The design of peptide-based substrates for the cdc2 protein kinase

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The substrate sequence specificity of the cdc2 protein kinase from *Pisaster ochraceus* has been evaluated. The peptide, Ac-Ser-Pro-Gly-Arg-Arg-Arg-Arg-Lys-amide, serves as an efficient cdc2 kinase substrate with a K_m of 1.50 ± 0.04 μM and a $V_{max.}$ of 12.00 ± 0.18 μmol/min per mg. The amino acid sequence of this peptide is not based on any sequence in a known protein substrate of the cyclin-dependent kinase, but rather was designed from structural attributes that appear to be important in the majority of cdc2 substrates. This cyclin-dependent enzyme is remarkably indiscriminate in its ability to recognize and phosphorylate peptides that contain an assortment of structurally diverse residues at the P−2, P−1 and P+2 positions. However, peptides that contain a free N-terminal serine or lack an arginine at the P+4 position are relatively poor substrates. These aspects of the substrate specificity of the cdc2 protein kinase are compared and contrasted with the previously reported substrate specificity of a cdc2-like protein kinase from bovine brain [Beaudette, Lew and Wang (1993) J. Biol. Chem. **268**, 20825–20830].

INTRODUCTION

The cdc2 protein kinase is the central control element that regulates the transition from the G_2 to M phase in the cell division cycle [1]. This serine/threonine-specific protein kinase is one of two subunits contained within maturation-promoting factor, a dimeric entity that achieved widespread prominence two decades ago for its ability to promote meiotic maturation in immature oocytes in the absence of hormonal stimulation [2,3]. The second component in maturation-promoting factor is a regulatory factor (cyclin) required for protein kinase activation. In the last few years, it has become evident that both the cdc2 protein kinase and its regulatory cyclin counterpart belong to separate yet burgeoning families of closely related proteins [4]. In animal cells, a series of cyclin-dependent kinases has now been described. These are activated at various stages in the cell division cycle. Similarly, there are several members of the cyclin family of regulatory proteins, the concentrations of which ebb and flow at specific points during the cell cycle.

The active cdc2–cyclin dimeric complex phosphorylates a variety of proteins [5], including the nuclear lamins, which reside on the inner membrane of the nuclear envelope [6–8]. Phosphorylation of these proteins is required for lamina disassembly, which in turn triggers nuclear envelope breakdown at mitosis. The cdc2–cyclin complex also phosphorylates histone H1, which is thought to promote chromosome condensation, an event that occurs at the onset of mitosis as well [9]. In addition, several other proteins serve (*in vivo* and/or *in vitro*) as substrates for the cyclin-dependent protein kinase, including $pp60^{c\text{-}src}$ [10], nucleolin [11], RNA polymerase II [12], p53 [13], protein synthesis elongation factors 1β and 1γ [14], the regulatory subunit of the cyclic AMP-dependent protein kinase [15], as well as the tyrosine phosphatase cdc25-C [16]. This list of protein substrates suggests that cyclin-dependent protein kinases serve as key enzymes responsible for implementing the underlying molecular events of the cell division cycle.

With a few exceptions, the amino acid sequence that encompasses the site of phosphorylation in cyclin-dependent protein kinase substrates generally conforms to S/T-P-X-Z, where X is a polar amino acid and Z is a basic amino acid residue [5]. Recently, this consensus sequence has been confirmed utilizing synthetic peptides for a cdc2-related protein kinase (a 33 kDa cdk2) isolated from bovine brain [17]. Almost simultaneously, a short report on the phosphorylation of synthetic vimentin peptides by starfish and mouse cdc2 kinases appeared [18]. The substrate specificity of a human cdc2 kinase has also been described [19]. Interestingly, several recent studies suggest that various cyclin-dependent kinases exhibit different substrate specificities [20–22]. Consequently, in order to address this issue, as well as to gain additional insight into the factors that control substrate recognition, we have undertaken a detailed analysis of the substrate specificity requirements of the cdc2 protein kinase isolated from *Pisaster ochraceus* (sea star).

