Specificity determinants for the AMP-activated protein kinase and its plant homologue analysed using synthetic peptides

Volume 334, number 3, 335–339 FEBS 13266 November 1993

Specificity determinants for the AMP-activated protein kinase and its plant homologue analysed using synthetic peptides

John Weekes[a], Kathryn L. Ball[a], F. Barry Caudwell[b], D. Grahame Hardie[a,*]

[a]*Department of Biochemistry, The University, Dundee, DD1 4HN, Scotland, UK*
[b]*MRC Protein Phosphorylation Unit, The University, Dundee, DD1 4HN, Scotland, UK*

Received 4 October 1993

Inspection of sequences around sites phosphorylated by the AMP-activated protein kinase (AMP-PK), and homologous sequences from other species, indicates conserved features. There are hydrophobic residues (M, V, L, I) at P-5 and P+4, and at least one basic residue (R, K, H) at P-2, P-3 or P-4. The importance of these residues has been established for AMP-PK and its putative plant homologue using a series of synthetic peptides. These results confirm the functional similarity of the animal and plant kinases, and suggest that the required motif for recognition of substrate by either kinase is M/V/L/I-(R/K/H,X,X)-X-**S/T**-X-X-X-M/V/L/I.

AMP-activated protein kinase; HMG-CoA reductase kinase; Synthetic peptide; Specificity determinant; Consensus sequence; Mammals; Higher plants

1. INTRODUCTION

The AMP-activated protein kinase (AMP-PK) is a multisubstrate protein kinase which phosphorylates and inactivates several key regulatory enzymes of lipid and carbohydrate metabolism, including acetyl-CoA carboxylase, HMG-CoA reductase, hormone-sensitive lipase and glycogen synthase [1]. AMP-PK is dramatically activated by elevation of AMP in intact cells, both via direct allosteric activation, and via AMP-promoted phosphorylation by an upstream kinase kinase. AMP is elevated and the kinase is activated in vivo under conditions which cause ATP depletion, such as incubation with fructose in isolated hepatocytes [2,3]. The protein kinase may be an important component of the cellular stress response, switching off ATP-consuming biosynthetic reactions whenever ATP is limiting. Recently an activity has been found in higher plants which, by functional criteria, appears to be a homologue of mammalian AMP-PK [4].

Identification of additional targets for AMP-PK, and its putative plant homologue, would be facilitated by the determination of the sequence motif for recognition by these kinases. In this paper we compare the sequence around several known sites of phosphorylation for AMP-PK, which allows identification of conserved features. To test whether these features are essential for recognition of a substrate by the kinase, we examined the phosphorylation of a series of synthetic peptides, both by AMP-PK and the plant protein kinase.

*Corresponding author. Fax: (44) (382) 201 063.

Abbreviations: AMP-PK, AMP-activated protein kinase.

2. MATERIALS AND METHODS

2.1. *Materials*

AMP-PK was purified from rat liver as far as the gel filtration step [5]. HMG-CoA reductase kinase was purified from cauliflower inflorescences [4]. Variants of the 'SAMS' peptide were synthesized by Cambridge Research Biochemicals (Warrington, UK) using their Multiple Peptide Synthesis service. This utilizes Fmoc polyamide chemistry on a solid phase Pepsyn KB resin. Variants of the glycogen synthase peptides were synthesized in Dundee using an Applied Biosystems 431 synthesizer. All peptides were purified and analysed in Dundee. They were purified by reversed phase HPLC on a Vydac 'Protein and Peptide' C18 column in 0.1% (v/v) trifluoroacetic acid using a gradient from water to acetonitrile. Peptides were analysed by positive mode FAB-mass spectrometry on a VG 70–250SE instrument using an 8 kV Xe gun and a matrix of dithiothreitol/dithioerythritol (3:1, w/w). This confirmed their purity and that they had the correct mass. Their concentrations were determined by amino acid analysis of phenylthiocarbamyl derivatives using a Millipore-Waters PICO-TAG system.

