Similar substrate recognition motifs for mammalian AMP-activated protein kinase, higher plant HMG-CoA reductase kinase-A, yeast SNF1, and mammalian calmodulin-dependent protein kinase I

Susan Dale[a], Wayne A. Wilson[a], Arthur M. Edelman[b], D. Grahame Hardie[a,*]

[a]*Department of Biochemistry, The University, Dundee, DD1 4HN, Scotland, UK*
[b]*Department of Pharmacology and Toxicology, State University of New York, Buffalo, NY, USA*

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Abstract **We have analysed phosphorylation of the synthetic peptide AMARAASAAALARRR, and 23 variants, by mammalian, higher plant and yeast members of the SNF1 protein kinase subfamily (AMP-activated protein kinase (AMPK), HMG-CoA reductase kinase (HRK-A), and SNF1 itself), and by mammalian calmodulin-dependent protein kinase I (CaMKI). These four kinases recognize motifs which are very similar, although distinguishable. Our studies define the following recognition motifs: AMPK: ΦΦ(X,β)XXS/TXXXΦ; HRK-A: Φ(X,β)XXSXXXΦ; Snf1: ΦXRXXSXXXΦ; CaMKI: ΦXRXXS/TXXXΦ; where Φ is a hydrophobic residue (M, V, L, I or F) and β is a basic residue (R, K or H).**

Key words: AMP-activated protein kinase; HMG-CoA reductase kinase; SNF1; Calmodulin-dependent protein kinase I; Specificity determinant; Consensus sequence

1. Introduction

AMP-activated protein kinase (AMPK) is involved in the response of mammalian cells to environmental stresses, particularly those which deplete ATP [1,2]. In response to conditions which elevate the AMP:ATP ratio, AMPK phosphorylates several targets, especially biosynthetic enzymes such as HMG-CoA reductase which are inactivated [3], thus providing a mechanism for conserving ATP. We have recently discovered activities in higher plants which, by a number of biochemical criteria, appear to be homologues of AMPK. Since they appear not to be directly activated by AMP, we have provisionally named them HMG-CoA reductase kinases (HRK-A and -B) [4,5].

Identification of novel targets for AMPK and HRK-A/B would be aided by identification of the sequence motif which they recognize on their target proteins. We recently proposed, by comparing sequences of natural substrates and by analysing phosphorylation of a series of variants of a synthetic peptide ('SAMS', based on the major sequence phosphorylated on rat acetyl-CoA carboxylase [6]) that hydrophobic side chains at P-5 and P + 4, and basic residues at P-4 or P-3 (where P represents the phosphorylated serine) were positive determinants [7].

In this paper we further examine the specificity of AMPK and HRK-A using a new series of peptides which were designed to define the optimal hydrophobic and basic side chains, and their optimal positions with respect to the phosphorylation site. We have also extended the analysis to two other protein kinases, i.e. SNF1 from the yeast *Saccharomyces cerevisiae*, and mammalian Ca^{2+}-calmodulin-dependent protein kinase I (CaMKI). The SNF1 protein kinase is a complex of at least two polypeptides, Snf1p and Snf4p [8]. The catalytic subunit of AMPK is closely related in sequence to Snf1p [9,10], while one of its accessory subunits [11] is related to Snf4p [12]. The *SNF1* and *SNF4* genes are both required for the response to glucose starvation, an acute stress for yeast [13]. Although SNF1 may share one physiological substrate with AMPK, i.e. acetyl-CoA carboxylase [10,14], nothing was known about how it recognizes its target sequences. CaMKI is a multifunctional Ca^{2+}-calmodulin-dependent protein kinase which is widely expressed in vertebrate tissues, but for which only a small number of potential physiological substrates have been identified to date [15–17]. Comparison of its specificity with AMPK was prompted by recent observations that its substrate recognition motif [18] was apparently very similar to that for AMPK [7].

2. Materials and methods

2.1. Synthesis and analysis of peptides

The SAMS peptide was synthesized using an ABI 431 synthesizer. The synapsin I peptide (LRRRLSDANF) was supplied by Y.-G. Kwon and D.S. Lawrence (SUNY, Buffalo). Other peptides were synthesized using the Multiple Peptide Synthesis service of Cambridge Research Biochemicals (Warrington, UK). Purification and analysis of peptides was as described previously [7].

