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Comparison of the specificities of p70 S6 kinase and MAPKAP kinase-1 identifies a relatively specific substrate for p70 S6 kinase: the N-terminal kinase domain of MAPKAP kinase-1 is essential for peptide phosphorylation

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Abstract **xxR/KxRxxSxx sequences were phosphorylated with high efficiency by both p70 S6 kinase ($p70^{S6K}$) and MAPKAP kinase-1. The best substrate for MAPKAP kinase-1 (KKKNRTLSVA) was phosphorylated with a K_m of 0.17 μM, and the best substrate for $p70^{S6K}$ (KKRNRTLSVA) with a K_m of 1.5 μM. The requirement of both enzymes for Arg/Lys at position *n*-5 could be partially replaced by inserting basic residues at other positions, especially by an Arg at *n* − 2 or *n* − 4. MAPKAP kinase-1 (but not $p70^{S6K}$) tolerated lack of any residue at *n* − 5 if Arg was present at *n* − 2 and *n* − 3. $p70^{S6K}$ (but not $p90^{S6K}$) tolerated Thr at position *n* and absence of any residue at *n* + 2. The peptide KKRNRTLTV, which combined these features, was relatively selective for $p70^{S6K}$ having a 50-fold higher V_{max}/K_m than MAPKAP kinase-1. Inactivation of the N-terminal kinase domain of MAPKAP kinase-1, which is 60% identical to $p70^{S6K}$, abolished activity towards all peptides tested, but the enzyme retained 30–40% of its activity if the C-terminal kinase domain was inactivated.**

Key words: S6 kinase; MAP kinase; Protein kinase; Ribosomal protein S6; Protein phosphorylation; Site-directed mutagenesis

1. Introduction

p70 ribosomal S6 kinase ($p70^{S6K}$) [1] and p90 ribosomal protein S6 kinase ($p90^{rsk}$) [2] were originally identified as enzymes which phosphorylate ribosomal protein S6 in vitro. These kinases both become activated within minutes after cell stimulation by insulin and growth factors, but via distinct signalling pathways. $p90^{rsk}$ is activated by the p42 and p44 mitogen-activated protein (MAP) kinases [3,4], but $p70^{S6K}$ is activated via a pathway that is blocked by rapamycin, a drug which does not inhibit the activation of MAP kinases or $p90^{rsk}$ [5,6]. Rapamycin prevents the growth factor-induced phosphorylation of S6 in several mammalian cells [7], indicating that $p70^{S6K}$ is responsible for S6 phosphorylation in vivo. For this reason, and to avoid confusion with $p70^{S6K}$ and the nuclear form $p85^{S6K}$ which is produced using an alternative initiation site [8,9], we have suggested that $p90^{rsk}$ be called **MAP** kinase-activated protein (MAPKAP) kinase-1 [10].

Rapamycin induces arrest of the eukaryotic cell division cycle in G1 [11] and prevents the interleukin-2 induced proliferation of T-cells [12], suggesting that S6 may not be the only in vivo substrate for $p70^{S6K}$. Similarly, MAPKAP kinase-1 phosphorylates many proteins in vitro, including tyrosine hydroxylase [10], the glycogen-binding (G_M) subunit of protein phosphatase-1 (PP1) in striated muscle [13] and glycogen synthase kinase-3 (GSK3) [14]. GSK3 may be a physiological substrate, because it is phosphorylated at the serine residue targetted by MAPKAP kinase-1 in vitro when A431 cells are stimulated by EGF [15] and in NIH 3T3 cells the phosphorylation of GSK3 is suppressed by transfection with dominant negative forms of MAP kinase kinase [16]. Coexpression with MAPKAP kinase-1 also enhances the phosphorylation (inactivation) of GSK3 in HeLa cells stimulated with phorbol esters [17]. $p70^{S6K}$ phosphorylates GSK3 at the same serine residue as MAPKAP kinase-1 in vitro, but rapamycin fails to prevent the inhibition of GSK3 by EGF in A431 cells [15] or by insulin in L6 or CHO cells [18,19]. Thus $p70^{S6K}$ is not essential for the inhibition of GSK3 in vivo.

