stokoe et al., 1992a) were isolated from rabbit skeletal muscle as described previously, and calmodulin-dependent protein kinase-II from rat brain (CaM kinase-II) was generously given by Dr. Angus Nairn, Rockefeller University, New York. Calmodulin was isolated from sheep brain (Stewart and Cohen, 1988). The 19-residue peptide inhibitor of cyclic AMP-dependent protein kinase (PKI) (Scott et al., 1986) and all other peptides were made on an Applied Biosystems 430A Peptide Synthesiser. Their purity was established by chromatography on a Vydac C₁₈ column (Separations Group, Hesperia, CA, U.S.A.) and by fast-atom-bombardment mass spectrometry (VG Analytical 250SE). Their concentrations were determined by amino acid analysis (Waters PICOTAG System).

Measurement of protein kinase activity

All assays were carried out at 30 °C with ATP of specific radioactivity 300 c.p.m./pmol. The data reported in the Results section represent mean values that are typical of at least three independent determinations. The standard error for all reported kinetic constants was $< \pm 20\%$.

MAPKAP kinase-2

The standard assay (0.05 ml) contained 50 mM sodium β -glycerophosphate, pH 7.0, 0.1 mM EDTA, 2.5 μ M PKI,

0.2 unit/ml MAPKAP kinase-2, the peptide KKPLNRT-LSVASLPGLamide (hereafter termed GS peptide-1; 30 μ M), 10 mM magnesium acetate and 0.1 mM [γ -³²P]ATP. After incubation for 10 min, a 0.04 ml sample was spotted on to a 2 cm $\times$ 2 cm square of phosphocellulose paper (Whatman P81), which was washed in 75 mM H₃PO₄ to remove ATP, and ³²P incorporated into the peptide was then analysed by counting the paper for radioactivity (Stokoe et al., 1992a). The phosphorylation of other peptides was carried out in an identical manner. One unit of MAPKAP kinase-2 was that amount which catalysed the phosphorylation of 1.0 nmol of GS peptide-1 in 1 min.

MAPKAP kinase-1

The standard assay (0.05 ml) contained 8 mM Mops, pH 7.0, 0.2 mM EDTA, 2.5 μ M PKI, 0.2 unit/ml MAPKAP kinase-1, the peptide [Gly-245,Gly-246]S6-(218–249) (30 μ M; Lavoie et al., 1991), 10 mM magnesium acetate and 0.1 mM [γ -³²P]ATP. After incubation for 10 min, incorporation of phosphate into peptide was determined as described for MAPKAP kinase-2. The phosphorylation of other peptides was carried out in an identical manner, except that the concentration of MAPKAP kinase-1 in the assay was increased to 0.5 unit/ml. One unit of MAPKAP kinase-1 was that amount which catalysed the phosphorylation of 1.0 nmol of the peptide [Gly-245,Gly-246]S6-(218–249) in 1 min.

CaM kinase-II

This was carried out as described for MAPKAP kinase-1, except that 0.2 mM EDTA was replaced by 1 mM CaCl₂ and 1 μ M calmodulin was included in the assays. One unit of activity was that amount which catalysed the phosphorylation of 1 nmol of GS peptide-1 (30 μ M) in 1 min.

PCR

Three degenerate oligonucleotides were designed to the combined amino acid sequences of peptides 5 and 6 of MAPKAP kinase-2 (Stokoe et al., 1992a) as shown in Figure 1. Oligonucleotides 1 and 3 were used as primers for the following PCR: 0.4 pmol of primers 1 and 3 were added to a portion of oligo(dT)-primed rabbit skeletal-muscle cDNA library in λ gt10 (da Cruz e Silva and Cohen, 1987) comprising 3×10^6 recombinant phage in a 20 μ l PCR reaction containing 1.5 units of thermostable DNA polymerase (Advanced Biotechnologies, London, U.K.) as described by Chen and Cohen (1993). PCR amplification was performed in a DNA thermal cycler (Perkin-Elmer Cetus) by using the following parameters: 94 °C for 5 min; 1 min at 94 °C,

1 min at 50 °C and 2 min at 72 °C for 40 cycles; 50 °C for 5 min and then 72 °C for 10 min. Examination of the PCR products by agarose-gel electrophoresis and ethidium bromide staining revealed a single 450-bp DNA fragment which, after Southern blotting on Hybond N+ (Amersham), was found to hybridize to primer 2 at 30 °C under conditions described previously (Cohen, 1991), with a final wash at 40 °C in 1.3 M NaCl/0.09 M sodium citrate/0.1 % SDS. The PCR fragment was excised and eluted from the gel by using a SpinX column (Costar, Cambridge, MA, U.S.A.), centrifuged for 15 min at 15000 *g*, precipitated in 0.3 M sodium acetate, pH 5.2, by addition of 2 vol. of ethanol and subcloned into plasmid pT7 Blue T (Novagen, supplied by AMS Biotechnology, Witney, Oxon., U.K.) for sequencing and amplification.

