Substrate specificity determinants of the checkpoint protein kinase Chk1

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Abstract The Chk1 protein kinase plays a critical role in a DNA damage checkpoint pathway conserved between fission yeast and animals. We have developed a quantitative assay for Chk1 activity, using a peptide derived from a region of *Xenopus* Cdc25C containing Ser-287, a known target of Chk1. Variants of this peptide were used to determine the residues involved in substrate recognition by Chk1, revealing the phosphorylation motif Φ-X-β-X-X-(S/T)*, where * indicates the phosphorylated residue, Φ is a hydrophobic residue (M > I > L > V), β is a basic residue (R > K) and X is any amino acid. This motif suggests that Chk1 is a member of a group of stress-response protein kinases which phosphorylate target proteins with related specificities.

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Key words: Cdc25; Chk1; Cell cycle checkpoint; Protein kinase specificity

1. Introduction

Eukaryotic cells possess checkpoint mechanisms which ensure that cell division is restrained in the presence of damaged or unreplicated DNA [1,2]. The Chk1 protein kinase is an essential component of the DNA damage checkpoint pathway conserved from yeast to animals that regulates entry into mitosis [3–5]. Chk1 phosphorylates the Cdc25 protein phosphatase, promoting the binding of a 14-3-3 protein to Cdc25 [6,7] and preventing nuclear translocation of Cdc25 [8–10]. Cdc25 is thus unable to activate the protein kinase Cdc2/cyclin B and the cell cycle is arrested prior to mitosis.

Chk1 may also execute its function through phosphorylation of other substrates. In vitro, Chk1 can phosphorylate *Schizosaccharomyces pombe* Wee1, the protein kinase that maintains inhibitory phosphorylation of Cdc2 [11]. In *Saccharomyces cerevisiae*, Chk1 phosphorylates Pds1, stabilising Pds1 and preventing the onset of anaphase [12]. However, the phosphorylation sites on these proteins have not been identified. It is also likely that other substrates remain to be discovered. In order to predict sites of phosphorylation on known substrates and to identify novel candidate substrates for a protein kinase, it is useful to determine minimal sequence requirements for recognition. The sequence specificity of Chk1 has not been reported, but a comparison of the sequences surrounding the known Chk1 phosphorylation sites in the human, *Xenopus laevis* and *S. pombe* isoforms of Cdc25 reveals conserved basic and hydrophobic residues N-terminal to the target serine, suggesting that these residues may be important for recognition by the kinase (Fig. 1). Here, we report the substrate specificity of human Chk1 determined using peptides derived from Cdc25C as in vitro substrates. We define a recognition motif that is present in other potential Chk1 substrates. This motif suggests that Chk1 is a member of a group of related kinases with similar substrate specificities that respond to different cellular stresses.

2. Materials and methods

2.1. Expression and purification of GST-hChk1

A human Chk1 cDNA [5] was sub-cloned as a fusion product with the glutathione *S*-transferase (GST) gene into the pFastBac1 vector, and recombinant baculoviruses were produced using the Bac-to-Bac system (Gibco). *Sf21* insect cells in suspension culture were infected with *GST-hChk1* baculoviruses and incubated at 27°C for 96 h. The following steps were carried out at 4°C: cells were harvested by centrifugation, the cell pellet was resuspended in lysis buffer (50 mM Tris–HCl, pH 8.5, 10 mM dithiothreitol (DTT), 1% (v/v) NP-40, 1 μg/ml each of leupeptin and aprotinin, 1 mM each of phenylmethylsulphonyl fluoride, pepstatin A, chymostatin and benzamidine), and sonicated four times for 10 s. The sonicate was centrifuged at $14\,000\times g$ for 10 min, and the supernatant mixed with 1 ml glutathione-Sepharose beads (Pharmacia) for 60 min. After washing with 50 mM Tris–HCl, pH 8.5, 150 mM NaCl, 5 mM DTT, 1%(w/v) NP-40, GST-hChk1 protein was eluted from the beads by successive incubations with 20 mM HEPES-KOH, pH 7.5, 50 mM reduced glutathione. Eluates containing GST-hChk1 protein (as judged by SDS-PAGE) were pooled, dialysed against 10 mM HEPES-KOH, pH 7.5, 1 mM DTT, aliquoted, snap-frozen, and stored at −70°C.

