J. Biochem. 122, 409-414 (1997)

Role of the Pyrrolidine Ring of Proline in Determining the Substrate Specificity of cdc2 Kinase or cdk5[1]

Shoji Ando,*,[2] Toshihiko Ikuhara,† Tomoko Kamata,‡ Yasuharu Sasaki,† Shin-ichi Hisanaga,§ Takeo Kishimoto,§ Hikaru Ito,‖ and Masaki Inagaki‖

**Laboratory of Chemistry, Saga Medical School, Saga 849; †Life Science Research Center and ‡Analytical Research and Computer Science Center, Asahi Chemical Industry Co., Ltd., Fuji 416; §Laboratory of Cell and Developmental Biology, Faculty of Bioscience, Tokyo Institute of Technology, Yokohama 227; and ‖Laboratory of Biochemistry, Aichi Cancer Center Research Institute, Nagoya 464*

Received for publication, April 7, 1997

To examine structural features of proline which are essential for the proline-directed phosphorylation by cdc2 kinase or cdk5, we prepared the peptide representing the cdc2 kinase phosphorylation site at Ser-55 in vimentin [Ser-Leu-Tyr-Ser-Ser-Ser^{55}-Pro^{56}-Gly-Gly^{58}-Ala-Tyr-NH_2], the peptide containing arginine in place of Gly-58, and their derivatives containing various *N*-methylamino acids or proline homologs in place of Pro-56, and tested them as substrates for the kinases. While substitution of the proline by proline homologs (L-pipecolic acid or L-azetidine-2-carboxylic acid) increased the K_m value 2- to 4-fold at utmost, substitution by *N*-methylamino acids (sarcosine, L-*N*-methylalanine, L-*N*-methylvaline, or L-*N*-methylleucine) increased the K_m value 7- to 40-fold for cdc2 kinase. For cdk5, these substitutions led to parallel effects on the K_m value to those found for cdc2 kinase; cdk5 recognized the peptides with a proline specificity similar to that for cdc2 kinase. These results suggest that the pyrrolidine ring of proline is important for substrate recognition by cdc2 kinase or cdk5. Molecular dynamics and molecular mechanics simulations indicated that the pyrrolidine ring of proline is optimal to stabilize a β-turn at the phosphorylation site and that the K_m values of the peptides for the enzymes might be related to the probability of the turn structure. The results obtained here also suggest that the pyrrolidine ring of proline is required to maintain a high V_{max} value for cdc2 kinase or especially for cdk5. These will aid in designing specific substrates or inhibitors for cdc2 kinase or cdk5.

Key words: cdc2 kinase, cdk 5, molecular dynamics and molecular mechanics simulations, peptide synthesis, phosphorylation.

cdc2 kinase is a Ser/Thr protein kinase which plays a pivotal role in controlling the cell cycle of all eukaryotic cells (*1*). The kinase is composed of a catalytic component p34^{cdc2} and a regulatory subunit cyclin essential for the kinase activity. In mammalian cells, p34^{cdc2}-related proteins which are closely related in size (35-40 kDa) and sequence (>40% identity) have been described. They have been referred to as cyclin-dependent kinases (cdks) and are designated individually as cdk1 (=p34^{cdc2}) to cdk8 by number (*2-5*). Cyclins are now defined as a family of structurally related proteins, and are categorized into subfamilies from A to H (*6, 7*). The transient activation of cdk-cyclin complexes drives cell cycle events; cdc2 kinase in a complex with cyclin B (p34^{cdc2}/cyclin B) promotes entry into mitosis (*3*), cdk4/cyclin D, and cdk6/cyclin D are involved in G1/S transition (*2*), and cdk2/cyclin E and cdk2/cyclin A in DNA replication (*8*).

Some cdks were found in tissues and cells where proliferative activity is very low, suggesting that cdks may not all be cell cycle regulators. A cdc2-like kinase (*9*) and tau protein kinase II (*10*) were purified from bovine brain. The catalytic subunit of these kinases has now been shown to be identical to cdk5, based on their amino acid sequences (*11-13*), and to be associated with a 23-25-kDa polypeptide (*9, 10*), which was shown to be a proteolytic fragment of the 35-kDa non-cyclin type activating subunit (*14-16*). The cdk5 has been implicated in the regulation of neurocytoskeleton dynamics and organization (*17*).