MATERIALS AND METHODS

All chemicals were obtained from Aldrich, except for [γ-^{32}P]ATP (New England Nuclear), protected amino acid derivatives (Advanced Chemtech, Bachem and U.S. Biochemical) and Liquiscint (National Diagnostics).

cdc2 protein kinase

The cdc2 protein kinase–cyclin B dimeric complex was purchased from Upstate Biotechnology. The enzyme–cyclin complex was isolated from *P. ochraceus* via sequential chromatography on hydroxyapatite, Cellex-P, $p13^{suc1}$ and Mono S as described previously [23]. The purified cdc2 complex is more than 95% homogeneous.

Peptide synthesis

Peptides were prepared on the benzhydrylamine resin with *t*-Boc amino acids using Merrifield's solid-phase methodology [24]. A solution of 90% HF/10% anisole was used to cleave the peptides from the resin. All of the crude peptides were subsequently partially purified via ion-exchange chromatography on SP-Sephadex C-25 {0.4–1.0 M KCl gradient in 50 mM potassium acetate, pH 3.5, on a preparative HPLC system using three Waters radial-compression modules (25 cm × 10 cm) connected in series [gradient (solvent A, 0.1% trifluoroacetic acid in water; solvent B, 0.1% trifluoroacetic acid in acetonitrile): 0–3 min (100% solvent A); a linear gradient from 3 min (100% solvent A) to 30 min (75% solvent A and 25% solvent B); a steep final

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linear gradient to 90 % solvent B for column-cleansing purposes]}. All peptides gave satisfactory fast-atom-bombardment mass-spectral analyses.

Assay of cdc2 protein kinase

Assays were performed in triplicate at pH 7.4 and thermostatically controlled in a water bath maintained at 30 °C. The final assay volume was 50 μl and contained 50 mM Tris/HCl, 12.5 mM $MgCl_2$, 0.125 mg/ml BSA, 62.5 mM β-glycerophosphate, 1 mM dithiothreitol and 100 μM [γ-^{32}P]ATP (200–400 c.p.m./pmol). Phosphorylation reactions were initiated by the addition of 10 μl of the protein kinase from a stock solution (0.067 ng/μl in 50 mM Tris/HCl, 12.5 mM $MgCl_2$, 0.125 mg/ml BSA, 62.5 mM β-glycerophosphate, 1 mM dithiothreitol and 500 μM ATP, at pH 7.4). Reactions were terminated after 5 min by spotting 25 μl aliquots on to 2.1 cm-diameter phosphocellulose paper disks. After 10 s the disks were immersed in 10 % acetic acid and allowed to soak with occasional stirring for at least 1 h. The acetic acid was decanted and the disks were collectively washed with 4 vol. of 0.5 % H_3PO_4, 1 vol. of water, followed by a final acetone rinse. The disks were air-dried and placed in plastic scintillation vials containing 6 ml of Liquiscint before scintillation counting for radioactivity.

Determination of kinetic constants

The apparent K_m (±S.D.) and $V_{max.}$ (±S.D.) values for all peptides were determined from initial-rate experiments that included five or six different substrate concentrations over a ten-fold range encompassing the K_m. The data from these experiments were plotted using the Lineweaver–Burk procedure, and the corresponding plots proved to be linear in all cases.