2.2. Purification of protein kinases

AMP-PK was purified from rat liver as far as the gel filtration step [19]. HRK-A was purified from cauliflower *(Brassica oleracea)* inflorescences [5]. SNF1 was purified from haploid cells of *S. cerevisiae* strain ABYS106, which is deficient in vacuolar proteinases *(pra1–1, prb1–1, prc1–1, cps1–3, his3, ura3, leu2)* essentially by the method of Mitchelhill et al. [10]. In some experiments we used a *snf1* disrupted strain *(snf1-Δ10)* and its congenic wild type [20]. CaMKI used in this study was the *a* isoform purified from rat brain [15], after activation by CaMKIa activator [21].

2.3. Enzyme assays

Peptide phosphorylation was measured using a P81 paper method [6]. Data were fitted to the Michaelis–Menten equation using a statistical method [22].

*Corresponding author. Fax: (44) (1382) 201063.
E-mail: d.g.hardie@dundee.ac.uk

Abbreviations: AMP-PK, AMP-activated protein kinase; HMG-, 3-hydroxy-3-methyl-; HRK—A, HMG-CoA reductase kinase-A; SNF, sucrose non-fermenting; CaMKI, calmodulin-dependent protein kinase I; PKA, cyclic AMP-dependent protein kinase.

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3. Results

3.1. Design of the synthetic peptide variants

For these specificity studies we designed the parent peptide AMARAASAAALARRR ('AMARA') using the following considerations:

(i) The phosphorylated serine and the P − 5 and P + 4 hydrophobic residues (bold type) were retained in the same relative positions as in the 'SAMS' peptide (HMRSAMSGLHLVKRR) used routinely for assay of AMPK. The arginine at P-4 was moved to P-3, so that we could examine the effect of moving the P—5 hydrophobic to P—4 without disturbing the basic residue. We already knew that some natural substrates for AMPK have the basic residue at P-3 rather than P-4 [7]. A basic residue at P-3 is a requirement for CaMKI [18].

(ii) All other residues were replaced by an amino acid with a small neutral side chain, alanine. This reduced the likelihood that an effect of moving a critical residue would be complicated by an effect of movement of the residue for which it was exchanged. Surprisingly, a peptide in which these residues were replaced by glycine, rather than alanine, was not a substrate for AMPK (not shown).

(iii) Three arginines were added to the C-terminus to ensure efficient binding to P81 paper, the basis of the kinase assay. These C-terminal basic residues are not essential for phosphorylation by AMPK [7] or CaMKI [18].

3.2. Purification of kinases and phosphorylation of parent 'AMARA' peptide

Table 1 shows that 'AMARA' was an excellent substrate for purified AMPK and HRK-A, with V/K_m values 3.8-fold and 4.5-fold higher respectively than for the 'SAMS' peptide. Phosphorylation by AMPK of 'AMARA' and of all variants tested was stimulated by AMP to the same extent as with the 'SAMS' peptide (not shown).

SNF1 was purified utilizing its naturally occurring polyhistidine sequence at the N-terminus, which allows binding to Ni^{2+}-agarose [10]. Purified SNF1 phosphorylated 'AMARA', but unlike AMPK or HRK-A it was not as good a substrate as 'SAMS' with a V/K_m 5-fold lower, due predominantly to a higher K_m (≈650 μM vs. 108 μM). It was important to demonstrate that phosphorylation of 'AMARA' and its variants was entirely due to SNF1. When assayed in crude extracts of cells grown in 2% glucose, there was a similar low level of phosphorylation of 'AMARA' and two of the variants (#3 and #4) in a *snf1-Δ* strain and in a congenic wild type. However in the wild type strain, phosphorylation of all three peptides was stimulated ≈10-fold by removal of glucose from the medium (not

Table 1
Kinetic parameters of 'SAMS' peptide and novel variant peptides determined using rat liver AMPK and cauliflower HRK-A