2.2. Synthesis of peptides

The *Xenopus* Cdc25 'SPS', 'APS', 'SPA' and 'human Cdc25C' peptides were synthesised and purified by Dr G. Bloomberg, Department of Biochemistry, University of Bristol, UK. All other peptides were synthesised on a 1.0 μmol scale as Cleaved PepSets (Chiron Technologies, Vic., Australia), dissolved in dimethylsulphoxide (DMSO), and stored at −70°C.

2.3. Phosphorylation of peptides by GST-hChk1

Peptides at 200 μM (unless stated otherwise) were phosphorylated in 50 mM HEPES, pH 7.5, 1 mM DTT, 100 μM γ[^{32}P]ATP (approx. 10 kBq/nmol), 10 mM $MgCl_2$ in a 10 μl reaction volume containing approximately 60 ng GST-hChk1. Reactions were carried out for 30 min at 37°C, then stopped by spotting 8 μl onto 15 mm×15 mm squares of Whatman P81 phosphocellulose cation-exchange paper. Squares were washed three times for 5 min in 500 ml 150 mM H_3PO_4, rinsed in ethanol, air-dried and Cerenkov counted for 1 min. Reactions were performed in triplicate and the mean rate of phosphorylation calculated.

2.4. Kinetic experiments

For assays in which Chk1 activity was determined at various peptide concentrations, serial dilutions of peptide were made in 50 mM HEPES-KOH (pH 7.5), 25% (v/v) DMSO to give a final concentration of 5%(v/v) DMSO in the kinase reaction, with the exception of the experiment shown in Fig. 2, where no DMSO was present. Reaction velocity and substrate concentration data were fitted to a Michaelis–Menten curve using GraphPad Prism software, which determined the kinetic constants V_{max} and K_M.

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3. Results

3.1. A quantitative assay for Chk1 activity

To generate a substrate for in vitro phosphorylation by Chk1, we synthesised the peptide, RLYRSPSMPEKLDRK, derived from residues 281–294 of the *Xenopus* Cdc25C protein and including the Ser-287 site phosphorylated by Chk1 [7]. A C-terminal Lys residue was added to promote binding to phosphocellulose paper. Phosphorylation of this peptide (hereafter referred to as the 'SPS peptide') was catalysed by human Chk1 expressed as a fusion protein with glutathione-*S*-transferase (Fig. 2). In addition to the Ser-287 site, this peptide contains a serine at position 285 (the numbering corresponding to the position in the parent protein), a residue reported to be phosphorylated by cyclin-dependent kinases [13]. A peptide containing a Ser-285 to Ala substitution (the APS peptide) was phosphorylated with similar kinetics to the SPS peptide (Fig. 2), showing that Ser-285 is not required for recognition by Chk1 and is not itself a site of phosphorylation. In contrast, a peptide in which Ser-287 was changed to an alanine residue (the SPA peptide) was not phosphorylated, confirming the site of phosphorylation as Ser-287. Since the kinase used in these studies was the human sequence, we also compared a peptide derived from human Cdc25C which has three amino acid changes compared to the APS peptide. The human-derived sequence was a slightly poorer substrate than the *Xenopus*-derived APS peptide and the latter was therefore used as the parent sequence for subsequent studies. Standard assay conditions were optimised such that the rate of phosphorylation was close to the V_{max} of the enzyme, linear up to 60 min, and proportional to the amount of Chk1 used (data not shown).