2.2. *Enzyme assays*

Peptide phosphorylation was measured using a P81 paper method as described previously [6]. Data were fitted to the Michaelis–Menten equation using a statistical method [7] implemented in Microsoft Excel on a Macintosh computer.

3. RESULTS

3.1. *Analysis of known and probable sites for the AMP-activated protein kinase*

Fig. 1 shows the sequences around serine residues (bold, underlined) known to be phosphorylated in cell-free assays by AMP-PK, and equivalent sequences from

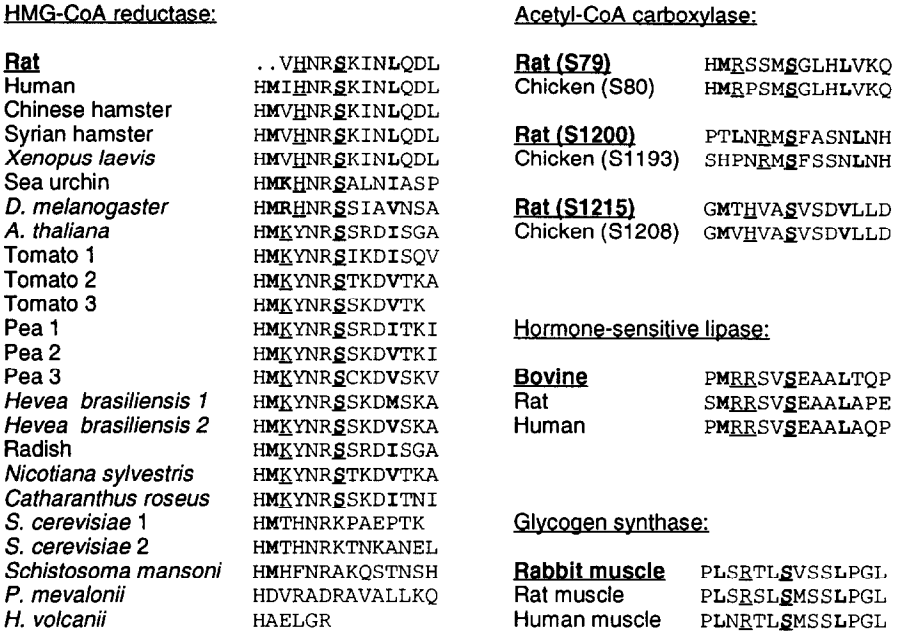

Fig. 1. Alignment of sequences known to be phosphorylated by AMP-PK (top sequence of each group) and homologous sequences from other species. The actual or putative phosphorylation sites are underlined and in bold type. Conserved hydrophobic residues mentioned in the text are in bold, and basic residues underlined. Apart from the peptide sequences of rat HMG-CoA reductase [11] and bovine hormone-sensitive lipase [15], all of the sequences can be obtained from the sequence databases.

other species. For each of the six groups, only the sequence at the top (species name underlined and in bold type) has been directly shown to be a substrate for the kinase. All of these six sites have also been shown to be phosphorylated in intact cells [1], and in the case of HMG-CoA reductase strong evidence has been obtained that this is catalysed by AMP-PK [3]. Although phosphorylation of the other aligned sequences has not yet been demonstrated directly, it is noticeable that the phosphorylated serine residue (bold, underlined) is generally conserved, as are several neighbouring residues. Sequences from a wide variety of species are particularly available for HMG-CoA reductase, where the phosphorylation site is at the extreme C-terminal end of the catalytic domain [8]. The phosphorylation site sequence on HMG-CoA reductase is well conserved in all mammals and higher plants, consistent with evidence that HMG-CoA reductases from animal phyla ranging from mammals to insects are regulated by phosphorylation [9,10], and our findings that HMG-CoA reductase