Group	No.	Peptide	AMPK			HRK-A		
			V (U/mg)	K_m (μM)	V/K_m**	V (U/mg)	K_m (μM)	V/K_m*
SAMS		HMRSAMSGLHLVKRR	1120 ± 90	26 ± 4	0.26 ± 0.05	3860 ± 280	119 ± 12	0.22 ± 0.03
	1	AMARAASAAALARRR	1670 ± 60	10 ± 1	1.00 ± 0.11	8220 ± 580	55 ± 7	1.00 ± 0.15
A	2	A**V**ARAASAAALARRR	543 ± 33	17 ± 4	0.19 ± 0.05	5430 ± 190	40 ± 3	0.91 ± 0.08
	3	A**L**ARAASAAALARRR	878 ± 44	11 ± 2	0.48 ± 0.09	3580 ± 240	19 ± 3	1.26 ± 0.22
	4	A**I**ARAASAAALARRR	678 ± 41	6.3 ± 1.9	0.64 ± 0.20	2410 ± 70	16 ± 1	1.01 ± 0.07
	5	A**F**ARAASAAALARRR	574 ± 18	10 ± 1	0.34 ± 0.04	3260 ± 190	20 ± 3	1.09 ± 0.18
	6	A**G**ARAASAAALARRR	640 ± 150	299 ± 74	0.01 ± 0.004	192 ± 37	248 ± 55	0.01 ± 0.002
B	7	**M**AARAASAAALARRR	566 ± 33	27 ± 3	0.13 ± 0.02	2350 ± 420	657 ± 127	0.02 ± 0.006
	8	AA**M**RAASAAALARRR	366 ± 32	48 ± 8	0.05 ± 0.01	613 ± 65	188 ± 22	0.02 ± 0.003
C	9	AMA**H**AASAAALARRR	622 ± 28	16 ± 2	0.23 ± 0.03	2980 ± 140	86 ± 6	0.23 ± 0.02
	10	AMA**K**AASAAALARRR	696 ± 44	12 ± 2	0.35 ± 0.06	2200 ± 60	42 ± 2	0.35 ± 0.02
	11	AMA**G**AASAAALARRR	78 ± 10	16 ± 4	0.03 ± 0.008	95 ± 7.2	56 ± 7	0.01 ± 0.002
D	12	AM**R**AAASAAALARRR	757 ± 34	16 ± 2	0.28 ± 0.038	2770 ± 90	69 ± 4	0.27 ± 0.018
	13	AMAA**R**ASAAALARRR	217 ± 4	24 ± 1	0.05 ± 0.002	448 ± 40	125 ± 16	0.02 ± 0.004
	14	AMAAA**R**SAAALARRR	442 ± 40	36 ± 6	0.07 ± 0.01	151 ± 8	11 ± 2	0.09 ± 0.02
E	15	AMARAASAAA**M**ARRR	882 ± 30	9.9 ± 1.1	0.53 ± 0.062	481 ± 51	2.5 ± 1.1	1.29 ± 0.582
	16	AMARAASAAA**V**ARRR	645 ± 46	20 ± 3	0.19 ± 0.032	209 ± 10	3 ± 0.7	0.47 ± 0.111
	17	AMARAASAAA**I**ARRR	1040 ± 30	12 ± 1	0.52 ± 0.046	6740 ± 240	86 ± 5	0.52 ± 0.036
	18	AMARAASAAA**F**ARRR	1150 ± 10	11 ± 1	0.63 ± 0.058	4830 ± 360	48 ± 7	0.67 ± 0.110
	19	AMARAASAAA**G**ARRR	109 ± 15	95 ± 18	0.01 ± 0.002	163 ± 42	518 ± 85	0.002 ± 0.001
F	20	AMARAASAA**L**AARRR	411 ± 19	69 ± 5	0.04 ± 0.003	906 ± 31	101 ± 5	0.06 ± 0.004
	21	AMARAASAAAA**L**RRR	*	*	*	*	*	*
	22	AA**M**RAASAAAA**L**RRR	534 ± 52	35 ± 7	0.09 ± 0.020	73 ± 7	13 ± 4	0.04 ± 0.01
G	23	AMARAA**T**AAALARRR	898 ± 19	15 ± 1	0.36 ± 0.025	256 ± 12	5.2 ± 0.9	0.33 ± 0.06
	24	AMARAA**Y**AAALARRR	*	*	*	*	*	*