3.2. Chk1 can phosphorylate serine or threonine, but not tyrosine residues

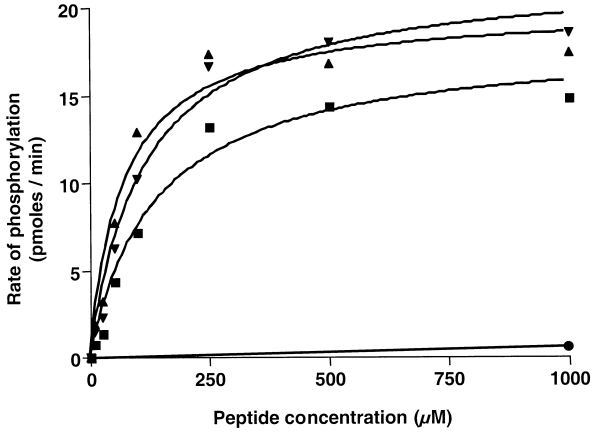
All of the phospho-acceptor sites for Chk1 identified thus far are serine residues. To determine whether threonine or tyrosine residues can also be phosphorylated by Chk1, variant APS peptides, in which Ser-287 was substituted for threonine or tyrosine, were tested in the Chk1 assay. The Thr-287 peptide was phosphorylated by Chk1 at a rate (28.6 pmol/min) slightly higher than the Ser-287 peptide (26.2 pmol/min), but the Tyr-287 peptide was not significantly phosphorylated. Chk1 is therefore a protein-serine/threonine kinase, but not a protein-tyrosine kinase.

3.3. Residues at positions −3 and −5 upstream of the target phosphorylation site are important for substrate recognition by Chk1

To determine which residues surrounding the phospho-acceptor site are important for recognition by Chk1, an alanine scan analysis of the APS peptide was performed. A panel of 12 APS peptide variants, each with one residue substituted for alanine, were assayed at 200 μM as substrates for Chk1 (Fig. 3A). Under these conditions, the majority of substitutions had little effect on the rate of phosphorylation of the peptide. Apart from the Ser-287 site, the only residues whose substitution to alanine significantly reduced the rate of phosphorylation were Leu-282 and Arg-284, five and three residues upstream of the phosphorylation site, respectively. None of the downstream residues, when substituted for alanine, caused a large reduction in the rate of phosphorylation by Chk1. More detailed kinetic experiments varying the concentration of substrate (Fig. 3B) showed that whereas substitution of Arg-281 or Met-288 to alanine resulted in an approximately two-fold reduction in catalytic efficiency (expressed as V_{max}/K_M), substitution of Leu-282 or Arg-284 to alanine reduced the catalytic efficiency by approximately 20-fold.

Protein	Sequence (↓ Chk1)	Site
Human Cdc25C	VSRSGLY**R**SPSMPENLNRPRL	Ser-216
***Xenopus* Cdc25C**	LNRSRLY**R**SPSMPEKLDRMPL	Ser-287
***S. pombe* Cdc25**	TPRRTLF**R**SLSCTVETPLANK	Ser-99
***S. pombe* Cdc25**	YLRPNVS**R**SRSSGNAPPFLRS	Ser-192
***S. pombe* Cdc25**	QDTPVVR**R**TQSMFLNSTRLGL	Ser-359

Fig. 1. Amino acid sequence alignment of known Chk1 substrates. Conserved hydrophobic residues are underlined, conserved basic residues are in bold.