Screening of cDNA libraries

The PCR fragment was excised from pT7 Blue T with *SacI* and *PstI*, separated from the vector on a gel and labelled with [α - 32 P]dATP (Amersham) by using a random-priming kit (Boehringer-Mannheim). This labelled PCR fragment was used as a probe to screen the λ gt10 rabbit skeletal-muscle cDNA library on duplicate filters. Three positive clones were isolated in 180000 recombinant phage. After purification of the phage and isolation of the DNA, all were found to contain 782-bp fragments, which were subcloned into the plasmid Bluescript KS+ (Stratagene, San Diego, CA, U.S.A.). Sequencing was performed either with an Applied Biosystems Inc. automated DNA sequencer, or manually with Sequenase 2.0 (United States Biochemical Corp., Cleveland, OH, U.S.A.). In the latter method, GC-rich regions were sequenced by using dITP or deaza-dGTP in place of dGTP. All three clones contained the same insert, which was cleaved with *BglII* to yield two fragments of 150 and 632 bp from the 5' and 3' regions, respectively. These two fragments were labelled and used to screen duplicate filters containing a combined oligo(dT)-primed and random-primed human skeletal-muscle cDNA library in λ gt10 (Clontech, Palo Alto, CA, U.S.A.). Two positive clones obtained from this library were used to screen duplicate filters containing an oligo(dT)-primed human teratocarcinoma cell line NTERA2-clone D1 cDNA library in λ gt10 (kindly provided by Dr. Jacek Skowronski, NIH, Bethesda, MD, U.S.A.). The sequences of the longest clones isolated from this library are presented in the Results section.

Northern blotting

A multiple-tissue Northern blot (Clontech) containing 2 μ g of poly(A)⁺ RNA from 8 human tissues was hybridized with a 522-bp *EcoRI*-*BbsI* fragment of the longest human MAPKAP kinase-2 clone, labelled with [α - 32 P]dATP by using the random-priming kit. The probe corresponded to the C-terminal portion of the protein beyond the catalytic domain, and also contained 150 bases of 3' non-coding region. Hybridization and washing were performed at high stringency according to the manufacturer's instructions.

RESULTS

Molecular basis for the substrate specificity of MAPKAP kinase-2

GS peptide-1 is a good substrate for MAPKAP kinase-2 (K_m = 12.5 μ M, V_{max} = 10 μ mol/min per mg) (Stokoe et al., 1992a). Its structure (KKPLNRTLSTVASLPGLamide) is closely related to the N-terminus of human glycogen synthase

(PLNRTLSTVASLPGL) (Browner et al., 1989), with two lysine residues being added to the N-terminus to facilitate its binding to phosphocellulose paper (see the Experimental section). In initial experiments amino acids were removed sequentially from either the C-terminal or N-terminal end of GS peptide-1 (but the two N-terminal lysine residues were always maintained). The removal of three residues from the C-terminus of GS peptide-1 had little effect on the kinetics of phosphorylation, the peptide KKPLNRTLSTVASL being phosphorylated with a K_m of 12 μ M and V_{max} of 10 μ mol/min per mg. The rate of phosphorylation (at a peptide concentration of 30 μ M) was unaffected when the N-terminal proline (equivalent to residue 1 of glycogen synthase) was removed, but deleting both the proline and the next leucine abolished phosphorylation by MAPKAP kinase-2 (peptides 2 and 3, Table 1). Further truncation experiments indicated that two amino acids must be present C-terminal to the phosphorylation site for efficient phosphorylation of peptides by MAPKAP kinase-2 (peptides 5–7 in Table 1).