Cdks catalyze proline-directed phosphorylation. Analysis of *in vivo* and *in vitro* phosphorylation sites of various proteins for cdc2 kinase in a complex with cyclin B has suggested that the kinase preferentially phosphorylates a Ser/Thr site which is followed by an adjacent proline, with or without a basic amino acid at the third residue (Ser/Thr-Pro-*Z*-Arg/Lys or Ser/Thr-Pro) (*18-22*). Systematic studies using synthetic peptides representing the phos-

[1] This research was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science, Sports and Culture of Japan.

[2] To whom correspondence should be addressed. Tel: +81-952-34-2192, Fax: +81-952-34-2022, E-mail: andoh-s@smsnet.saga-med.ac.jp

Abbreviations: Aze, azetidine-2-carboxylic acid; Boc, *t*-butyloxycarbonyl; cdk, cyclin-dependent protein kinase; MeAla, *N*-methylalanine; MeLeu, *N*-methylleucine; MeVal, *N*-methylvaline; MD and MM simulations, molecular dynamics and molecular mechanics simulations; Pip, pipecolic acid or piperidine-2-carboxylic acid; *Pt*, probability of β-turn; Sar, sarcosine or *N*-methylglycine; Vim(50-60), peptide fragment of vimentin consisting of positions 50-60.

phorylation sites have shown that proline acts as the primary substrate specificity determinant for cdc2 kinase and that the basic residue on the carboxyl-terminal side of the site enhances the site recognition (*23-26*). Similar sequence specificity has been observed for cdk2 associated with cyclin A (*26*). Cdk5 purified from brain phosphorylates neurofilament M, H, and tau proteins at multiple sites which are identical to sites phosphorylated *in vitro* by cdc2 kinase (*12, 27, 28*). It was, however, observed that cdk5 phosphorylates more preferentially the sequence Ser/Thr-Pro-*Z*-Arg/Lys over the sequence Ser/Thr-Pro rather than does cdc2 kinase (*24, 28*), suggesting that cdk5 has a stronger dependence on basic amino acid residues on the carboxyl-terminal side of the site.

Charged amino acids around the site are assumed to interact as determinants with oppositely charged residues in the catalytic site of a protein kinase, as observed in the complex between the catalytic subunit of cAMP-dependent protein kinase and its inhibitory peptide (*29*). However, the molecular basis of the proline-directed phosphorylation by cyclin-dependent protein kinases has remained unknown. In this respect, we reported that a proline in a peptide substrate for cdc2 kinase or cdk5 can be replaced partly by sarcosine (*24*), a finding which suggested that the *N*-substituted structure of proline is important for substrate recognition by the kinases. To obtain more information on the structural factors of proline required for the phosphorylation, the peptides representing the *in vivo* and *in vitro* phosphorylation site in vimentin (*19-22*) and containing various *N*-methylamino acids or proline homologs in place of proline were prepared in this study, and tested as substrates for cdc2 kinase or cdk5. The kinetic data, together with molecular dynamics (MD) and molecular mechanics (MM) simulations, substantiated the importance of the 5-membered (pyrrolidine) ring structure of proline in the substrate specificity of cdc2 kinase or cdk5, as well as highlighting differences in specificity between the kinases.

EXPERIMENTAL PROCEDURES

Preparation of Protein Kinases—cdc2 kinase in a complex with cyclin B was prepared from mouse FM3A mammary tumor cells at the logarithmic growth phase by the method of Kusubata *et al.* (*21, 24*). Cdk5 in a complex with the 23-kDa regulatory subunit was prepared from the microtubule fraction of porcine brain by the method of Hisanaga *et al.* (*28, 30*). Protein concentrations were measured according to Bradford (*31*) with bovine serum albumin as standard.

Peptide Synthesis and Purification—Amino acids except glycine and sarcosine used in this study are all in L-form. Azetidine-2-carboxylic acid and pipecolic acid were purchased from Sigma (St. Louis, MO) and derivatized into the Boc-amino acids by the conventional method. Other Boc-amino acid derivatives were from Kokusan Kagaku (Tokyo). Peptides were synthesized as the carboxyl-terminal amide using 4-methylbenzhydrylamine-resin on an Applied Biosystems 431A peptide synthesizer. Coupling reactions of the imino acids and the next serine to the growing peptide chain were carried out at least twice. After deprotection and cleavage from the resin with 1 M trifluoromethanesulfonic acid/trifluoroacetic acid, peptides were purified by gel chromatography on Sephadex G-25 and then by reversed-phase HPLC. Identity of the peptides was confirmed by amino acid analysis, gas-phase sequencing and fast atom bombardment mass spectrum measurement. Concentration of the peptide solution was determined by amino acid analysis.