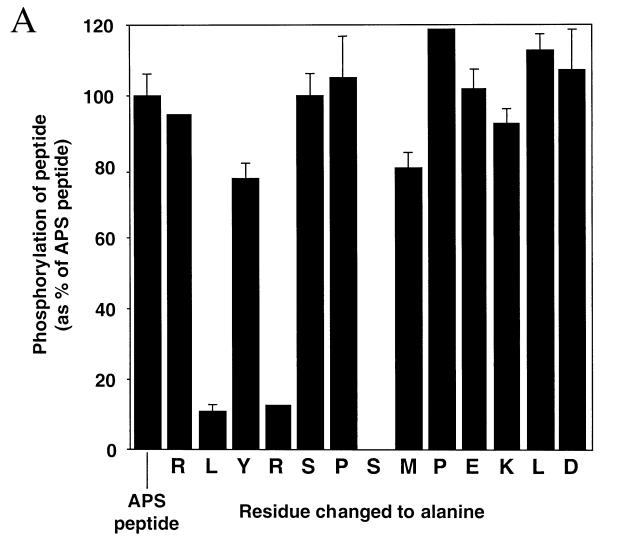


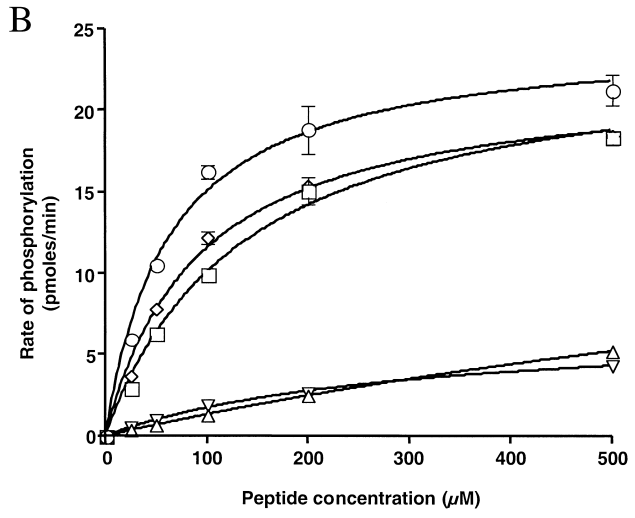
↓Chk1	V_{max} (pmoles/min)	K_M (μM)	V_{max}/K_M (pmoles/min/μM)
▼ RLYRSPSMPEKLDRK	21.88	115.8	0.189
▲ RLYRAPSMPEKLDRK	19.87	71.8	0.277
■ GLYRAPSMPENLNRK	17.99	140.4	0.128
● RLYRSPAMPEKLDRK			

Fig. 2. Comparison of the rate of phosphorylation of Cdc25C peptides by Chk1: (▼) *Xenopus* SPS peptide; (▲) *Xenopus* APS peptide; (■) Human Cdc25C peptide; (●) *Xenopus* SPA peptide.

3.4. Variations in position and identity of residues surrounding the targeted serine affect the rate of phosphorylation by Chk1.

To investigate in more detail the contribution of the critical residues for recognition by Chk1, we designed a peptide, RLARAASMAAALARK, based on the APS peptide, but with seven of the non-essential residues substituted by alanine. This minimal peptide maintained a high rate of phosphorylation by Chk1 (Fig. 4A). When Leu^{-5} was substituted with Met or Ile, the rate of phosphorylation increased, but with Val or Arg, and particularly Ala substitutions, the rate was decreased. When Arg^{-3} was substituted with Lys, the peptide was phosphorylated at 80% of the rate of the parent peptide, whereas substitution to Ala greatly reduced the rate. Peptides in which Leu^{+5} was substituted or moved relative to the phosphorylated serine residue showed a slight increase in the rate of phosphorylation, the largest being when Leu was moved from the +5 to the +4 position. However, a peptide in which





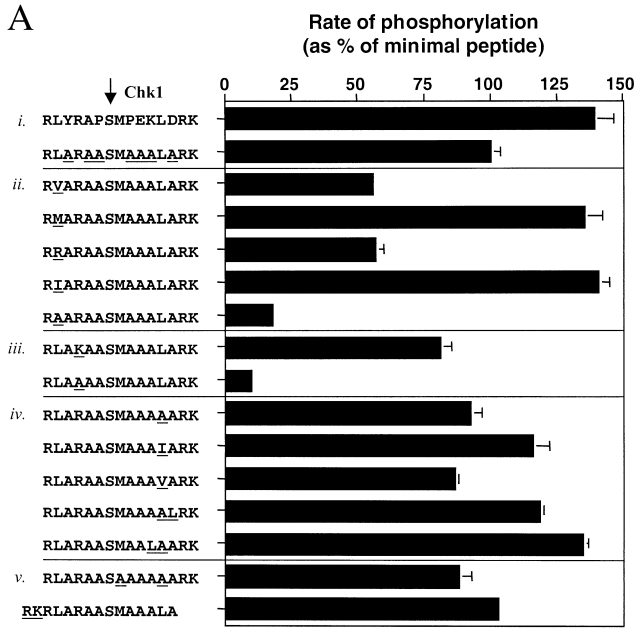
↓ Chk1	V_{max} (pmoles/min)	K_M (μM)	V_{max}/K_M (pmoles/min/μM)	%APS
○ RLYRAPSMPEKLDRK	24.5	63	0.388	100
□ ALYRAPSMPEKLDRK	23.8	136	0.175	45
△ RAYRAPSMPEKLDRK	19.9	1422	0.014	4
▽ RLYAAPSMPEKLDRK	6.8	294	0.023	6
◇ RLYRAPSAPEKLDRK	22.1	92	0.241	62