The above experiments indicated that KKLNRTLSTVA was the minimum sequence required for efficient phosphorylation by MAPKAP kinase-2, and all further analogues examined were variants of this decapeptide. Since the data (Table 1) suggested that the leucine located five residues N-terminal to the phosphorylation site (position $n-5$) might be critical for phosphorylation, this residue was changed to other amino acids in the next series of experiments (Tables 2 and 3). The results confirmed that the presence of a large hydrophobic residue at position $n-5$ was essential for efficient phosphorylation, the rate decreasing progressively with decreasing hydrophobicity of the residue at this position (Phe > Leu > Val $\gg$ Ala) (Table 3). More detailed kinetic analyses indicated that a hydrophobic residue at

Table 1 The decapeptide KKLNRTLSTVA is the smallest peptide phosphorylated efficiently by MAPKAP kinase-2

The concentration of each protein kinase in the assays was 0.2 unit/ml towards GS peptide-1.

Peptide (30 μ M)	Rate of phosphorylation relative to peptide-1 (%)		
	MAPKAP kinase-2	CaM kinase-II	MAPKAP kinase-1
1 KKPLNRTLSTVASL	100	110	100
2 KKLNRTLSTVASL	122	97	152
3 KKNRTLSTVASL	< 1	4	170
4 KKRTLSTVASL	< 1	3	165
2 KKLNRTLSTVASL	122	97	152
5 KKLNRTLSTVA	98	81	83
6 KKLNRTLSTV	15	5	10
7 KKLNRTLST	< 1	< 1	< 1

Table 2 Effect of the amino acid situated five residues N-terminal to the phosphorylated serine on the kinetic parameters of MAPKAP kinase-2

Peptide	V_{max} (μ mol/min per mg)	K_m (μ M)	V_{max}/K_m
1 KKFNRTLSTVA	10.1	9.3	1.1
2 KKLNRTLSTVA	12.7	18.4	0.69
3 KKVNRTLSTVA	10.3	36.4	0.28
4 KKANRTLSTVA	10.4	435	0.02
5 KKPNRTLSTVA	12.5	612	0.02

Table 3 Identification of several key specificity determinants for MAPKAP kinase-2

The concentration of each protein kinase in the assays was 0.2 unit/ml towards GS peptide-1. Amino acid replacements are shown by **bold** letters.

Peptide (30 μ M)	Rate of phosphorylation relative to peptide-1 (%)		
	MAPKAP kinase-2	CaM kinase-II	MAPKAP kinase-1
1 KKLNRTLSVA	100	100	100
2 K K FNRTLSVA	105	95	69
3 K K VNRTLSVA	67	71	120
4 K K ANRTLSVA	9	16	120
5 K K PNRTLSVA	6	37	68
6 K K KNRTLSVA	< 1	17	186
7 K K ENRTLSVA	< 1	1	52
8 KKLNRTLS L A	95	115	71
9 KKLNRTLS A A	63	106	48
10 KKLNRTLS E A	62	9	42
11 KKLNRTLS K A	< 1	2	< 1
12 KKLNRTLS P A	2	1	< 1
13 KKLN K TLSPA	2	31	< 1
14 KKLNRTLS T VA	3	94	4

position $n-5$ was required for peptide binding (Table 2). The K_m increased progressively as the hydrophobicity of the amino acid decreased. Phosphorylation became undetectable when the leucine at position $n-5$ was replaced by either glutamic acid or lysine (Table 3). Replacement of the arginine at position $n-3$ by a lysine residue also abolished phosphorylation, as did replacement of the serine by a threonine residue (peptides 13 and 14, Table 3). Thus the minimum sequence for efficient phosphorylation by MAPKAP kinase-2 is HydXRXSXX, where Hyd is a bulky hydrophobic residue.

Changing the valine at position $n+1$ to leucine, alanine or glutamic acid did not affect the rate of peptide phosphorylation by MAPKAP kinase-2 significantly (peptides 8–10, Table 3), but changing it to either proline or lysine abolished phosphorylation (peptides 11 and 12, Table 3). The two N-terminal lysine residues added to facilitate the assays were not critical for the specificity of MAPKAP kinase-2, since placing them at the C-terminus of the peptide (instead of the N-terminus) only decreased the rate of phosphorylation slightly (results not shown).