Several studies originally suggested that the substrate specificities of $p70^{S6K}$ and MAPKAP kinase-1 were distinct. The motif **RxRxxS** was found to be critical for the specificity of $p70^{S6K}$ [20], whereas MAPKAP kinase-1 was found to phosphorylate tyrosine hydroxylase and the G_M subunit of PP1 at **RR**xS motifs [10,13,21]. Moreover, small peptides of the type **RR**xSxx were phosphorylated with quite low (50 μM) K_M values by MAPKAP kinase-1 [22]. In contrast, several substrates containing these motifs were not phosphorylated by $p70^{S6K}$ [23]. However, despite these differences MAPKAP kinase-1 and $p70^{S6K}$ both phosphorylate the same serine residue in GSK3 [16] and target the same serine in S6 preferentially in vitro [20,22], both of which lie in **R**x**R**xx**S** motifs. These observations, and further experiments with a different panel of synthetic peptides [24], indicated that although an Arg located three residues N-terminal to the phosphorylated serine (position *n* − 3) is essential for phosphorylation by MAPKAP kinase-1, an Arg at *n* − 2 is not. These findings have led us to compare the specificities of $p70^{S6K}$ and MAPKAP kinase-1 in greater detail. We now show that, although phosphorylation can be enhanced if Arg is present at *n* − 2, MAPKAP kinase-1 greatly prefers Arg at *n* − 5 and the requirements of $p70^{S6K}$ and MAPKAP kinase-1 for basic residues are very similar. Nevertheless, we have been able to exploit other differences in the specificity requirements of these enzymes to generate the first peptide substrate which shows a high degree of selectivity for $p70^{S6K}$ over MAPKAP kinase-1. We also demonstrate that it is the N-terminal kinase

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**The first two authors have made similar contributions to this study.

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domain of MAPKAP kinase-1 which is essential for phosphorylating peptides with either **RxRxxS** or **RRxS** motifs.

2. Material and methods

2.1. Materials

The peptide LRRASLG (Kemptide) was purchased from Sigma Chemical Co. (Poole, UK), and the 19-residue peptide inhibitor of cyclic AMP-dependent protein kinase (termed PKI) [25] and other peptides synthesised at Dundee on an Applied Biosystems 431A peptide synthesiser. Their purity was established by high performance liquid chromatography and fast-atom-bombardment mass spectrometry and their concentrations were determined by quantitative amino acid analysis. MAPKAP kinase-1β ($p90^{rsk-2}$) from rabbit skeletal muscle [4] was provided by Dr. C. Sutherland at Dundee. Reagents for cell culture were purchased from GIBCO (Paisley, UK), antibody 12CA5 from Boehringer (Lewes, UK), Protein G-Sepharose from Sigma (Poole, UK) and microcystin from Calbiochem. PD 98059 was a generous gift from Dr. Alan Saltiel (Parke-Davis, MI, USA).