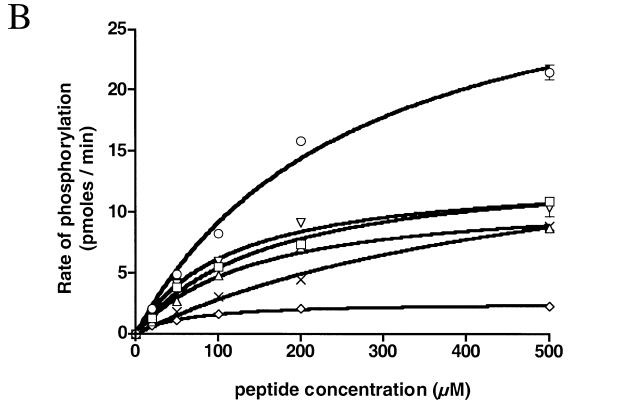
Fig. 3. Effect of substitution of residues in the *Xenopus* Cdc25C APS peptide on phosphorylation by Chk1. A: Alanine scan analysis. B: Comparison of phosphorylation kinetics.

all of the downstream residues were substituted by alanine was phosphorylated at 89% of the rate of the parent peptide, showing that residues C-terminal to the phospho-acceptor site do not play a strong role in substrate recognition by Chk1. A peptide in which the C-terminal Arg and Lys residues were swapped onto the N-terminal end of the molecule increased the rate of phosphorylation by 52%, indicating that N-terminal basic residues are positive determinants. More detailed kinetic experiments were performed on minimal peptide variants in which the positions of the upstream Leu and Arg residues were altered (Fig. 4B). Whereas movement of Arg^{-3} to the −4 or −2 positions resulted in 63% and 92% of the catalytic efficiency being retained, respectively, movement of Leu^{-5} and Arg^{-3} together relative to the phosphorylated Ser resulted in the reduction of the V_{max}/K_M to less than 35% of that of the minimal peptide. Together these data indicate a minimal consensus for recognition by Chk1 as Φ-X-β-X-X-(S/T)*, where * indicates the phosphorylated residue, Φ is a hydrophobic residue (M > I > L > V), β is a basic residue (R/K) and X is any amino acid.

3.5. *Serines 104 and 117 of S. pombe Wee1 are putative sites of phosphorylation by Chk1*

S. pombe Wee1 protein kinase is phosphorylated on one or





↓ Chk1	V_{max} (pmoles/min)	K_M (μM)	V_{max}/K_M (pmoles/min/μM)	% min
○ RLARAASMAAALARK	33.54	266.0	0.126	100.0
□ ALARAASMAAALARK	14.04	157.7	0.089	70.6
△ RLAARASMAAALARK	11.49	142.9	0.080	63.5
▽ RLRAAASMAAALARK	13.19	113.2	0.116	92.1
◇ RALARASMAAALARK	2.66	60.8	0.044	34.9
× LARAAASMAAALARK	18.15	534.6	0.034	27.0

Fig. 4. Comparison of minimal peptides as Chk1 substrates. A: Effect of substituting specific residues (underlined) on rate of phosphorylation: (*i*) APS peptide and minimal parent peptide; (*ii*) varying Leu-282; (*iii*) varying Arg-284; (*iv*) varying Leu-292; (*v*) substitution of residues 288–293 to Ala, and swapping C-terminal RK onto N-terminus. B: Effect of changing the position of upstream residues on the kinetics of phosphorylation.

more serine residues by Chk1 in vitro [11]. Examination of the contexts of all the serine residues present in *S. pombe* Wee1 revealed two sites, Ser-104 and Ser-117, which contained a basic residue at position −3 and a small hydrophobic residue at position −5. We found that two peptides containing these serine residues, PVPRRPS[104]LFDRPNRK and LVSRSSS[117]RLGDSPRK were phosphorylated at rates comparable to the APS peptide (Fig. 5). Substitution Ser-104 with Ala completely abolished phosphorylation by Chk1, confirming this residue as the site of phosphorylation. Substitution of Ser-117 with Ala reduced the rate of phosphorylation by 64%, indicating that this residue is a target for Chk1, although other residues in this peptide are also phosphorylated by Chk1.