Table I

Kinetic parameters of rat AMP-activated protein kinase and cauliflower HMG-CoA reductase kinase using a series of variants of the 'SAMS' peptide

Peptide	Animal kinase		Plant kinase	
	V (U/ml)	K_m (μM)	V (U/ml)	K_m (μM)
(1) HMRSAMSGLHLVKRR	10026 ± 1085	59 ± 13	1467 ± 32	95 ± 4
(2) HMKSAMSGLHLVKRR	14564 ± 1289	112 ± 16	1570 ± 67	133 ± 9
(3) HMHSAMSGLHLVKRR	8847 ± 498	114 ± 11	1165 ± 95	117 ± 15
(4) HMGSAMSGLHLVKRR	5626 ± 1313	429 ± 111	590 ± 116	573 ± 126
(5) HGRSAMSGLHLVKRR	6044 ± 1200	999 ± 208	38 ± 2	40 ± 4
(6) HMRSAGSGLHLVKRR	7947 ± 590	42 ± 6	1004 ± 37	70 ± 5
(7) HMRSAMSGLHGVKRR	2643 ± 707	165 ± 49	45 ± 3	19 ± 3
(8) HMRSAMSGLHLGKRR	6728 ± 292	38 ± 4	735 ± 81	38 ± 9
(9) HMRSAMSGLHGGKRR	1240 ± 286	174 ± 60	49 ± 4	48 ± 10
(10) HGRSAMSGLHGGKRR	0	–	0	–
(11) HMRSAMTGLHLVKRR	7069 ± 232	56 ± 4	114 ± 6	34 ± 5
(12) HMRSAMYGLHLVKRR	24 ± 5	57 ± 15	0	–

Changes from the SAMS peptide (no. 1) are indicated by underlining, while the phosphorylated residue is in bold type. Values are presented ± standard errors [7].

in potato microsomes is inactivated by both mammalian AMP-PK and the putative higher plant homologue [4]. Significantly, the phosphorylation site sequence is *not* conserved in HMG-CoA reductases from the yeast *S. cerevisiae* (two genes: *HMG1/HMG2*), the parasite *S. mansoni*, the eubacterium *P. mevalonii*, or the archaebacterium *H. volcanii*. However in all of these cases the catalytic domains themselves are conserved, indicating that the phosphorylation site sequences conserved in higher animals and plants are not required for HMG-CoA reductase activity. Yeast HMG-CoA reductase was reported not to be inactivated by incubation of a crude extract with MgATP [9], while we have shown that HMG-CoA reductase in homogenates of yeast strains which overexpress the C-terminal catalytic fragments of Hmg1p or Hmg2p was not inactivated by purified AMP-PK (W. Wilson, R. Hampton, D.G.H., unpublished).

Assuming that all of the sequences listed in Fig. 1 from vertebrates, insects and plants are substrates for AMP-PK, one can frame several hypotheses regarding the mechanism of recognition by the kinase:

(1) In every case the actual or putative phosphorylation site (bold, underlined) is a serine residue.
(2) With one exception the phosphorylation sites have hydrophobic residues (bold type) at P-5 (M, V, L) and at P+4 (M, V, I or L; numbering with respect to the phosphorylated serine). The exception is S1200/S1193 on acetyl-CoA carboxylase, where in the rat sequence the hydrophobic residues are displaced by one residue and occur at P-4 and P+5, and in the chicken the N-terminal hydrophobic residue appears to be missing. S1200 is however a secondary site on acetyl-CoA carboxylase, the S79 site being phosphorylated much more rapidly [11].
(3) There is a basic residue (underlined; R, H or K) on the N-terminal side of the phosphorylated serine, although the spacing is variable (P-2, P-3 or P-4).

We synthesized two series of synthetic peptides to test the importance in substrate recognition of the conserved residues described above.