2.2. Partial purification of p70 S6 kinase

Ten rats were injected with cycloheximide (5 mg/100 g of body weight) to activate $p70^{S6K}$. One hour later, the rats were killed by cervical dislocation, the livers removed and homogenised in 2.5 vols. of 15 mM sodium pyrophosphate, 5 mM EDTA, 25 mM NaF, 1 mM benzamidine, 1 mM sodium orthovanadate, 0.1 mM phenylmethylsulphonyl fluoride (PMSF) and 0.1% (by vol.) 2-mercaptoethanol, pH 6.8, and centrifuged for 30 min at $38,000 \times g$. The supernatant was passed through a buchner funnel containing 10 g (dry weight) QAE-Sephadex equilibrated in 25 mM Tris-HCl, 1 mM EDTA, 25 mM NaF, 1 mM sodium pyrophosphate, 1 mM benzamidine, 1 mM sodium orthovanadate, 0.1 mM PMSF and 0.1% (by vol.) 2-mercaptoethanol, pH 7.0 (Buffer A). After washing with Buffer A containing 0.1 M NaCl until the absorbance at 280 nm was < 0.1, $p70^{S6K}$ was eluted with Buffer A containing 0.5 M NaCl. The eluate was dialysed against 20 mM triethanolamine-HCl, 1 mM EDTA, 1 mM benzamidine, 5% (by vol.) glycerol, 0.03% (by mass) Brij-35 and 0.1% (by vol.) 2-mercaptoethanol, pH 7.4 (Buffer B), and chromatographed on a column of Q-Sepharose (10×1.6 cm) equilibrated in Buffer B. The column was developed with a 400 ml linear gradient to 0.7 M NaCl and the most active fractions, eluting between 300 and 350 mM NaCl, were pooled, dialysed against 20 mM Tris-HCl, 1 mM EDTA, 0.03% (by mass) Brij-35, 5% (by vol.) glycerol and 0.1% (by vol.) 2-mercaptoethanol, pH 7.0 (Buffer C) and chromatographed on a 5 ml HiTrap column of heparin-Sepharose (Pharmacia) equilibrated in Buffer C. The column was developed with a 200 ml linear NaCl gradient to 1 M NaCl and the most active fractions, eluting between 300 and 360 mM NaCl were pooled, dialysed against Buffer C containing 50% (by vol.) glycerol and stored at −20°C. The preparation was free of MAPKAP kinase-1 and other known kinases, because it was devoid of activity towards the peptide LRRASLG (Kemptide) [23], inactivated by incubation with PP2A, and all the activity towards the substrates $(G^{245}/G^{246})S6^{218-249}$ [26] and KRRRLASLAA [20] was immunoprecipitated by the anti-$p70^{S6K}$ antibodies described in [27].

2.3. Assay of $p70^{S6K}$ and MAPKAP kinase-1

$p70^{S6K}$ and MAPKAP kinase-1 were diluted to 2.0 U/ml in 50 mM MOPS, pH 7.0, 0.1 mM EGTA, 0.1% (by vol.) 2-mercaptoethanol, 0.03% (by mass) Brij 35. The standard assays (50 μl) contained 50 mM MOPS, pH 7.0, 0.1 mM EGTA, 0.1% (by vol.) 2-mercaptoethanol, 2.5 μM PKI, 0.2 U/ml $p70^{S6K}$ or MAPKAP kinase-1, the peptide $(G^{245}/G^{246})S6^{218-249}$ (30 μM), 10 mM $MgCl_2$ and 0.1 mM [γ-^{32}P]ATP (200–1000 cpm/pmol). After incubation for 10–15 min at 30°C, reactions were terminated and analysed as in [27]. When assaying immunoprecipitated MAPKAP kinase-1, the tubes were agitated continuously throughout the assay to keep the enzyme in suspension. One unit of activity was that amount which catalysed the phosphorylation of 1 nmol of S6 peptide in 1 min. The phosphorylation of other peptides was carried out in an identical manner. Michaelis constants (K_m) and V_{max} values were determined from double reciprocal plots of $1/V$ against $1/S$, where S was the substrate concentration. The standard errors for all reported kinetic constants was < ± 20%, and the data reported are given as mean values for at least three independent determinations.