3.6. Serine 343 of Xenopus Cdc25C is a good in vitro substrate for Chk1

Chk1 phosphorylates *Xenopus* Cdc25C on multiple sites, although only one of these, Ser-287, has been identified [7]. Inspection of the *Xenopus* Cdc25C sequence identified a potential Chk1 phosphorylation site, Ser-343, within the motif L-X-K-X-X-S*. This residue is located in a region of Cdc25C highly conserved between the *Xenopus* and human forms, and is equivalent to Ser-263 of human Cdc25C, shown to be a residue targeted by the kinase C-TAK1 [14]. The *Xenopus* Cdc25C-derived peptide SLKKTLS[343]LCDVDIRK was a good substrate for Chk1, with a phosphorylation rate 56% that of the APS peptide (Fig. 5). Phosphorylation of the peptide by Chk1 was abolished when Ser-343 was substituted with Ala, but 92% of the original rate was maintained when both Thr-341 and Ser-337 were substituted with Ala, confirming Ser-343 as the residue targeted by Chk1 in this peptide.

3.7. Chk1 can phosphorylate peptide substrates of related protein kinases

The requirement of Chk1 for upstream hydrophobic and basic residues is similar to that reported for mammalian AMP-activated protein kinase (AMPK), plant 3-hydroxy-3-methylglutaryl-CoA reductase kinase (HRK-A), SNF1 from *S. cerevisiae* and the Ca^{2+}/calmodulin-dependent protein kinase I (CaMKI) [15]. We therefore wished to determine whether Chk1 could phosphorylate two peptides used as substrates for these kinases, the 'SAMS' peptide, HMRSAMS[79]GLHLVKRR, derived from the sequence surrounding Ser-79 of rat liver acetyl-CoA carboxylase [16], and the 'AMARA' peptide, AMARAASAAALARRR, which contains the minimal consensus sequence [15]. The SAMS peptide was phosphorylated at a reasonable rate (33%) compared to the APS peptide (Fig. 5). A peptide with a Ser-79 to Ala substitution was not phosphorylated, confirming this residue as the target of Chk1. The AMARA peptide was a better substrate for Chk1 than the SAMS peptide, being phosphorylated at 92% of the rate of the APS peptide.

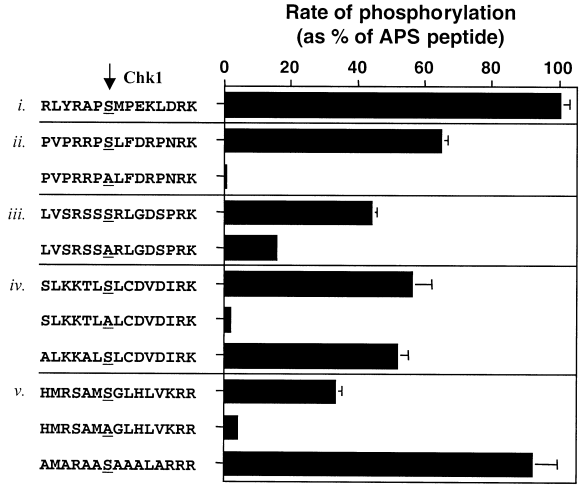


Fig. 5. Rate of phosphorylation of peptides predicted to be substrates of Chk1: (*i*) APS (*Xenopus* Cdc25C Ser-287) peptide; (*ii*) *S. pombe* Wee1 (Ser-104); (*iii*) *S. pombe* Wee1 (Ser-117); (*iv*) *Xenopus* Cdc25C (Ser-343); (*v*) SAMS [16] and AMARA [15] peptides.

4. Discussion

We have determined the substrate specificity of the checkpoint protein kinase Chk1 using a series of synthetic peptides derived from a known substrate, Cdc25C. We show that the most important determinants for substrate recognition by Chk1 are a hydrophobic residue at position −5 (M > I > L > V) and a basic residue (R > K) at position −3 relative to the phospho-acceptor site, which can be a serine or threonine residue. These data allow us to define the minimal consensus motif for phosphorylation by Chk1 phosphorylation as Φ-X-β-X-X-(S/T)*. This motif is consistent with the primary sequence context of known Chk1 sites in Cdc25 from different species (Fig. 1), which all show a conserved basic residue (Arg) in the−3 position, and a small hydrophobic residue (Leu or Val) in the −5 position. It seems likely therefore that the substrate specificity of Chk1 homologues from *S. pombe* to vertebrates is conserved.