Comparison of the substrate specificities of MAPKAP kinase-2 and CaM kinase-II

Like MAPKAP kinase-2, CaM kinase-II phosphorylates tyrosine hydroxylase at Ser-19 and Ser-40 (Campbell et al., 1986) and glycogen synthase at Ser-7 (Woodgett et al., 1982). Since CaM kinase-II also phosphorylates GS peptide-1 ($K_m = 8.3 \mu\text{M}$, $V_{max} = 8.5 \mu\text{mol/min per mg}$), it was decided to make a more detailed comparison of the specificities of these protein kinases. The specificity requirements of CaM kinase-II were similar to those of MAPKAP kinase-2 in several respects. Firstly, the effect of truncating the C-terminus of the peptide was almost identical (Table 1). Secondly, CaM kinase-II was unable to tolerate either a lysine or a proline residue at position $n+1$ (Table 3). Thirdly, a hydrophobic residue was preferred at position $n-5$ (Table 3).

However, the specificities of MAPKAP kinase-2 and CaM kinase-II also differed in several respects. The most striking differences were the ability of CaM kinase-II to tolerate replacement of the phosphorylation-site serine by a threonine

residue and the arginine at position $n-3$ by a lysine residue (peptides 13 and 14 in Table 3). In addition, replacement of the leucine at position $n-5$ by proline was much less deleterious to CaM kinase-II than to MAPKAP kinase-2 (peptide 5 in Table 3), whereas replacement of this leucine by lysine greatly decreased, but did not abolish, phosphorylation by CaM kinase-II (peptide 6 in Table 3). Finally, a glutamic acid residue at position $n+1$ was detrimental to the activity of CaM kinase-II, but not of MAPKAP kinase-2 (peptide 10 in Table 3).

Comparison of the substrate specificities of MAPKAP kinase-2 and MAPKAP kinase-1

GS peptide-1 is phosphorylated by MAPKAP kinase-1 with K_m of $36 \mu\text{M}$ and $V_{max} = 1.1 \mu\text{mol/min per mg}$. Like MAPKAP kinase-2, MAPKAP kinase-1 could not tolerate replacement of the arginine at position $n+3$ by a lysine, the phosphorylation-site serine by a threonine, or the replacement of valine at position $n+1$ by either proline or lysine (Table 3), and it also required the presence of at least two amino acids C-terminal to the phosphorylation site for efficient phosphorylation (Table 1). However, deletion of the N-terminal leucine residue in GS peptide-1 did not abolish phosphorylation by MAPKAP kinase-1, but actually increased the rate of phosphorylation (peptides 2–4 in Table 1). The lack of importance of leucine at position $n-5$ for the specificity of MAPKAP kinase-1 was confirmed by changing it to other amino acids, which either failed to affect the rate of phosphorylation significantly, or even increased it (Table 3).

Sequence of rabbit and human MAPKAP kinase-2

Six tryptic peptides from rabbit skeletal muscle MAPKAP kinase-2 were isolated and sequenced (Stokoe et al., 1992a), of which three (peptides 2, 5 and 6) showed similarities to sub-domains VI and XI of the catalytic subunits of other protein kinases (Hanks et al., 1988). By using degenerate oligonucleotides, designed from the combined amino acid sequence of peptides 5 and 6 (Figure 1), a DNA fragment of 450 bp was directly amplified by PCR from a rabbit skeletal muscle cDNA library. The PCR product hybridized to a degenerate oligonucleotide corresponding to the internal amino acid sequence of peptides 5 + 6, confirming that it originated from MAPKAP kinase-2 cDNA. The fragment, encoding 150 amino acids, included three of the six peptides sequenced. By using the PCR fragment as a probe, one 782-bp partial cDNA clone encoding sub-domains I–X was obtained by screening of the same library. Cleavage of this 500-bp rabbit cDNA at a unique *Bgl*II site yielded a 150-bp 5' fragment and a 632-bp 3' fragment. On screening of a human skeletal-muscle cDNA library with these rabbit cDNA fragments, three clones were identified from 180 000 phage that were positive with both the 5' and 3' probes. DNA sequencing of the two largest clones confirmed that they encoded human MAPKAP kinase-2, but, since neither was full length, the most 5' region of the longest clone was used to screen a human teratocarcinoma cDNA library. The three largest clones (3 kb) from the teratocarcinoma library were purified, and both DNA strands were sequenced in the coding region. The sequence of the longest clone is shown in Figure 2. The others were identical, except that they lacked 90 and 110 bases at the 5' end. The 3' non-coding region, which comprised 1.5 kb, was not sequenced in its entirety.