2.4. Construction of site-specific mutant plasmids

pBluescript phagemid containing DNA encoding a nonapeptide that corresponds to an epitope in the influenza haemagglutinin (HA 1) immediately followed by cDNA encoding the rat MAPKAP kinase-1α ($p90^{rsk-1}$) [28] was modified by PCR mutagenesis to mutate Asp^{205} or Asp^{557} to Ala. The Ala^{205} mutant was produced by digestion of pBluescript HA-MAPKAP kinase-1α with *Bgl*II and *Eco*NI and ligation of a mutated *Bgl*II-*Eco*NI digested PCR fragment. The PCR fragment was produced using pBluescript HA- MAPKAP kinase-1α as template and two primers, one primer that contained a unique *Bgl*II restriction site (underlined) 5′-CAA GGA GAT CTC TAT CAC ACA CCA CGT CAA GG-3′, and the other that contained both a unique *Eco*NI restriction site (underlined) and the mutation (bold type), 5′-AAT GGC CTC CTT GCT CAG GCC AAA **GGC** AGT GAG-3′). The reaction conditions and subsequent purification of DNA were as described previously [29], except that oligonucleotides were annealed for 1 min at 65°C. The Ala^{557} mutant was produced by digestion of pBluescript HA-MAPKAP kinase-1 with *Sna*I and *Nde*I and ligation of a mutated *Sna*I-*Nde*I digested PCR fragment that was created by a two-step PCR. In the first PCR, two overlapping fragments, termed here A and B, were prepared using pBluescript HA-MAPKAP kinase-1 as template. Fragment A was amplified with an outer forward primer (5′-CGA CAG TAA GCA CGT ATA CCT GGT G-3′) covering a unique *Sna*I site and an inner reverse primer (5′-GCA AAG CCA AAG **GCG** CAT ATT CGT AGG-3′). Fragment B was amplified with an inner forward primer (5′-CCT ACG AAT ATG **CGC** CTT TGG CTT TGC-3′) and an outer reverse primer (5′-CCC AGG CTC CAT ATG TCA CAG CC-3′) that covers a unique *Nde*I site. The reaction conditions and isolation of Fragments A and B were as described above. The two fragments were then annealed and used as a template for second-round PCR amplification as described [29]. The DNA was digested with *Sna*I and *Nde*I, purified on a 1% agarose gel using Geneclean (Stratech Ltd., Luton, UK) and subcloned into the *Sna*I-*Nde*I digested pBluescript phagemid containing wild type DNA.

The sequences of the MAPKAP kinase-1α mutants were checked using an Applied Biosystems automatic DNA sequencer using specific oligonucleotide primers. The mutant and wild type MAPKAP kinase-1α DNA constructs were expressed by subcloning from the pBluescript phagemid vector into the pMT2 vector using the unique *Eco*RI site.

3. Results

3.1. Comparison of the substrate specificity of MAPKAP kinase-1β and $p70^{S6K}$

We have previously analysed the substrate specificity of the stress-activated enzyme MAPKAP kinase-2 using a set of synthetic peptides related to the N-terminal sequence of glycogen synthase, and compared its specificity to that of MAPKAP kinase-1 [24]. Like MAPKAP kinase-1, MAPKAP kinase-2 is activated by the p42 and p44 MAP kinases in vitro, but in vivo it is activated by a distinct MAP kinase homologue which responds to cellular stresses and the cytokine interleukin-1 [30]. The minimum sequence required for phosphorylation by MAPKAP kinase-2 is **L/FxRxxSxx** [24]. The 'parent' peptide of this series, KKLNRTLSVA, was phosphorylated by MAPKAP kinase-2 with a V_{max} of 13 μmol $min^{-1} \cdot mg^{-1}$ and a K_m of 18 μM, but phosphorylation was abolished if the Leu at position $n-5$ was changed to Lys or Glu. In contrast, substitution of the Leu by Lys was not deleterious to MAPKAP kinase-1, and actually doubled the rate of phosphorylation at a peptide concentration of 30 μM [24]. More detailed kinetic analysis has now revealed that replacing the Leu at $n-5$ with Lys, not only abolishes phosphorylation by MAPKAP kinase-2, but drastically decreases the K_m for phosphorylation by MAPKAP kinase-1 from 30 μM to < 0.2 μM (Table 1). The resulting peptide, KKKNRTLSVA, is therefore the best substrate for MAPKAP kinase-1 identified so far, with a similar V_{max}, but a six-fold lower K_m, than $(G^{245},G^{246})S6^{218-249}$ (Table 1), a 32 residue pep-

Table 1
Comparison of the substrate specificities of MAPKAP kinase-1 and p70 S6 kinase