3.2. *Peptides based on acetyl-CoA carboxylase*

The first series of peptides were based on the SAMS peptide (HMRSAMSGLHLVKRR) which is derived from the sequence around Ser-79 on rat liver acetyl-CoA carboxylase, and which we have previously shown is a specific substrate for the kinase in rat liver [6]. The primary structures of these peptides and the kinetic parameters obtained using rat liver AMP-activated protein kinase are presented in Table I. In order to confirm that the peptides were being phosphorylated by AMP-PK and not a contaminating protein kinase, we selected 3 of the variants (3, 6 and 11) and showed that their phosphorylation was stimulated by AMP to the same extent (2- to 3-fold) as with the parent 'SAMS' peptide

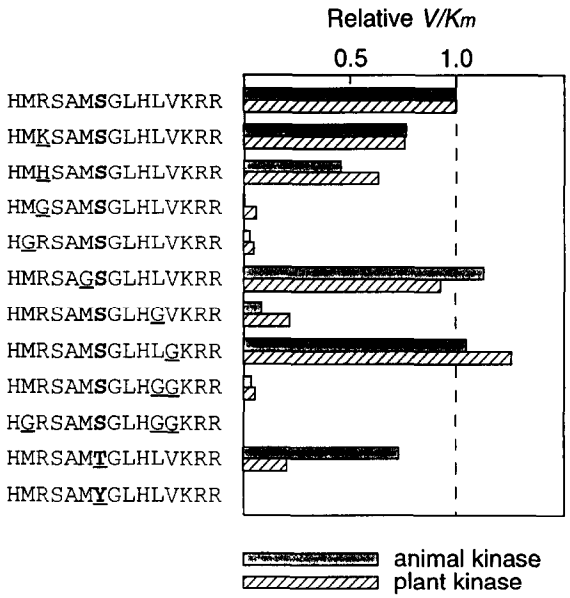

Fig. 2. V/K_m values for the 'SAMS' peptide and variants, obtained using rat AMP-PK or cauliflower HMG-CoA reductase kinase. V/K_m values are expressed relative to that of the parent SAMS peptide, and were calculated from the V and K_m values in Table I. The phosphorylation site is in bold type, and variations from the parent sequence underlined.

(not shown). Table I also shows parameters obtained using the putative cauliflower homologue of AMP-PK. Although both protein kinases preparations were highly purified, neither was homogeneous, so the V values obtained using the plant and animal systems cannot be directly compared. To allow easier comparison, we have also presented the data graphically in Fig. 2 as V/K_m values, relative to those of the parent 'SAMS' peptide.

3.3. *Peptides based on glycogen synthase*

Another series of peptides tested with mammalian AMP-PK were based on the sequence around Ser-7 on rabbit muscle glycogen synthase (GS) (Table II). The peptides all had a KK sequence at the N-terminus to facilitate binding to phosphocellulose paper. Different batches of AMP-PK were used for the experiments in Tables I and II, so the V values cannot be directly compared. However, we analysed the 'SAMS' peptide with both batches, so the relative V/K_m values are comparable. The parent peptide (GS_{1-14}) had a higher V/K_m than the 'SAMS' peptide, and truncating the C-terminus down to the leucine at P+4 actually increased this parameter. Subsequent removal of the proline at P-6 had only a small detrimental effect, but removal of the leucine at P-5 caused a large reduction in V/K_m.

4. DISCUSSION

Fig. 2 graphically illustrates that rat AMP-PK, and the cauliflower HMG-CoA reductase kinase which we