RESULTS AND DISCUSSION

The cdc2 protein kinase phosphorylates a wide variety of proteins, both *in vitro* and *in vivo* [5–16]. A representative list of sequences that are recognized as sites of phosphorylation in intact proteins is provided in Table 1. Based on these studies, S/T-P-X-Z (where Z is typically a basic residue) has been proposed to be the minimal sequence necessary for substrate recognition [5]. However, even this rudimentary primary sequence is not found in all of the sites demonstrated to serve as cdc2 kinase substrates. For example, both vimentin and desmin are phosphorylated at serine residues that do not precede a proline moiety [27,28]. In addition, several of the sequences identified in Table 1 do not contain arginine or lysine residues on the C-terminal side of the residue that serves as the site of phosphorylation. Nevertheless, the majority of sequences phosphorylated by the cdc2 protein kinase possess the Ser-Pro dyad followed by one or more basic amino acid residues. With these features in mind, we have designed a series of cdc2 peptide substrates utilizing a strategy that differs from the large number of previously described studies that have focused on the creation of peptide-based protein kinase substrates. Typically, short sequences surrounding the site of phosphorylation in protein substrates are synthesized and then evaluated as protein kinase substrates in their own right. As the sequences that undergo cdc2 protein kinase-catalysed phosphorylation differ dramatically from one protein substrate to the next, we decided to design a potential peptide substrate based on the structural attributes that appear to be important in the majority of cdc-2 recognition sites. The target peptide evolved from a starting Ser-Pro sequence. We used serine rather than its secondary-alcohol-containing counterpart, threonine, as protein kinases generally appear to phosphorylate the former much more effectively than the latter [30,31].

Table 1 Phosphorylation sites in known protein substrates of the cdc2 protein kinase

Substrate	Sequence	Reference
Histone H1	K/R-S/T-P-X-K	9
pp60$^{c\text{-}src}$	S-Q-T-P-N-K-T	–
	H-R-T-P-S-R-S	10
	V-T-S-P-Q-R-S	
Nucleolin	T-P-A/G-K-K	11
p53	S-S-S-P-Q-P-K-K-K	13
c-abl	P-D-T-P-E-L-L-H	25
	A-V-S-P-L-L-P-R	
Lamin C	R-L-S-P-S-P-T-S-Q-R	26
Vimentin	L-G-S-A-L-R-R-R	27
Desmin	Y-S-S-S-Q-R-R-R	28
SV40 large T	H-A-T-P-P-K-K-R	29

Ser-Pro-Gly

Owing to the well-known physical constraints imposed by the cyclic ring system of the proline moiety, this residue is often found at the $i+1$ position of a β-turn [32]. Consequently, it is possible that a reverse turn is a critical structural feature required for recognition by the cdc2 kinase. Therefore we incorporated a glycine residue adjacent to the proline moiety, as glycine at this position is especially prevalent in β-turns [32].

Ser-Pro-Gly-Arg-Arg-Arg-Arg

Because of the presence of basic residues on the C-terminal side of the Ser-Pro dyad in many of the sequences illustrated in Table 1, we positioned a contiguous series of four basic residues adjacent to the glycine moiety. Although these could be either lysine or arginine residues, we chose the latter as arginine appears to enhance substrate efficacy to a much greater extent than lysine in peptide substrates developed for other protein kinases, including the cyclic AMP-dependent protein kinase [33].

Ac-Ser-Pro-Gly-Arg-Arg-Arg-Arg

We recently differentiated between two forms of protein kinase substrate specificity: (i) sequence specificity and (ii) active-site specificity [34,35]. The latter refers to the ability of a protein kinase to recognize and phosphorylate various hydroxyl-bearing residues, differing in both steric and stereochemical features. For reasons outlined previously, an active-site substrate-specificity study requires that the phosphorylatable residue reside at either the N- or C-terminus [34]. Consequently, we made the strategic decision to retain serine as the N-terminal amino acid. However, it is likely that a free (i.e. positively charged) N-terminal serine so closely positioned to a presumably hydrophobic active site would have deleterious consequences with respect to substrate efficiency. In short, the peptide should prove to be a more efficient substrate if it were N-acetylated.

Ac-Ser-Pro-Gly-Arg-Arg-Arg-Arg-Lys-amide (peptide 1)

N-acetylation precludes the ready quantification of peptide concentration by the ninhydrin method. Consequently, we inserted a lysine moiety at the P+6 position to overcome this minor inconvenience. In addition, this peptide, as well as all of

Table 2 K_m, $V_{max.}$ and $k_{cat.}/K_m$ values for peptides that differ at the P−1 position

Kinetic constants were determined as described in the Materials and methods section. Values are given as the mean ± S.D.