Number	Peptide	MAPKAP kinase-1			p70 S6 kinase		
		V_{max} (μmol·min^{-1}·mg^{-1})	K_m (μM)	V_{max}/K_m	V_{max} (μmol·min^{-1}·mg^{-1})	K_m (μM)	V_{max}/K_m
	KKKNRTLSVA	1.55	0.17	9.3	0.89	3.3	0.27
2	KKRNRTLSVA	1.80	0.71	2.5	1.52	1.5	1.01
3	KKLNRRLSVA	1.17	1.5	0.78	0.21	9.7	0.02
4	KKRNRRLSVA	1.73	2.8	0.62	1.29	1.6	0.80
5	KKLRRTLSVA	1.35	2.3	0.59	0.38	2.3	0.16
6	AARNRTLSVA	0.88	1.9	0.46	1.01	7.3	0.14
7	KKRNKTLSVA	0.84	17	0.05	0.76	34	0.02
8	KKLNRTLSVA	0.86	30	0.03	0.27	47	0.006
9	KKPNRTLSVA	1.85	59	0.03	0.41	>500	<0.001
10	AALNRRLSVA	0.29	9.2	0.03	0.12	140	<0.001
11	AAKNRTLSVA	0.52	28	0.02	0.58	350	0.002
12	KKENRTLSVA	1.08	96	0.01	0.05	130	<0.001
13	KKLNKTLSVA	0.16	>500	<0.001	0.03	49	<0.001
14	GRPRTSSFAEG	0.79	0.76	1.0	1.27	0.9	1.41
15	KRRRLASLAA	1.30	0.27	4.8	1.53	1.9	0.81
16	$(G^{245}/G^{246})S6^{218-249}$	1.76	0.93	1.9	1.24	1.9	0.65
17	LRRASLG	1.94	23	0.09	0	–	0
18	SPQPS**RR**GSESSEEVYV	1.07	25	0.04	0	–	0

Peptides 1–13 are variants of the peptide KKKNRTLSVA, peptide 14 corresponds to the sequence in GSK3 phosphorylated by both MAPKAP kinase-1 and p70 S6 kinase, while peptide 15 is related to the sequence surrounding the serine in ribosomal protein S6 phosphorylated by both enzymes. Peptide 16 is the standard peptide substrate which has been used to assay MAPKAP kinase-1 and p70 S6 kinase. Peptide 17 (Kemptide) is the standard substrate for cyclic AMP-dependent protein kinase and peptide 18 corresponds the sequence surrounding the serine residue (bold type) in the G-subunit of PP1 which is phosphorylated by MAPKAP kinase-1 in vitro. K_m values were determined from Lineweaver-Burke plots and V_{max} values were compared to that reported for peptide 16 in [23]. Further experimental details are given in section 2.

ide related to the C-terminus of ribosomal protein S6 which has been used to assay MAPKAP kinase-1 previously [23]. The K_m for KKKNRTLSVA was at least two orders of magnitude lower than the synthetic peptide substrates for MAPKAP kinase-1 studied previously which contain **RRxS** motifs [22].

The Leu-to-Lys substitution creates a **KxRxxS** sequence, similar to the **RxRxxS** motif which is critical for substrate recognition by $p70^{S6K}$ [20]. We therefore decided to compare the specificities of MAPKAP kinase-1 and $p70^{S6K}$ using variants of the peptide **KKKNRTLSVA**. These experiments (Table 1) confirmed the importance of Arg at position $n-3$ and Arg or Lys at position $n-5$ for both protein kinases, peptides containing a **K/RxRxxS** motif being phosphorylated with very low K_m values (see peptides 1, 2, 4, 6, 14 and 15). Changing the Arg at position $n-3$ to Lys increased the K_m for either kinase over 20-fold (compare peptides 2 and 7 in Table), while substitution of a basic residue at $n-5$ by Leu, Pro or Glu increased the K_m for peptide phosphorylation even more and also reduced the V_{max}, particularly with $p70^{S6K}$ (compare peptides 1 and 2 with peptides 8, 9 and 12). The presence of Lys at positions $n-6$ and $n-7$ also improved phosphorylation by both MAPKAP kinase-1 and $p70^{S6K}$, especially when the residue at position $n-5$ was Lys rather than Arg (compare peptide 1 with 11, 2 with 6, and 3 with 10). Another similarity between the two protein kinases was that the large rise in K_m resulting from lack of an Arg at $n-5$ could be avoided by inserting Arg at positions $n-2$ (compare peptides 2, 3 and 8) or $n-4$ (compare peptides 2, 5 and 8). However, the presence of Arg at positions $n-2$ or $n-4$ could not alleviate the large decrease in the V_{max} of $p70^{S6K}$ associated with lack of a basic residue at position $n-5$.