believe to represent its higher plant homologue [4], have a very similar substrate specificity. Table I and Fig. 2 show that replacement of the arginine at P-4 with glycine caused 7- and 6-fold increases in K_m, and 13- and 15-fold reductions in V/K_m, for the mammalian and plant kinases respectively. This indicates that a basic residue on the N-terminal side of the phosphorylated serine is critical in recognition for both kinases. This requirement is shared with other protein kinases such as calmodulin-dependent kinase II and cAMP-dependent protein kinase [12], although in the latter case two adjacent basic residues, believed to form salt bridges with 4 glutamate side chains in the protein kinase [13], are required for efficient phosphorylation. Replacement of the arginine at P-3 in the SAMS peptide with lysine or histidine caused only a small decrease (2-fold or less) in V/K_m for either the animal or plant kinase. Interestingly, all higher plant HMG-CoA reductases have lysine at this position, rather than the arginine found in animal HMG-CoA reductases, while the S1215/S1208 site on acetyl-CoA carboxylase has histidine at P-3 (Fig. 1). AMP-PK appears to be much more tolerant than cAMP-dependent protein kinase with respect to the nature of the N-terminal basic residue(s). In our peptide study we did not address the importance of the spacing of the basic residue, but inspection of the known substrates in Fig. 1 suggests that it is also quite tolerant in this respect, the basic residues being found at P-2, P-3 or P-4.

This study also reveals the key importance of the P+4 and P-5 hydrophobic residues in substrate recognition by both the animal and plant kinases. Replacement of the leucine residue at P+4, or of the methionine at P-5, with glycine caused large (5- to 30-fold) reductions in V/K_m. In contrast, replacement of the methionine at −1 or of the valine at +5 had only marginal effects. Replacement of two or more of these hydrophobic residues was even more deleterious: we could not detect any phosphorylation of a peptide in which the hydrophobic residues at −5, +4 and +5 were all replaced with glycine. The importance of the P-5 hydrophobic residue was also demonstrated by the study using peptides based on glycogen synthase (Table II). Truncation of the C-terminus down to the leucine at P+4 actually increased V/K_m (this peptide was the best substrate of any tested). Truncation of the N-terminus had only a small effect on V/K_m when the P-6 proline was removed, but a large effect when the P-5 leucine was removed. Once again we have not studied the importance of the spacing of these residues, although consideration of the Ser-1200 site on rat acetyl-CoA carboxylase (Fig. 1) suggests that a P-4, P+5 layout, which preserves the 9 residue spacing between hydrophobic residues, is permissible. The peptide HLPPRIHRASDPGLPAEEPK (based on the sequence around Ser-1859 on the multifunctional protein CAD, phosphorylated by cyclic AMP-dependent protein kinase [14]) was not a substrate, indicating that isoleucine at P-4 and leucine at P+4 do not fulfill the requirement. The absolute requirement for hydrophobic residues for recognition is at present unique to AMP-PK and the plant HMG-CoA reductase kinase, although cyclic AMP-dependent protein kinase has a preference for a hydrophobic residue at P+1 [12], and this is thought to bind to a hydrophobic pocket in the kinase active site [13].

Table II

Kinetic parameters of rat AMP-activated protein kinase using a series of variants of the glycogen synthase peptide (based on Ser-7 of rabbit muscle glycogen synthase)

Peptide	V (U/ml)	K_m (μM)	$*V/K_m$
(13) KKPLNRTL**S**VASLPGL	1853 ± 100	32 ± 7	1.66
(14) KKPLNRTL**S**VASL	1809 ± 34	26 ± 2	2.00
(15) KKLNRTL**S**VASL	1322 ± 38	24 ± 3	1.57
(16) KKNRTL**S**VASL	621 ± 69	71 ± 5	0.25

The phosphorylated residue is shown in bold type. $*V/K_m$ ratios are expressed relative to the parent 'SAMS' peptide. Values for V and K_m are presented ± standard errors [7].