	Peptide	K_m (μM)	$V_{max.}$ (μmol · min^{-1} · mg^{-1})	$k_{cat.}/K_m$ (min^{-1} · μM^{-1})
1	Ac-Ser-Pro-Gly-Arg-Arg-Arg-Arg-Lys-amide	1.5 ± 0.1	12.0 ± 0.2	272 ± 2
2	Ac-**Arg**-Ser-Pro-Gly-Arg-Arg-Arg-Arg-Lys-amide	1.9 ± 0.1	10.3 ± 0.3	186 ± 2
3	Ac-**Gly**-Ser-Pro-Gly-Arg-Arg-Arg-Arg-Lys-amide	7.9 ± 0.8	12.3 ± 0.6	53 ± 4
4	Ac-**Asp**-Ser-Pro-Gly-Arg-Arg-Arg-Arg-Lys-amide	16.2 ± 3.5	12.3 ± 1.4	26 ± 2
5	Ac-**Asn**-Ser-Pro-Gly-Arg-Arg-Arg-Arg-Lys-amide	14.8 ± 2.8	16.5 ± 2.5	38 ± 2
6	Ac-**Pro**-Ser-Pro-Gly-Arg-Arg-Arg-Arg-Lys-amide	9.2 ± 0.6	8.6 ± 0.2	32 ± 1

the others described in this study, contained an amide-capped C-terminus. As is apparent from Table 2, peptide **1** is an outstanding synthetic substrate for the cdc2 protein kinase, with a K_m in the low micromolar range.

Recently, peptide libraries have been used to assess kinase substrate specificity [36]. This provides a means of rapidly identifying a peptide substrate for a target enzyme. One disadvantage of the combinatorial method is that it is not possible to extract kinetic parameters from peptide mixtures. In short, the contribution of a specific amino acid residue to the efficacy of a particular substrate can only be inferred in a qualitative fashion. In marked contrast, it is simply not practical to synthesize, purify and assay a series of peptides in which each of the 20 standard amino acids has been inserted into every position on the peptide. In order to overcome these inherent difficulties, we have developed a strategy that allows us to quantitatively assess the structural class of residue most readily accommodated at a specific site on the substrate, without resorting to the preparation of a vast array of peptides. This is illustrated by the approach that we have taken to investigate the effect of residues at the P−1 position on phosphorylation kinetics. With peptide **1** as the parent compound, we analysed the activity of five P−1 analogues (**2–6**). These contain a positively charged residue (peptide **2**), a non-polar residue (peptide **3**), a negatively charged residue (peptide **4**), a residue that can serve as a hydrogen bond donor/acceptor (peptide **5**) and a proline moiety (peptide **6**) at the P−1 site. Interestingly, all proved to exhibit slightly higher K_m values than peptide **1**. Of the five structurally disparate residues examined, arginine (i.e. peptide **2**) is apparently the most readily tolerated at the P−1 site, displaying a K_m value that is just slightly higher than that exhibited by peptide **1**. In contrast, an aspartic acid or asparagine residue at P−1 provides peptides with K_m values an order of magnitude higher than that of peptide **1**. Although the K_m values in this series exhibit some variability, the $V_{max.}$ values displayed by these peptides are remarkably constant, implying that the rate-determining step is identical for these substrates.

We employed a strategy identical with that described above to investigate the effect of residues at the P−2 position on phosphorylation kinetics (Table 3). Peptides containing a positively charged residue (**7**), a non-polar residue (**8**), a negatively charged residue (**9**), a residue that can act as a hydrogen bond donor/acceptor (**10**) and a proline moiety (**11**) at the P−2 site were synthesized. All of these peptides possess an arginine at P−1, as this appears to be the residue most readily tolerated at this position (Table 2). Once again, the K_m values associated with this series of peptides are slightly higher than that exhibited by the corresponding truncated analogue (i.e. peptide **2**). As with the P−1 site, an aspartic acid residue at P−2 produces the greatest increase in K_m. Furthermore, the maximal velocities associated with the cdc2 kinase-catalysed phosphorylation of the peptides listed in both Tables 2 and 3 are relatively constant. However, whereas arginine is clearly favoured at P−1, proline earns this distinction at P−2. The results for peptides **1–11** are intriguing in the light of the fact that the majority of peptide substrates that have been described for protein kinases contain the phosphorylatable residue at a non-terminal position within the peptide chain. In other words, protein kinases appear to interact favourably with residues on both sides of the phosphorylation site. Not surprisingly, peptides containing a serine/threonine/tyrosine moiety at a terminal position are generally poorer substrates than their untruncated counterparts [34,37,38]. In marked contrast, the cdc2 protein kinase does not appear to interact with residues positioned on the N-terminal side of the phosphorylatable residue. Indeed, in terms of $k_{cat.}/K_m$, peptide **1** is a superior substrate to all of its N-terminally substituted analogues. However, it is important to bear in mind that we have limited our survey to only five distinct types of