Using these substrate specificity data, we have predicted likely additional sites targeted by Chk1 in *Xenopus* Cdc25C and *S. pombe* Wee1, and we have confirmed that peptides derived from these sites are indeed phosphorylated by Chk1. Database searches reveal many other proteins containing potential Chk1 phosphorylation sites. One of these, Pds1, is a protein involved in controlling the timing of anaphase that has been proposed recently to be a Chk1 substrate in *S. cerevisiae* [12]. Examination of the amino acid sequence reveals that Pds1 contains six sites conforming to the Chk1 motif that are putative sites of phosphorylation. Of course, the recognition motif for a protein kinase is only the minimal requirement for phosphorylation. Whether or not a protein is a substrate in cells may depend upon interactions with this motif and localisation to the same sub-cellular compartment as the kinase.

The Ser-343 site of *Xenopus* Cdc25C that we have identified as a putative target for Chk1 lies within a region of this phosphatase highly conserved between frogs and humans. The equivalent residue in human Cdc25C, Ser-263, is phosphorylated by C-TAK1 [14]. This kinase also phosphorylates Ser-216 [14], the major site phosphorylated by Chk1 [5] and Chk2 [17]. In *S. pombe*, Cds1 and Chk1 phosphorylate Cdc25 in an identical manner on three serine residues in vitro [18]. Taken together, these results suggest that the three kinases Chk1, Cds1 and C-TAK1 have very similar phosphorylation motifs.

The catalytic domains of Chk1, Cds1 and C-TAK1 is most closely related in sequence to the Ca^{2+}/calmodulin-dependent protein kinase (CaMK) group [19]. Other members of this

group, AMPK, SNF1 and CaMKI, similarly require N-terminal basic and hydrophobic residues, although they have a stronger preference for C-terminal hydrophobic residues than Chk1 [15,20]. Consistent with these related specificities, Chk1 phosphorylates two peptide substrates designed for AMPK. How then can the sequence specificities of these Ser/Thr protein kinases be rationalised, based on their structures? X-ray crystallographic studies of the catalytic domains of several protein kinases have identified residues which form the 'specificity pockets' that determine substrate recognition [21,22]. In the autoinhibited form of CaMKI [23], the active site of the kinase is occupied by a pseudosubstrate region (^{297}NFAKSKW*KQAFN^{308}) containing the Φ-X-β-X-X-(S/T)*-X-X-X-Φ motif found in substrates of this kinase [24], but with a non-phosphorylatable Trp residue in the phospho-acceptor position. There is an electrostatic interaction between Glu-102 in the catalytic domain and Lys-300 in the pseudosubstrate. A hydrophobic pocket formed by residues Phe-104, Ile-210 and Pro-216 accommodates a hydrophobic residue at position −5 in the pseudosubstrate. Sequence alignment shows that these contact-making residues are conserved in the catalytic domains of kinases which share similar phosphorylation motifs. In an analysis of 24 protein- Ser/Thr kinases, we find that the residue in the position equivalent to Glu-102 in CaMKI is acidic (either Glu or Asp) in kinases (including Chk1) which recognise an upstream basic residue in their substrates. Similarly, all three of the residues Phe-104, Ile-210 and Pro-216 are retained or conservatively substituted in those kinases (including Chk1) which require a hydrophobic residue at the −5 position. Both the Glu-102 equivalent residue and the hydrophobic pocket-forming triplet are also conserved in the kinases C-TAK1, Cds1 and Chk2, further suggesting that they share with Chk1 the Φ-X-β-X-X-S/T* substrate recognition motif.

While this group of protein-serine/threonine kinases have related catalytic domains with similar substrate specificities, they have distinct regulatory domains that respond to different cellular stresses, e.g. ATP depletion (AMPK), glucose starvation (SNF1), elevated cytoplasmic Ca^{2+} concentration (CaMKI), DNA damage (Chk1) or DNA replication arrest (Cds1/Chk2). While it remains to be determined if these kinases have common substrates in vivo, it may be interesting to examine the possible effects of stresses other than DNA damage or DNA replication arrest on the phosphorylation of Cdc25 and the timing of mitotic initiation. It may also be interesting to examine whether Chk1 can phosphorylate known substrates of these related kinases, which include metabolic enzymes and transcription factors [25].

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