We also addressed the nature of the phosphorylated amino acid itself. For rat AMP-PK, replacement of the phosphorylated serine by threonine reduced V without markedly affecting K_m. This was the one peptide where the animal and plant protein kinases gave somewhat different results, as V was more significantly depressed for the plant kinase when serine was replaced by threonine. For both animal and plant kinases, phosphorylation of the equivalent tyrosine containing peptide was barely detectable. Thus AMP-PK and the plant HMG-CoA reductase kinase are serine/threonine-specific, rather than dual specificity, protein kinases. AMP-PK phosphorylates the 'SAMS' peptide exclusively on S7 [6]. We did not directly show that S7 (T7) was the site phosphorylated for all of the variants, but the fact that the Y7 peptide was not detectably phosphorylated renders it very unlikely that S4 is phosphorylated in any of the variants.

The strong similarity in substrate specificity between the animal and plant kinases reinforces the view that the plant HMG-CoA reductase kinase is a functional equivalent of mammalian AMP-PK. From our studies we propose that the minimal recognition sequence for both kinases is M/V/L/I-(R/K/H,X,X)-X-**S/T**-X-X-X-M/V/L/I (where '/' indicates alternatives, and commas indicate that the order of residues is not important). However, the importance of the positioning of the hydrophobic and basic residues remains to be determined. Perusal of the natural substrates in Fig. 1 indicates that the X residues can be almost anything, although acidic residues are not seen between −4 and −1 and, given the requirement for a basic residue in this region, it is possible that acidic residues would be inhibitory. Clearly,

the target sequence is unlikely to be phoshorylated unless it is exposed on the surface of the protein. At least in the cases of acetyl-CoA carboxylase, HMG-CoA reductase and glycogen synthase, the phosphorylation site is located at, or near the N- or C-terminus, regions that on proteins of known structure are often found to be exposed.

Acknowledgements: This study was supported by an SERC studentship (to J.W.), by a programme grant from the Wellcome Trust, and by a project grant from the AFRC. F.B.C. is a staff member of the MRC Unit. We are very grateful to Randolph Hampton for yeast strains over-expressing HMG1 and HMG2, and Elizabeth Carrey for the CAD peptide.

REFERENCES

[1] Hardie, D.G. and MacKintosh, R.W. (1992) BioEssays 14, 699–704.
[2] Moore, F., Weekes, J. and Hardie, D.G. (1991) Eur. J. Biochem. 199, 691–697.
[3] Gillespie, J.G. and Hardie, D.G. (1992) FEBS Lett. 306, 59–62.
[4] MacKintosh, R.W., Davies, S.P., Clarke, P.R., Weekes, J., Gillespie, J.G., Gibb, B.J. and Hardie, D.G. (1992) Eur. J. Biochem. 209, 923–931.
[5] Carling, D., Clarke, P.R., Zammit, V.A. and Hardie, D.G. (1989) Eur. J. Biochem. 186, 129–136.
[6] Davies, S.P., Carling, D. and Hardie, D.G. (1989) Eur. J. Biochem. 816, 123–128.
[7] Wilkinson, G.N. (1961) Biochem. J. 80, 324–332.
[8] Clarke, P.R. and Hardie, D.G. (1990) EMBO J. 9, 2439–2446.
[9] Hunter, C.F. and Rodwell, V.W. (1980) J. Lipid Res. 21, 399–405.
[10] Brown, K., Havel, C.M. and Watson, J.A. (1983) J. Biol. Chem. 258, 8512–8518.
[11] Munday, M.R., Campbell, D.G., Carling, D. and Hardie, D.G. (1988) Eur. J. Biochem. 175, 331–338.
[12] Kemp, B.E. (1990) Trends Biochem. Sci. 15, 342–346.
[13] Knighton, D.R., Zheng, J., Ten Eyck, L.F., Xuong, N.H., Taylor, S.S. and Sowadski, J.M. (1991) Science 253, 414–420.
[14] Carrey, E.A., Campbell, D.G. and Hardie, D.G. (1985) EMBO J. 4, 3735–3742.
[15] Garton, A.J., Campbell, D.G., Carling, D., Hardie, D.G., Colbran, R.J. and Yeaman, S.J. (1989) Eur. J. Biochem. 179, 249–254.