Table 3 K_m, $V_{max.}$ and $k_{cat.}/K_m$ values for peptides that differ at the P−2 position

Kinetic constants were determined as described in the Materials and methods section. Values are given as the mean ± S.D.

	Peptide	K_m (μM)	$V_{max.}$ (μmol · min^{-1} · mg^{-1})	$k_{cat.}/K_m$ (min^{-1} · μM^{-1})
2	Ac-Arg-Ser-Pro-Gly-Arg-Arg-Arg-Arg-Lys-amide	1.9 ± 0.1	10.3 ± 0.3	186 ± 2
7	Ac-**Arg**-Arg-Ser-Pro-Gly-Arg-Arg-Arg-Arg-Lys-amide	5.2 ± 1.0	7.4 ± 0.8	48 ± 5
8	Ac-**Gly**-Arg-Ser-Pro-Gly-Arg-Arg-Arg-Arg-Lys-amide	6.1 ± 0.1	10.3 ± 0.2	58 ± 1
9	Ac-**Asp**-Arg-Ser-Pro-Gly-Arg-Arg-Arg-Arg-Lys-amide	7.9 ± 0.7	7.9 ± 2.7	34 ± 1
10	Ac-**Asn**-Arg-Ser-Pro-Gly-Arg-Arg-Arg-Arg-Lys-amide	5.6 ± 0.7	9.1 ± 0.6	56 ± 3
11	Ac-**Pro**-Arg-Ser-Pro-Gly-Arg-Arg-Arg-Arg-Lys-amide	2.6 ± 0.3	11.0 ± 0.7	144 ± 9

Table 4 K_m, $V_{max.}$ and $k_{cat.}/K_m$ values for peptides that differ at the P+2 position

Kinetic constants were determined as described in the Materials and methods section. Values are given as the mean ± S.D.

	Peptide	K_m (μM)	$V_{max.}$ (μmol · min^{-1} · mg^{-1})	$k_{cat.}/K_m$ (min^{-1} · μM^{-1})
1	Ac-Ser-Pro-**Gly**-Arg-Arg-Arg-Arg-Lys-amide	1.5 ± 0.1	12.0 ± 0.2	272 ± 2
12	Ac-Ser-Pro-**Pro**-Arg-Arg-Arg-Arg-Lys-amide	5.0 ± 0.8	13.3 ± 1.1	91 ± 5
13	Ac-Ser-Pro-**Phe**-Arg-Arg-Arg-Arg-Lys-amide	5.2 ± 0.1	8.8 ± 0.1	58 ± 2
14	Ac-Ser-Pro-**Arg**-Arg-Arg-Arg-Arg-Lys-amide	2.2 ± 0.1	9.7 ± 0.2	151 ± 1
15	Ac-Ser-Pro-**Asp**-Arg-Arg-Arg-Arg-Lys-amide	6.0 ± 2.0	5.5 ± 1.1	31 ± 5
16	Ac-Ser-Pro-**Asn**-Arg-Arg-Arg-Arg Lys-amide	7.0 ± 1.4	10.8 ± 1.3	53 ± 4

Table 5 K_m, $V_{max.}$ and $k_{cat.}/K_m$ values for peptides 12 and 17–21

Kinetic constants were determined as described in the Materials and methods section. Values given as the mean ± SD.