3.2. A peptide substrate with a high degree of selectivity for $p70^{S6K}$ over MAPKAP kinase-1

The similar requirement of MAPKAP kinase-1 and $p70^{S6K}$ for basic residues suggested that it might be difficult to develop

Table 2
Development of a peptide substrate with a high degree of selectivity for p70 S6 kinase over MAPKAP kinase-1

	Peptide	MAPKAP kinase-1			p70 S6 kinase		
		V_{max} (μmol·min^{-1}·mg^{-1})	K_m (μM)	V_{max}/K_m	V_{max} (μmol·min^{-1}·mg^{-1})	K_m (μM)	V_{max}/K_m
	KKRNRTLSVA	1.80	0.73	2.5	1.52	1.5	1.0
	KKRNRTLSV	2.03	16	0.13	1.43	1.4	1.0
	KKRNRTLTV	0.27	40	0.007	1.47	4.8	0.31
	KKRNRTLTK	0.07	180	<0.001	1.53	31	0.05
	RNRTLTV	0.20	53	0.004	1.10	11	0.10
	KKRNRALTV	0.27	82	0.003	1.22	38	0.03
	KKRNRGLTV	0.13	96	0.001	0.75	119	0.006
	KKRNRNLTV	0.04	100	<0.001	0.98	62	0.02
	KKRNRTLSPA	0.02	>500	<0.001	0.10	>500	<0.001

Changes from the parent peptide KKRNRTLSVA are underlined. K_m values were determined from Lineweaver-Burke plots and V_{max} values were determined relative to peptide 16 in Table 1, whose V_{max} was reported in [20]. Further experimental details are given in section 2.

a specific peptide substrate for $p70^{S6K}$ by making amino acid substitutions N-terminal to the site of phosphorylation, and prompted a comparison of the C-terminal requirements of each enzyme. With the peptide series used previously, we found that for efficient phosphorylation by MAPKAP kinase-1, the residue at position $n + 1$ must not be Pro or Lys, a residue must be present at position $n + 2$, and the residue at position n must be Ser and not Thr [24]. The effect of making these replacements was therefore examined using KKRNRTLSVA as the parent peptide, since this was the best substrate for $p70^{S6K}$ identified in Table 1 (K_m = 1.5 μM). Like MAPKAP kinase-1, $p70^{S6K}$ greatly disliked Pro at position $n + 1$ (compare peptide 1 with peptide 9 in Table 2). However, elimination of the Ala at position $n + 2$ had no effect on phosphorylation by $p70^{S6K}$, whereas it increased the K_m for MAPKAP kinase-1 over 20-fold (compare peptides 1 and 2 in Table 2). Moreover, substituting the Ser in peptide 2 by Thr decreased the V_{max} of MAPKAP kinase-1 by 7–8-fold, but did not affect the V_{max} of $p70^{S6K}$ and only increased the K_m to 4.8 μM (compare peptides 2 and 3 in Table 2). Thus two changes converted KKRNRTLSVA from a peptide with a 2.5-fold higher V_{max}/K_m for MAPKAP kinase-1, to one (KKRNRTLTV) which had a 40–50-fold higher V_{max}/K_m for $p70^{S6K}$. Changing the valine residue in KKRNRTLTV to Lys produced a peptide with a similar selectivity for $p70^{S6K}$ over MAPKAP kinase-1 in terms of V_{max}/K_m, but the K_m for $p70^{S6K}$ was much higher (compare peptides 1 and 4 in Table 2). MAPKAP kinase-1 phosphorylated both Thr residues in the peptide KKRNRTLTV (data not shown), but substitution of the first Thr by Ala, Gly or Asn did not improve selectivity, because these changes were deleterious to $p70^{S6K}$ as well as to MAPKAP kinase-1 (peptides 6–8 in Table 2). Changing the first threonine in KKRNRTLTV to Arg or Lys made this peptide a slightly worse substrate for $p70^{S6K}$ and a better substrate for MAPKAP kinase-1 and therefore decreased selectivity for $p70^{S6K}$. Removing both Lys residues at the N-terminus of KKRNRTLTV also failed to improve selectivity for $p70^{S6K}$ (peptide 5 in Table 2).