Variations to the parent sequence are indicated by underlining and bold type. Results are quoted ± standard errors determined by a statistical method [22].
*Phosphorylation barely detectable.
**V/K_m values are relative to those obtained with AMARAASAAALARRR.

shown), as previously shown for 'SAMS' phosphorylation [14]. There was no increase in activity in the *snf1-Δ* strain. The low level of activity in the *snf1* disrupted strain was therefore due to slow phosphorylation by other yeast protein kinases that are not activated by glucose starvation. These activities were removed by the purification procedure, because when an extract from the *snf1* disrupted strain was taken through an identical procedure, it did not phosphorylate 'AMARA' (not shown).

Purified CaMKI also phosphorylated 'AMARA' although V/K_m was 6× less than for the synapsin I peptide LRRRLSDANF. Phosphorylation of 'AMARA' and all variants tested (#3, #4, #16, #18) was completely dependent on prior activation by CaMKIa activator (not shown).

3.3. Comparison of phosphorylation of 'AMARA' and 23 variants

The initial rates of phosphorylation of 'AMARA' and its 23 variants were compared for all four kinases at a single peptide concentration (40 μM, Table 2). For AMPK and HRK-A we also determined the parameters V and K_m for all variants where the rate of phosphorylation was significant (Table 1).

4. Discussion

'AMARA' turned out to be an excellent substrate for AMPK and HRK-A, with V/K_m values ≈4-fold higher than for the 'SAMS' peptide (Table 1). The new peptide may turn out to be useful for routine assay of these kinases, although its specificity in crude extracts would have to be tested. It was not as good a substrate of SNF1 as the 'SAMS' peptide, due principally to a high K_m (650 μM). SNF1 also had a rather high K_m (108 μM) for the 'SAMS' peptide itself (c.f. K_m of 26 μM with AMPK), perhaps reflecting the fact that leucine rather than methionine is the optimal residue at the P-5 position (Table 2). 'AMARA' was not as good a substrate for CaMKI as the synapsin I peptide (LRRRLSDANF) used previously, with V/K_m being 6-fold lower. This may be because CaMKI also has a strong preference for leucine rather than methionine at P-5 (Table 2), and also because additional arginines at P-4 and P-2, although not essential, appear to lower the K_m of CaMKI for peptide substrates [18].

The general conclusion that may be drawn from the studies with 'AMARA' variants is that AMPK, HRK-A, SNF1 and CaMKI have very similar, but distinguishable, substrate recognition requirements. We will now discuss the more specific conclusions about recognition motifs resulting from these studies. The lettering below refers to the lettered groups of variant peptides in Tables 1 and 2. The numbering refers to the peptide number, where #1 was the parent peptide:

(A) Replacement of the hydrophobic residue (M) at P—5 by glycine (#6) had a very large deleterious effect on phosphorylation by all kinases. For AMPK and HRK-A this was associated with large increases in K_m, and for HRK-A there was

Table 2
Initial rates of phosphorylation of variant peptides at a single concentration (40 μM) by rat AMPK, *B. oleracea* HRK-A, *S. cerevisiae* SNF1 and rat CaMKI