	Peptide	K_m (μM)	$V_{max.}$ (μmol · min^{-1} · mg^{-1})	$k_{cat.}/K_m$ (min^{-1} · μM^{-1})
12	Ac-Ser-Pro-Pro-Arg-Arg-Arg-Arg-Lys-amide	5.0 ± 0.8	13.3 ± 1.1	91 ± 5
17	Ac-Ser-Pro-Pro-**Ala**-Arg-Arg-Arg-Lys-amide	6.3 ± 0.3	8.8 ± 0.2	48 ± 2
18	Ac-Ser-Pro-Pro-Arg-**Ala**-Arg-Arg-Lys-amide	44.7 ± 4.2	5.4 ± 0.3	4.1 ± 0.2
19	Ac-Ser-Pro-Pro-Arg-Arg-**Ala**-Arg-Lys-amide	6.2 ± 0.5	7.7 ± 0.1	42 ± 3
20	Ac-Ser-Pro-Pro-Arg-Arg-Arg-**Ala**-Lys-amide	4.0 ± 0.2	5.7 ± 0.1	49 ± 3
21	Ac-Ser-Pro-Pro-**Ala**-Arg-**Ala**-**Ala**-Lys-amide	3500*	1.5*	

* Since this peptide is a poor substrate, we were unable to achieve the requisite peptide concentrations to obtain an accurate assessment of K_m and $V_{max.}$

amino acid residues. For example, it is possible that a hydrophobic residue or one that bears a hydroxyl moiety may enhance substrate efficacy when positioned at P−1 or P−2.

We next turned our attention to the effect on phosphorylation kinetics by residues on the C-terminal side of the phosphorylatable serine moiety. As noted above, a proline at P+1 is thought to be extremely important for substrate recognition by the cdc2 protein kinase. As a consequence, we decided not to explore the ramifications of proline replacement on substrate efficacy. The effect of residues at the P+2 position on phosphorylation kinetics is illustrated in Table 4. Peptide **12** contains the Pro-Pro dyad present in the sequence adjacent to the phosphorylation site in SV40 large T antigen [29]. Although the $V_{max.}$ associated with the phosphorylation of peptide **12** is nearly identical with that exhibited by peptide **1**, the K_m is 3-fold higher. From the series of P+2 variants displayed in Table 4, it is evident that glycine is favoured at this position, followed closely by arginine. However, it is also evident that a variety of amino acid residues, differing in both charge and size, are relatively well tolerated at this site.

We explored the importance of positively charged residues at P+3 through P+6 by sequentially replacing the arginine residues at these sites with alanine. In these series of experiments, peptide **12** served as the archetype peptide substrate. In all cases, the resulting peptides (i.e. **17–20**; Table 5) are phosphorylated nearly as rapidly as the parent peptide **12**. Indeed, in the worse case, no more than a 60% reduction in $V_{max.}$ relative to that of peptide **12** is observed. However, replacement of the arginine at the P+4 position with an alanine (i.e. peptide **18**) does produce a cdc2 protein kinase substrate that is significantly poorer than peptide **12**. In this case, the K_m is an order of magnitude larger than that for peptide **12**. In contrast, there is only a 25% increase in K_m with peptides **17** and **19**, and no increase at all with peptide **20**. One interpretation of these results is that positively charged residues at P+3, P+5 and P+6 only marginally contribute to substrate efficacy, whereas the arginine at P+4 is much more critical. In order to test the validity of this supposition, we prepared peptide **21**, which contains a single arginine residue at the putatively hypersensitive P+4 site. Much to our surprise, we found that this peptide is an extraordinarily inefficient cdc2 protein kinase substrate. On the basis of these results, we conclude that an arginine at P+4 significantly enhances substrate efficacy, but a single arginine at this position is not sufficient for substrate recognition. However, it is important to note that these comments only apply to peptides of the sequences provided in Table 5. From Table 1 it is evident that certain sites in intact proteins undergo cdc2 protein kinase-catalysed phosphorylation even in the absence of arginine or lysine residues on the C-terminal side of the phosphorylation site.