All the six tryptic peptides sequenced previously are present in the longest human MAPKAP kinase-2 cDNA isolated. This clone contains the entire catalytic domain and extends to the C-terminus. However, the lack of an initiating ATG and its

Human DNA	5'-TCCCAGGGCCAGAGCCCGCGTGCCTTC	CCGCCCCCGCCCCCGCCGCGCAGCCCCC	ACCCCTGCCCTGCCGCAACCCCGCGCAG	90
Human a.a.	S Q G Q S P P V P F	P A P A P P P Q P P	T P A L P H P P A Q	30
Rabbit DNA	5'-.....G...G.....	A.....C.....		
Human DNA	CCGCCCCCGCCCCCGCAGAGTTC	CAGTCCACGTCAAGTCCGCGCTGCAGATC	AAGAAGAAGCCATCATCGATGACTACAAG	180
Human a.a.	P P P P P P Q Q F P	Q F H V K S G L Q I	K K N A I I D D Y K	60
Rabbit a.a.		R.....		
Subdomain				--
Rabbit DNA	C.....G....	A...G.....G....		
Human DNA	GTCACGACCGAGTCTCGGGCTGGGCATC	AACGGCAAAGTTTTCAGATCTTCAACAAG	AGGACCCAGGAGAAATTCGCCCTCAAAATG	270
Human a.a.	V T S Q V L G L G I	N G K V L Q I F N K	R T Q E K F A L K M	90
Rabbit a.a.		S.....	K.....	
Subdomain	-----I-----	-----II-----		
Rabbit DNA	T.....T	..AC.....G..C.....C..C..T		
Human DNA	CTTCAGGACTGCCCAAGCCCGCAGGGAG	GTGGAGCTGCAGTGGCGGCTCCAGTGC	CCGACATCGTACGGATCGTGGATGTGTAC	300
Human a.a.	L Q D C P K A R R E	V E L H W R A S Q C	P D I V R I V D V Y	120
Rabbit a.a.				
Subdomain	-----III-----	-----IV-----		
Rabbit DNA	C..C.....G.....	C.....G.....T.....T	CTG.....C..T....	
Human DNA	GAGAATCTGTACGAGGAGGAAGTGCCTG	CTGATTGTGATGGAATGTTGGACGGTGA	GAACCTTTAGCCGAATCCAGGATCGAGGA	450
Human a.a.	E N L Y A G R K C L	L I V M E C L D G G	E L F S R I Q D R G	150
Rabbit a.a.			L.....	
Subdomain	-----V-----			
Rabbit DNA	A..G.....T..G.....G..A	C..A..G..A	C.....C	
Human DNA	GACCAGGCATTCACAGAAAGAGAAGCATCC	GAAATCATGAAGAGCATCGGTGAGGCCATC	CAGTATCTGCATTCAATCAACATTGCCCAT	540
Human a.a.	D Q A F T E R E A S	E I M K S I G E A I	Q Y L H S I N I A H	180
Rabbit a.a.				
Subdomain	-----VIA-----			
Rabbit DNA	C.....C.....C.....T.....	C..T.....A.....		
Human DNA	CGGGATGTCAAGCCTGAGAATCTCTATAC	ACCTCCAAAGGCCCAACGCCATCTCGTAA	CTCACTGACTTTGGCTTTGCCAAGGAAACC	630
Human a.a.	R D V K P E N L L Y	T S K R P N A I L K	L T D F G F A K E T	210
Rabbit a.a.		K.....		
Subdomain	-----VIB-----	-----VII-----		
Rabbit DNA	CC.....C	G..C.....C.....C..G..G..	C..C.....A...	
Human DNA	ACCAGCCACAACCTTTTGACCACTCCTGT	TATACACCGTACTATGTGGCTCCAGAAGTG	CTGGGTCAGAGAAGTATGACAAGTCTCTGT	720
Human a.a.	T S H N S L T T P C	Y T P Y Y V A P E V	L G P E K Y D K S C	240
Rabbit a.a.				
Subdomain	-----VIII-----			
Rabbit DNA	G.TC.T....C.....	C..C.....	C..C....	
Human DNA	GACATGTTGGTCTGGGTGTCATCATGTAC	ATTCTGCTGTGGGTATCCCCCTTCTAC	TCCAACCCAGGCCTTGCCATCTCTCCGGGC	810
Human a.a.	D M L V L G V I M Y	I L L C G Y P P F Y	S N H G L A I S P G	270
Rabbit a.a.	W S.....			
Subdomain	-----IX-----			
Rabbit DNA	G.....C	G-3'		
Human DNA	ATGAAGACTCGCATCCGAATGGCCAGTAT	GAATTTCCCAACCCAGAATGGTCAGAAGTA	TCAGAGGAAGTGAAGATGTCATTCGGAAT	900
Human a.a.	M K T R I R M G Q Y	E F P N P E W S E V	S E E V K M L I R N	300
Rabbit a.a.				
Subdomain	-----X-----			
Human DNA	CTGCTGAAACAGAGCCACCCAGAGAATG	ACCATCACCGAGTTTATGAACCCCTTGG	ATCATGCAATCAACAAAGTCCCTCAAAACC	990
Human a.a.	L L K T E P T Q R M	T I T E F M N H P W	I M Q S T K V P Q	330
Subdomain	-----XI-----			
Human DNA	CCACTGCACACCGCGGTCTGAAGGAG	GACAAGGAGCGGTGGGAGGATGTCAAGGAG	GAGATGACCACTGCCTTGCCCAATGCGC	1080
Human a.a.	P L H T S R V L K E	D K E R W E D V K E	E M T S A L A T M R	360
Human DNA	GTGACTACGAGCAGATCAAGATAAAAAAG	ATTGAAGATGCATCAACCTCTGCTGCTG	AAGAGGCGGAAGAAAGCTCGGGCCCTGGAG	1170
Human a.a.	V D Y E Q I K I <u>K K</u>	I E D A S N P L L L	<u>K R R K K</u> A R A L E	390
Human DNA	GCTGCGCTCTGGCCACTGAGCCACCGCG	CCCTCTGCCACGGGAGGACAAGCAATAA	CTCTCTACAGGAATATATTTTAAACGAA	1260
Human a.a.	A A A L A H *			396
Human DNA	GAGACAGAAGTGTCCACATCTGCCTCTCT	CCTCTCAGCTGCATGGAGCTGGAAGTGC	ATCAGTACTGAATT-3'	1335