No.	Group	Peptide	AMPK	HRK-A	SNF1	CaMKI
1		AMARAASAAALARRR	1.000 ± 0.443	1.000 ± 0.018	1.000 ± 0.046	1.000 ± 0.026
2	A	A**V**ARAASAAALARRR	0.329 ± 0.103	0.698 ± 0.018	0.418 ± 0.019	1.287 ± 0.044
3		A**L**ARAASAAALARRR	0.561 ± 0.176	0.693 ± 0.017	2.164 ± 0.084	4.427 ± 0.089
4		A**I**ARAASAAALARRR	0.434 ± 0.136	0.452 ± 0.008	1.126 ± 0.045	3.179 ± 0.066
5		A**F**ARAASAAALARRR	0.374 ± 0.117	0.568 ± 0.014	1.183 ± 0.065	2.800 ± 0.056
6		A**G**ARAASAAALARRR	0.051 ± 0.016	0.008 ± 0.000	0.046 ± 0.002	0.031 ± 0.003
7	B	**M**AARAASAAALARRR	0.164 ± 0.052	0.035 ± 0.001	0.130 ± 0.008	0.200 ± 0.005
8		AA**M**RAASAAALARRR	0.135 ± 0.042	0.026 ± 0.001	0.098 ± 0.005	0.104 ± 0.004
9	C	AMA**H**AASAAALARRR	0.303 ± 0.095	0.243 ± 0.004	0.100 ± 0.006	0.053 ± 0.003
10		AMA**K**AASAAALARRR	0.363 ± 0.114	0.300 ± 0.009	0.171 ± 0.008	0.099 ± 0.006
11		AMA**G**AASAAALARRR	0.038 ± 0.012	0.011 ± 0.000	0.034 ± 0.006	0.012 ± 0.002
12	D	AM**R**AAASAAALARRR	0.369 ± 0.116	0.280 ± 0.008	0.080 ± 0.009	0.017 ± 0.004
13		AMAA**R**ASAAALARRR	0.101 ± 0.034	0.031 ± 0.001	0.057 ± 0.006	0.007 ± 0.002
14		AMAAA**R**SAAALARRR	0.144 ± 0.045	0.030 ± 0.001	0.053 ± 0.003	0.018 ± 0.001
15	E	AMARAASAAA**M**ARRR	0.537 ± 0.168	0.108 ± 0.003	0.228 ± 0.008	0.716 ± 0.013
16		AMARAASAAA**V**ARRR	0.329 ± 0.103	0.050 ± 0.001	0.178 ± 0.006	0.248 ± 0.013
17		AMARAASAAA**I**ARRR	0.561 ± 0.176	0.597 ± 0.010	0.760 ± 0.028	1.509 ± 0.040
18		AMARAASAAA**F**ARRR	0.661 ± 0.207	0.652 ± 0.012	0.372 ± 0.017	1.424 ± 0.034
19		AMARAASAAA**G**ARRR	0.025 ± 0.008	0.002 ± 0.000	0.011 ± 0.003	0.014 ± 0.001
20	F	AMARAASAA**L**AARRR	0.108 ± 0.035	0.072 ± 0.001	0.007 ± 0.001	0.066 ± 0.004
21		AMARAASAAAA**L**RRR	0.002 ± 0.001	0.000 ± 0.000	0.002 ± 0.001	0.059 ± 0.002
22		AA**M**RAASAAAA**L**RRR	0.184 ± 0.058	0.014 ± 0.001	0.005 ± 0.002	0.008 ± 0.001
23	G	AMARAA**T**AAALARRR	0.461 ± 0.144	0.060 ± 0.001	0.053 ± 0.006	0.645 ± 0.015
24		AMARAA**Y**AAALARRR	0.007 ± 0.003	*	*	*

Results are expressed relative to the rates obtained for each enzyme with the parent peptide (No. 1) (±standard error of the mean for 3–6 determinations). Variations to the parent sequence are indicated by underlining and bold type.
*Phosphorylation not detectable.

also a large decrease in V. Replacement of the methionine by other hydrophobic residues with either aliphatic (V, L, I; #2–4) or aromatic (F; #5) side chains had much smaller effects. The preferences for specific hydrophobic side chains differed for the four kinases (based on Table 2, AMPK: M > L > I ≈ F ≈ V; HRK-A: M > V ≈ L > F > I; SNF1: L > F≈I≈M > V; CaMKI: L > I > F > V > M. The order of preference of these protein kinases does not correlate in a simple manner with hydropathy index [23], indicating that other features such as the shape of the side chain are also important.