Finally, we tested the notion that the presence of a free N-terminal serine would have deleterious consequences on the kinetics of phosphorylation. Indeed, the N-acetylated peptide **12** exhibits a K_m (5.0 ± 0.8 μM) that is sixfold lower than that obtained for the non-acetylated analogue, H_2N-Ser-Pro-Pro-Arg-Arg-Arg-Arg-Lys-NH_2 (31.8 ± 2.6 μM). In addition, the $V_{max.}$ for the free N-terminal peptide (4.8 ± 0.2 μmol · min^{-1} · mg^{-1}) is less than half that exhibited by its acetylated counterpart (13.3 ± 1.1 μmol · min^{-1} · mg^{-1}).

Wang and his co-workers recently evaluated the sequence substrate specificity of cdc2-like protein kinase isolated from bovine brain [17]. Several similarities associated with the substrate-sequence-dependence of the cdc2-like protein kinase and the cdc2 protein kinase evaluated here are apparent. First, a free N-terminus at the phosphorylatable residue (compare peptides **12** and H_2N-Ser-Pro-Pro-Arg-Arg-Arg-Arg-Lys-NH_2) has a deleterious effect on K_m values for substrates of both enzymes. Secondly, both enzymes prefer peptide substrates that bear basic residues on the C-terminal side of the site of

phosphorylation. Third, the maximal velocities associated with both the cdc2 and cdc2-like kinase-catalysed phosphorylations are relatively invariant. Indeed, although the peptides described here contain significant structural and electronic differences, the $V_{max.}$ values associated with these substrates exhibit just over 3-fold variability overall. However, the cdc2 protein kinase examined by us and the cdc2-like protein kinase investigated by Wang and co-workers do appear to exhibit at least two intriguing differences in their substrate specificities. Whereas both enzymes display a preference for sequences that contain a cluster of basic residues on the C-terminal side of the phosphorylatable residue, the cdc2-like protein kinase from bovine brain is especially sensitive to the loss of a basic residue at the P+3 position. In contrast, the cdc2 kinase from *P. ochraceus* exhibits, at most, a minor dependence on a basic residue at this position (compare peptides **12** and **17**). There is, however, a notable increase in K_m in the absence of a basic residue at P+4 (compare peptides **12** and **18**). More significantly, the cdc2-like protein kinase does not tolerate an aspartic acid residue at the P+2 position (the K_m increases by two orders of magnitude). However, the insertion of an aspartic acid residue at P+2 for a cdc2 protein kinase substrate (i.e. peptide **15**) produces a negligible effect on substrate efficacy. Although these observations are striking, they are tempered by the fact that the primary sequences of the substrates employed by Wang and co-workers and by us differ to some extent. Consequently, any conclusions drawn from these comparisons must await full verification using identical peptide substrates for both enzymes.

In summary, we have examined the substrate specificity of the cdc2 protein kinase. The primary sequence in the lead substrate (i.e. peptide **1**) is not based on any naturally occurring sequence present in a known substrate of the enzyme, but was designed from structural attributes that are common to the majority of cdc2-recognition sites. To the best of our knowledge, peptide **1** is the most efficient substrate ever reported for the cdc2 protein kinase. Perhaps the most surprising aspect of cdc2 protein kinase substrate specificity is that residues on the N-terminal side of the phosphorylation site decrease substrate efficacy. The ability of the cdc2 protein kinase to catalyse peptide substrates is most severely compromised by (1) the presence of a free N-terminal serine and (2) the absence of an arginine at P+4. Finally, with efficient substrates such as Ac-Ser-Pro-Gly-Arg-Arg-Arg-Arg-Lys-amide in hand, it is now possible to consider evaluating the range of alcohol-bearing residues that can be both accommodated within the active site and phosphorylated by the enzyme [34,35]. As noted by others [17,18], substrate-specificity studies such as these may ultimately lead to species capable of discriminating between members of the cyclin-dependent family of protein kinases.

Note added in proof (received 9 June 1995)

A peptide library was recently employed to assess the sequence-specificity of serine/threonine-specific kinases, including cyclin-dependent protein kinases [39].

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