Figure 2 Comparison of the nucleotide (DNA) and protein (a.a.) sequences encoding human and rabbit MAPKAP kinase-2

In the rabbit sequences, identities with the human sequences are indicated by dots. The six tryptic peptides from rabbit MAPKAP kinase-2 sequenced previously (Stokoe et al., 1992a) are underlined and are identical in the human sequence. The position of the phosphorylation site (Thr-330) is circled, and the termination codon is marked with an asterisk. The XI protein kinase subdomains are marked, and the putative nuclear localization signal is boxed.

calculated molecular mass (~44 kDa) indicate that it is not full-length. The threonine phosphorylated by MAP kinase (Stokoe et al., 1992a) lies immediately C-terminal to the catalytic domain (Figure 2), whereas a putative nuclear localization sequence starts 28 amino acid residues before the C-terminus (Figure 2). The rabbit and human MAPKAP kinase-2 sequences show 90% nucleotide and 97% amino acid sequence identity over the

region where they can be compared, indicating a high degree of conservation between these species.

Expression of human MAPKAP kinase-2 in different tissues

Northern-blot analysis detected a single 3.3 kb transcript in human skeletal muscle (Figure 3) and other human tissues

Figure 3 The mRNA encoding MAPKAP kinase-2 is contained within a single 3.3 kb transcript

The figure shows an autoradiograph of a Northern blot containing poly(A)⁺ RNA from human skeletal muscle hybridized with a MAPKAP kinase-2 clone as described in the Experimental section. The positions of relevant λ HindIII fragments are indicated

Table 4 Relative levels of MAPKAP kinase-2 expression in various human tissues, determined by densitometric analysis of Northern blots

The 3.3 kb mRNA encoding MAPKAP kinase-2 was present in all human tissues examined. The blot was also hybridized with a control protein phosphatase 1 β probe, which showed that the mRNA on the Clontech Northern blot was extremely variable from tissue to tissue. The mRNA encoding protein phosphatase 1 β is present at similar levels in rat (Sasaki et al., 1990) and rabbit (P. T. W. Cohen, unpublished work) tissues. The ratio MAPKAP kinase-2 mRNA:protein phosphatase 1 β mRNA was therefore measured in each human tissue, and values are expressed relative to testis.