(B) Shifting the methionine at P—5 to P—6 (#7) or P—4 (#8) dramatically reduced the rate of phosphorylation, especially for HRK-A where both shifts produced 50-fold decreases in V/K_m. The hydropathy index assigned to alanine is only just lower than that assigned to methionine [23]. Nevertheless, the side chain of alanine is much smaller than those of the other hydrophobic amino acids, and it is clear that it will not fulfil the requirement for a hydrophobic side chain at the P—5 position.

(C) Replacement of the basic residue (R) at P—3 by other basic residues (H or K; #9–10) was tolerated by AMPK and HRK-A but reduced the rate of phosphorylation (R ≫ K > H). It was less well tolerated by SNF1 and CaMKI, which can be regarded as essentially arginine-specific. Replacement with glycine (#11) almost abolished phosphorylation in every case.

(D) AMPK and HRK-A tolerated movement of the arginine at P—3 to P—4 (#12) reasonably well. SNF1 and CaMKI have a stringent requirement for an arginine at P-3. Peptides containing the arginine at P—2 or P—1 (#13–14) were poor substrates for all kinases.

(E) Replacement of the leucine at P + 4 by glycine (#19) almost abolished phosphorylation in every case: for AMPK and HRK-A there were decreases in V and and increases in K_m. Other hydrophobic amino acids can replace leucine. The preferences for specific hydrophobic side chains differed for the four kinases, although valine was the worst in every case (based on Table 2, AMPK: L > F ≈ I ≈ M > V; HRK- A: L > F ≈ I ≫V SNF1: L > I > F > M > V; CaMKI: I ≈ F > L > M ≫ V).

(F) Shifting the leucine at P + 4 to P + 5 (#21) dramatically reduced the rate of phosphorylation by all of kinases. Shifting it to P + 3 (#20) also has a large deleterious effect, particularly for SNF1. These results show that an alanine does not fulfil the requirement for a hydrophobic residue at P + 4. For AMPK but not for the other kinases, the effect of shifting the leucine to P + 5 can be partially compensated by also shifting the methionine at P—5 to P—4 (#22).

(G) The peptide containing threonine in place of serine (#23) was a reasonably good substrate for AMPK and CaMKI, but a very poor substrate for HRK-A and SNF1. The peptide containing tyrosine in place of serine (#24) was not significantly phosphorylated by any of the kinases.

These results confirm our more limited conclusions for the comparison of AMPK and HRK-A using a smaller series of variants of the 'SAMS' peptide [7], and now also encompass the yeast homologue of AMPK, SNF1. They also confirm the previously suggested recognition motif for CaMKI, determined using variants of the synapsin I peptide [18]. The present results also now establish for all four kinases the optimal hydrophobic side chains at the P-5 and P + 4 positions, and the effect of varying the distance from the phosphorylated serine. For CaMKI, they also establish the order of preference for basic amino acids at the P-3 position, and for the AMPK/SNF1 family the preferred position of the N-terminal basic residue.

The consensus sequences for substrate recognition by the four kinases can be represented as: Φ(X,β)XX**S/T**XXXΦ (AMPK), Φ(X,β)XX**S**XXXΦ (HRK-A), ΦX**R**XX**S**XXXΦ (SNF1) and ΦX**R**XX**S/T**XXXΦ (CaMKI). Φ indicates a hydrophobic residue (M, V, L, I or F), β a basic residue (R > K > H), and parentheses indicate those cases where the order of amino acids at the P-4 and P-3 positions is not critical. The four kinases differ in their preference for the shape of the side chains at P-5 and P + 4, but all have stringent requirements for hydrophobic residues at these positions. It is interesting to note that cyclic AMP-dependent protein kinase (PKA) has a preference, although not a strict requirement, for a hydrophobic residue at the P + 1 position, and in the crystal structure of the catalytic subunit, the P + 1 side chain of the complexed pseudosubstrate peptide is accommodated in a hydrophobic pocket formed by L198 and L205 [24]. One can envisage that each of the four kinases in this study have two hydrophobic pockets which accomodate the P-5 and P + 4 side chains. The shapes of these pockets may be subtly different, explaining the varying preferences for individual hydrophobic side chains.