3.3. *The N-terminal kinase domain of MAPKAP kinase-1α is essential for peptide phosphorylation*

MAPKAP kinase-1 possesses two protein kinase domains in the same polypeptide [31]. The N-terminal or C-terminal kinase domains of MAPKAP kinase-1α ($p90^{rsk\text{-}1}$) were inactivated by mutating the Asp residue of the conserved DFG motif in subdomain 7 to Ala. Wild-type and mutant MAPKAP kinase-1 tagged at the N-terminus with a peptide from haemagglutinin were transiently transfected into COS-1 cells. The cells were either incubated for 90 min with PD 98059 (an inhibitor of the activation of MAP kinase kinase [32]) to suppress the basal level of MAPKAP kinase-1 activity, or stimulated with phorbol myristate acetate to activate MAPKAP kinase-1. The enzymes were then immunoprecipitated from the lysates with antibody 12CA5 that recognises the peptide tag, and assayed. As shown in Fig. 1A, the mutant in which the C-terminal kinase domain had been inactivated retained about 30% of the activity of the wild-type enzyme towards $(G^{245}/G^{246})S^{218-249}$ and about 40% of the activity towards KKKNRTLSVA and two peptide substrates with **RR**x**S** motifs, LRRASLG and **RR**LSSLRA (Fig. 1B). In contrast, the mutant in which the N-terminal domain of MAPKAP kinase-1 was inactivated failed to phosphorylate any peptide tested (Fig. 1).