Phosphorylation of Synthetic Peptides—Phosphotransferase assays were performed for 5 min at 25°C in a reaction mixture with a final volume 50 μl containing 25 mM Tris-HCl buffer (pH 7.5), 3 mM $MgCl_2$, 0.1 mM [γ-^{32}P]ATP, peptide substrate, and 0.4 μg/ml of cdc2 kinase or cdk5. For determination of kinetic constants, each peptide was used over a 15-fold range of concentration around the apparent K_m value. All reactions were initiated by addition of ATP, and terminated by addition of 30% acetic acid. Phosphopeptide was separated from [γ-^{32}P]-ATP by ion exchange chromatography on AG 1-X8 (acetate form) and counted as described by Kemp *et al.* (*32*). To determine kinetic constant values, Lineweaver-Burk plots were prepared. All experiments were repeated at least twice.

Analysis of Phosphorylation Site—Phosphopeptides were isolated from assay mixtures using reversed-phase HPLC. The peptides were treated with ethanethiol in an alkaline condition to convert a phosphorylated serine into an *S*-ethylcysteine, the location of which was determined by gas-phase sequencing, as described (*33, 34*).

Conformational Analysis—Molecular dynamics (MD) and molecular mechanics (MM) simulations were performed to analyze the structures of peptides. Calculations were done using the Consistent Valence Force Field (CVFF) of the program, Discover (MSI, San Diego, CA), on a Silicon Graphics IRIS INDIGO/R3000 workstation. Simulated systems comprised tetrapeptides, acetylseryl-*X*-glycyl-arginylmethane (Ac-Ser-*X*-Gly-Arg-CH_3) in which *X* is proline, pipecolic acid, *N*-methylleucine, or sarcosine. Initial structures for each peptide were constructed using the standard fragments prepared in INSIGHT-II (MSI, San Diego, CA). To obtain various structures, high temperature MD calculations were carried out. A dielectric constant of 80 was used to minimize long-range Coulomb interactions that are overemphasized in vacuum simulations. The time step used for the integration of the dynamic equation was 1 fs. Pre-run of 20 ps was performed at 300 K, followed by simulations with a duration 20 ps at 600 K and 900 K. Total run time at a temperature of 900 K was 120 ps. The structures were sampled over 100 ps every 1 ps and, hence, totally 100 structures were generated for each initial structure. Finally, full optimization of the sampled structures was carried out from the energetic point of view.

Probability of β-turn structures, *Pt* (%), was estimated with an assumption that the Boltzmann distribution is held:

$$Pt(\%) = \frac{\sum_{\text{all}} \exp(-E_j/k_B T)}{\sum_{i=1} \exp(-E_i/k_B T)} \times 100$$

where k_B is the Boltzmann constant, T is temperature, and E_j stands for the energy of conformations leading to β-turn structures. Structures possessing appropriate set of backbone dihedral angles (ϕ, ψ) ranging ($-90°$ to $-30°$, $-60°$ to $0°$) or ($-90°$ to $-30°$, $+90°$ to $+150°$) at the $i+1$-th residue were adopted as β-turns (*35*).

RESULTS

Preparation of Peptides—Structures of the peptides synthesized in this study are shown in Fig. 1. The peptide Vim(50-60) represents the amino acid sequence around the *in vivo* and *in vitro* phosphorylation site, Ser-55, of vimentin by cdc2 kinase (*19-22*). Glycine-58 was replaced by arginine in [Arg^{58}]Vim(50-60); otherwise cdk5 [which critically requires a basic amino acid at the third residue on the carboxyl-terminal side of the site (*24, 28*)] does not phosphorylate Ser-55 in vimentin. To evaluate the significance of the ring structure of Pro-56, this residue was replaced by sarcosine (Sar), *N*-methylalanine (MeAla), *N*-methylvaline (MeVal), or *N*-methylleucine (MeLeu). To evaluate the significance of the ring size of Pro-56, the residue was replaced by azetidine-2-carboxylic acid (Aze) or pipecolic acid (Pip). Peptides were synthesized and purified as described in "EXPERIMENTAL PROCEDURES." The yields of peptides containing MeVal were very low (~10%), since the incorporation of MeVal and the next serine residues into the growing peptide chain did not proceed quantitatively. The identity of the peptides was confirmed by mass spectrum measurements.