The similarities in the recognition of substrates by AMPK and SNF1 are consistent with the ≈60% sequence identity of their kinase domains [9], although before this study very little was known about the substrate specificity of SNF1. The specificity of SNF1 is, if anything, more stringent than that of AMPK, but whether this will be reflected in a more restricted specificity for physiological substrates remains to be determined. HRK-A is likely [2] to be the *B. oleracea* homologue of the product of the rye *RKIN1* gene, which complements *snf1* mutations when expressed in yeast [25]. The similarities in specificity between the AMPK/SNF1 family and CaMKI were more surprising, and suggest that these kinases may share some physiological substrates. The SNF1 subfamily, while not themselves calmodulin-dependent, do fall within the calmodulin-dependent protein kinase subfamily according to the sequences of their kinase domains [26]. Another non-calmodulin-dependent protein kinase that falls by this criterion into the same subfamily is MAP kinase-activated protein kinase-2 (MAPKAP kinase-2 [27]). MAPKAP kinase-2 also requires a hydrophobic residue at P-5 and a basic residue at P-3, although the P + 4 hydrophobic residue is not required [27]. The same authors reported that calmodulin-dependent protein kinase II, which has a requirement for a P-3 arginine residue, also prefers a hydrophobic residue at P-5, although in this case it is not essential. Therefore all of these proteins which fall, by kinase domain sequence comparison, into the calmodulin-dependent protein kinase subfamily [26] may have a hydrophobic pocket which binds the P-5 side chain.

It is also interesting to compare the requirement for basic residues in substrates of these protein kinases with that for PKA. The optimal motif for PKA is RRXS, and in the crystal structure the pair of arginines in the bound pseudosubstrate peptide interact with the side chains of four glutamates, i.e. E127 and E331 (P—3) and E170 and E230 (P—2) [24]. Interestingly, E127 and E170 are conserved in the sequence [9] of rat AMPK (E99 and E142). Acidic residues (E or D) are also conserved in these two positions in Snf1p [13], all four sequenced members of the plant Rkin1 family of which HRK-A may be a member [2], and bovine CaMKI [17].

Of the four kinases studied, information about specific sites phosphorylated on protein substrates is only available for AMPK and CaMKI. With one exception the known or likely physiological substrates for AMPK [7] contain the motif $\Phi(X,\beta)XXS/TXXX\Phi$. The exception is the S1200 site on rat acetyl-CoA carboxylase [28]. This site has L at P—4, R at P—2, and L at P + 5. It therefore has all the key residues with the correct spacing, but they are displaced by one residue in the C-terminal direction with respect to the serine. Possibly these recognition determinants can bind to the kinase, and although the serine is not ideally positioned it can still be phosphorylated. In this respect it is interesting that peptide #21, which has the hydrophobics at P—5 and P + 5, is not phosphorylated at all, but that peptide #22, where they are P—4 and P + 5, is a substrate, albeit a poor one, for AMPK. By contrast, HRK-A, SNF1 and CaMKI are intolerant of shifts of the hydrophobic residues away from the P—5 and P + 4 positions, even when the 9 residue spacing is maintained. For CaMKI, the recognition motif defined in this and the previous study [18] is consistent with the two protein substrates for which sequence information is available, i.e. synapsin I (**LRRRLSDSNF** [29]) and the cAMP-response element binding protein, CREB (**LSRRPSYRKI** [30]). Indeed, L at P-5, R at P-3 and I or F at P + 4 is the optimal combination of residues for CaMKI as judged by the results in Table 2.

In conclusion the results described here provide:

(i) the first report of the substrate recognition motif for SNF1, which is similar although distinguishable from those of its mammalian and higher plant homologues;

(ii) further refinement of the substrate recognition motifs of AMPK, HRK-A and CaMKI, information which will be invaluable in the identification of additional physiological substrates;

(iii) evidence that sequence similarity within kinase domains can be reflected in similarities in substrate recognition, even when the regulatory ligands acting on the protein kinases are quite different;

(iv) a rationale for focussing on intracellular targets, e.g. HMG-CoA reductase, which could integrate multiple physiological signals (e.g. Ca^{2+} and stress) converging at a single regulatory phosphorylation site.

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