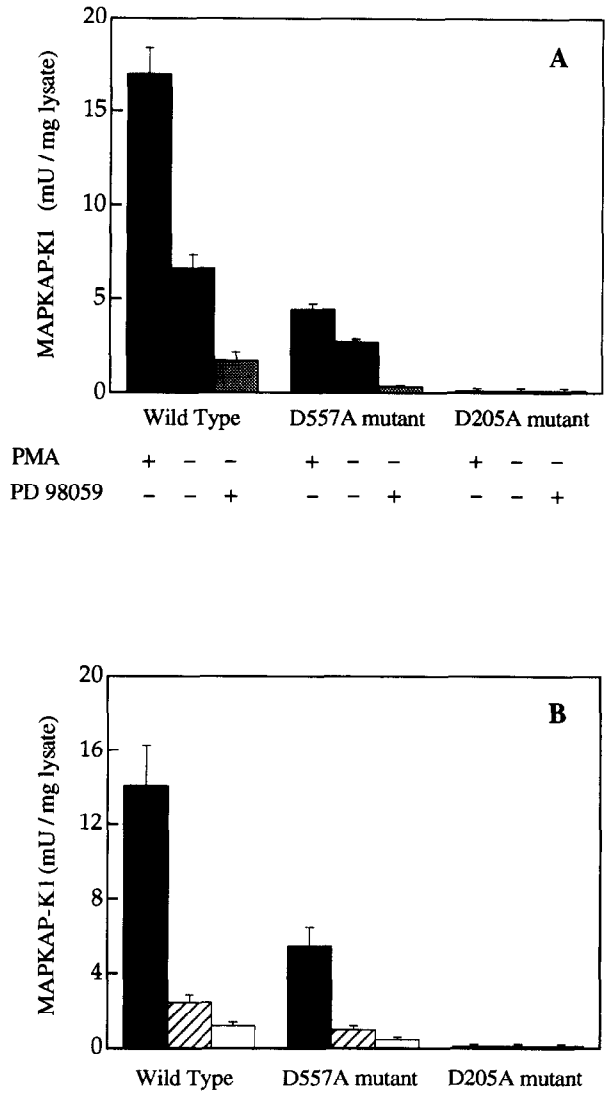

Fig. 1. Effect of inactivating the N-terminal or C-terminal kinase domains of MAPKAP kinase-1α on its activity towards different peptide substrates. (A) COS-1 cells were grown on 10 cm plates, and transiently transfected with wild type (WT) MAPKAP kinase-1α or the D205A or D557A mutants as in [36]. Cells were then grown in DMEM, 10% FCS for 48 h, serum starved in DMEM, 0.5% FCS for 3 h, the drug PD 98059 being added after 1.5 h where indicated. Cells incubated in the absence of PD 98059, were stimulated for 15 min with 0.1 μM phorbol myristate acetate (PMA) and lysed in buffer [37] supplemented with 2 μM microcystin. After centrifugation for 5 min at 13,000 × *g*, aliquots of the supernatants were immunoprecipitated with antibody 12CA5 prebound to Protein G-Sepharose beads.The immunoprecipitates were washed twice with lysis buffer containing 0.5 M NaCl, twice with 50 mM MOPS, pH 7.0, 0.1 mM EGTA, 0.1% (by vol.) 2-mercaptoethanol, 0.03% (by mass) Brij 35, 2 μM microcystin, and assayed for MAPKAP kinase-1 activity with the peptide $(G^{245}/G^{246})S6^{218-249}$. Control experiments were carried out in parallel in which the cells were transfected with expression vector alone. These values were typically 5% of the PMA-stimulated activity. Immunoblotting experiments showed that the levels of expression of MAPKAP kinase-1 were similar in all transfections. (B) The experiment was carried out as in (A), and the figure shows the activity of immunoprecipitates from PD 98059-treated cells subtracted from the activity in immunoprecipitates from PMA stimulated cells towards the peptides KKKNRTLSVA (filled bars), LRRASLG (hatched bars) or RRLSSLRA (open bars).

4. Discussion

The present results establish that the specificities of MAPKAP kinase-1 and p70^{S6K} are far more similar than realised previously. Both enzymes require Arg at position $n-3$ and Arg (or Lys) at position $n-5$, explaining why both enzymes phosphorylate the same serine residues in GSK3 and S6 (see section 1). However, the requirement for a basic residue at position $n-5$ can be overcome, at least partially, by the presence of Arg or Lys at other positions N-terminal to the site of phosphorylation. This presumably explains why MAPKAP kinase-1 is able to phosphorylate serine residues in the G_M-subunit of PP1 [13], tyrosine hydroxylase [10] and peptides [22] that lie in **RRxS** sequences, while p70^{S6K} was reported to phosphorylate this motif in the transcription factor CREM [33].

The structure of MAPKAP kinase-1 is unusual in that two protein kinase domains are present in a single polypeptide [31]. The N-terminal domain is most similar to p70^{S6} kinase (nearly 60% sequence identity) [34], and the present results suggest that this is the domain which phosphorylates both **RxRxxS** and **RRxS** motifs, since mutation of the Asp in the conserved N-terminal DFG motif in subdomain VII abolished activity towards all peptides examined. In contrast, mutation of the corresponding Asp in the C-terminal DFG motif decreased activity by 60–70%. As this paper was about to be submitted, Bjørbaek et al. [35] also reported that inactivation of the N-terminal domain of the isoform MAPKAP kinase-1γ (p90^{rsk-3}) by mutation of Lys^{91} abolished activity, and that inactivation of the C-terminal domain by mutation of Lys^{444} reduced activity by a similar amount to that reported here. The partial loss of MAPKAP kinase-1 activity towards peptide substrates resulting from inactivation of the C-terminal domain may be explained if the C-terminal kinase domain is required to enhance the activity of the N-terminal kinase domain by autophosphorylating one or more residues.

p70^{S6K} cannot phosphorylate peptides which have less than five residues N-terminal to the phosphorylated serine, explaining its failure to phosphorylate several substrates for MAPKAP kinase-1, such as LRRASLG ([20] and see Table 1). However, p70^{S6K} is much more tolerant of Thr at the site of phosphorylation than MAPKAP kinase-1 and, in contrast to MAPKAP kinase-1, no residue is required at position $n+2$ (Table 2). These differences have allowed us to develop the first peptide substrate which shows a high degree of selectivity for p70^{S6K} over MAPKAP kinase-1, and which should prove useful for future studies of this enzyme.

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