Tissue	Relative mRNA levels
Kidney	1.4
Skeletal muscle	1.1
Liver	1.0
Testis	1.0
Lung	0.7
Spleen	0.5

(results not shown), similar to the size of the longest human cDNA isolated. The level of the 3.3 kb transcript only varied about 3-fold in the six human tissues examined (Table 4).

DISCUSSION

The finding that MAPKAP kinase-2 phosphorylates peptides with the sequence HydXXXXXX, where Hyd is a large hydrophobic residue (Phe, Leu, Ile), explains all previous data on the specificity of this enzyme. Thus the two serine residues in murine hsp25 that are phosphorylated by MAPKAP kinase-2 both lie in LXXXXS sequences, whereas the two residues in human tyrosine hydroxylase phosphorylated by MAPKAP kinase-2 lie in FXRXXS (Ser-19) and IXRXXS (Ser-40) sequences. The lack of a suitable hydrophobic residue at position $n-5$ explains why MAPKAP kinase-2 does not phosphorylate the glycogen-binding subunit of protein phosphatase-1, peptides related to the C-

terminus of ribosomal protein S6, the standard peptide substrate for cyclic AMP-dependent protein kinase ('Kemptide', LRRASLG) and other substrates commonly used to assay protein kinases such as casein, phosvitin, the smooth-muscle myosin P-light chain, histone H1 and myelin basic protein (Stokoe et al., 1992a; D. Stokoe and P. Cohen, unpublished work). In contrast, a hydrophobic residue at position $n-5$ is not required for phosphorylation by MAPKAP kinase-1, and changing it to a lysine residue actually increases the rate of phosphorylation (Table 3). This activation is not explained by phosphorylation of the threonine residue in the peptide (directed by the cluster of N-terminal lysine residues), since after phosphorylation only phosphoserine is detected by phosphoamino acid analysis (results not shown). Rather, the increased rate of phosphorylation resulting from replacement of Leu by Lys appears to be caused by a marked decrease in the K_m for peptide phosphorylation (I. Leighton and P. Cohen, unpublished work). The presence of lysine at position $n-5$ is also likely to explain the enhanced rate of phosphorylation of peptides 3 and 4 in Table 1. Sequences of the type RRXSXX are also phosphorylated readily by MAPKAP kinase-1 (Donella-Deana et al., 1993), explaining why it phosphorylates 'Kemptide' (Lavoigne et al., 1991), the glycogen-binding subunit of protein phosphatase-1 (Dent et al., 1990), and Ser-40 of tyrosine hydroxylase (Sutherland et al., 1993a), which all contain this motif. The lack of two arginine residues at positions $n-2$ and $n-3$ can account for the failure of MAPKAP kinase-1 to phosphorylate either serine in hsp25 or Ser-19 of tyrosine hydroxylase.

The presence of a lysine residue at position $n+1$ virtually abolished peptide phosphorylation by MAPKAP kinase-1 and MAPKAP kinase-2 (Table 3). Since basic residues are present at this position in some of the best substrates for protein kinase C, such as the MARCKS protein (Graff et al., 1991), the specificities of MAPKAP kinases and protein kinase C may not overlap *in vivo*. Of even greater potential significance is the observation that a proline at position $n+1$, which is a prerequisite for peptide/protein phosphorylation by MAP kinase (Clark-Lewis et al., 1991; Gonzalez et al., 1991), prevents peptide phosphorylation by both MAPKAP kinases. This may be important in ensuring that the specificities of MAPKAP kinases, which are thought to lie downstream of MAP kinase in the same protein-phosphorylation cascade, never overlap with that of MAP kinase.

Peptide phosphorylation by CaM kinase-II, like MAPKAP kinase-2, requires a basic amino acid (preferably an arginine) at position $n-3$ (Pearson et al., 1985; Hanson and Schulman, 1992), but the present study also suggests that a hydrophobic residue is preferred at position $n-5$, although the requirement is not quite as stringent as it is for MAPKAP kinase-2 (Table 3). This finding can also explain why MAPKAP kinase-2 and CaM kinase-II both phosphorylate glycogen synthase at Ser-7 and tyrosine hydroxylase at Ser-19 and Ser-40 (although, in contrast with MAPKAP kinase-2, CaM kinase-II phosphorylates Ser-19 preferentially). The importance of a particular residue at position $n-5$ has recently been demonstrated for another growth-factor-stimulated protein kinase, termed p70 S6 kinase, which recognizes the sequence RXRXXSXX (Flotow and Thomas, 1992).

Replacement of the valine at position $n+1$ by glutamic acid inhibited phosphorylation by CaM kinase-II. In this respect CaM kinase-II differs from MAPKAP kinase-2, but resembles cyclic AMP-dependent protein kinase (Meggio et al., 1981; Donella-Deana et al., 1993). However, the most striking difference between MAPKAP kinase-2 and CaM kinase-II was the ability of the latter to tolerate a lysine at position $n-3$ and

to phosphorylate threonine as well as serine (Table 3). Replacement of the phosphorylation-site serine by a threonine residue greatly decreases phosphorylation by MAPKAP kinase-1 and MAPKAP kinase-2, largely due to a greatly increased K_m for the peptide. At least two physiological substrates of CaM kinase-II are phosphorylated at threonine residues, namely phospholamban (Simmerman et al., 1986) and CaM kinase-II itself. 'Autophosphorylation' of CaM kinase-II converts it into a form which is active in the absence of Ca^{2+} ions and calmodulin (Hanson and Schulman, 1992).

Searches of current protein-sequence databases with the region of MAPKAP kinase-2 containing sub-domains I–XI revealed sequence similarities to many protein kinases. However, the most similar enzymes were four types of calmodulin-dependent protein kinase, CaM kinase-IV (40% identity, 60% similarity), CaM kinase-II (38% identity, 56% similarity), phosphorylase kinase (35% identity, 58% similarity) and myosin light-chain kinase (29% identity, 55% similarity). The catalytic domain of MAPKAP kinase-2 also showed 37% identity (60% similarity) with the C-terminal protein kinase domain of ribosomal S6 kinase-II (a MAPKAP kinase-1 homologue) and 'putative serine kinase H1' (37% identity, 59% similarity). These six protein kinases, which form one branch of the protein kinase phylogenetic tree (Hanks and Quinn, 1991), show similar identities when compared with each other. Because the sequence of MAPKAP kinase-2 resembled that of several calmodulin-dependent protein kinases and its specificity showed some similarities to that of CaM kinase-II (Table 3), we examined the effect of Ca^{2+} ions and calmodulin on MAPKAP kinase-2 activity. However, no effect of Ca^{2+} /calmodulin could be detected by using either the dephosphorylated (inactive) or phosphorylated (active) form of the enzyme (D. Stokoe, unpublished work).

The region immediately N-terminal to the catalytic domain contains 21 proline residues in a sequence of 35 amino acids. This region contains two examples of Pro-Pro-Pro-Xaa-Pro-Pro sequences which may interact with SH3-domain-containing proteins (Cicchetti et al., 1992; Ren et al., 1993). The threonine residue phosphorylated by MAP kinase lies immediately C-terminal to the catalytic domain of MAPKAP kinase-2, just after sub-domain XI (Figure 2). In contrast, the phosphorylation sites of most other protein kinases which are activated by phosphorylation (e.g. cyclin-dependent protein kinases, MAP kinase, cyclic-AMP-dependent protein kinase and MAPKAP kinase-1) are located 8–10 residues N-terminal to the conserved APE motif present in sub-domain VIII of the catalytic domain (see Sutherland et al., 1993b). However, the most intriguing structural feature is the sequence KKK₁₀RKKRK just before the C-terminus of MAPKAP kinase-2, which conforms exactly to the consensus nuclear localization signal (Dingwall and Laskey, 1991) found in many well-characterized nuclear proteins such as nucleoplasmin, steroid-hormone receptors and the transcription factors fos and jun. Heat-shock protein (hsp) 25 is located in the nucleus as well as the cytoplasm, and nuclear hsp25 is reported to be more highly phosphorylated than cytoplasmic hsp25 (Arrigo et al., 1988). It will clearly be important to study the subcellular localization of MAPKAP kinase-2 and to investigate whether, like MAP kinase (Chen et al., 1992; Traverse et al., 1992) and MAPKAP kinase-1 (Chen et al., 1992), it is translocated from the cytoplasm to the nucleus after growth-factor stimulation.

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