Phosphorylation of Peptides Representing the Ser-55 Site in Vimentin—The phosphotransferase assay conditions were chosen as optimal for phosphorylation of the peptides containing proline, as described in "EXPERIMENTAL PROCEDURES." Peptide Vim(50-60) or [Arg^{58}]Vim(50-60) was readily phosphorylated by cdc2 kinase with a

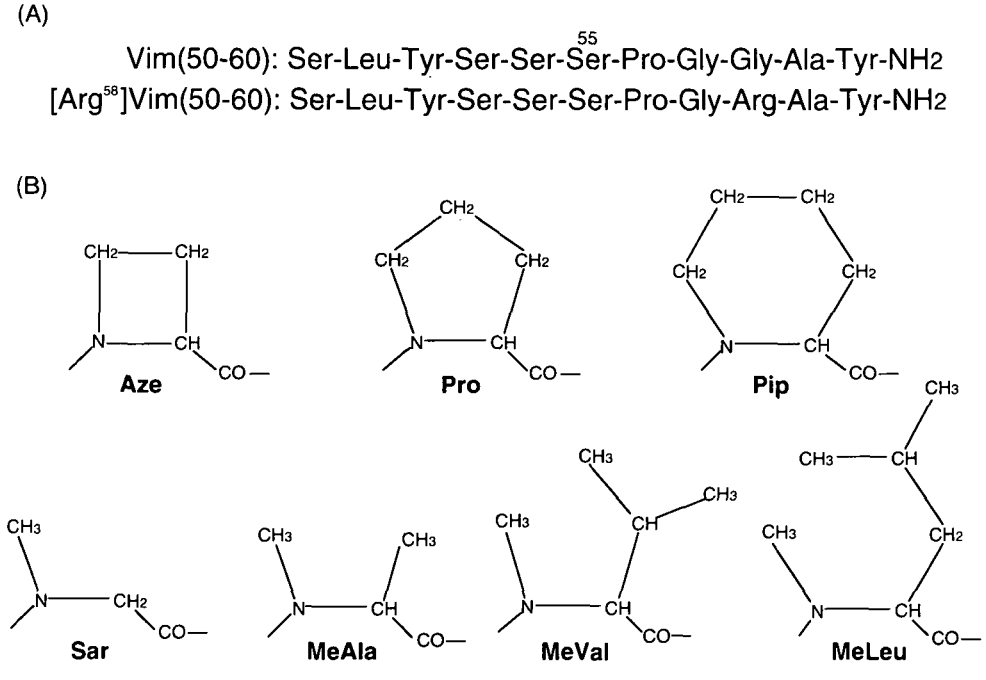

Fig. 1. **Structures of synthetic peptides.** (A) Amino acid sequences. (B) Structures of proline homologs and *N*-methylamino acids. Amino acids except glycine and sarcosine are all in L-form. Aze, azetidine-2-carboxylic acid; Pip, pipecolic acid or piperidine-2-carboxylic acid; Sar, sarcosine or *N*-methylglycine; MeAla, *N*-methylalanine; MeVal, *N*-methylvaline; MeLeu, *N*-methylleucine.

TABLE I. **Kinetic constants for phosphorylation of synthetic peptides by cdc2 kinase and cdk5.** Peptide phosphorylation was determined as described under "EXPERIMENTAL PROCEDURES."

Peptide	cdc2 kinase			cdk5		
	Apparent K_m (mM)	V_{max} (μmol·min^{-1}·mg^{-1})	V_{max}/K_m (% control)	Apparent K_m (mM)	V_{max} (μmol·min^{-1}·mg^{-1})	V_{max}/K_m (% control)
Vim(50-60)	0.886	0.481	100		NP	
[Aze^{56}]Vim(50-60)	0.851	0.224	48.4		NP	
[Pip^{56}]Vim(50-60)	0.998	0.178	32.8		NP	
[Sar^{56}]Vim(50-60)	4.23	0.125	5.5		NP	
[MeAla^{56}]Vim(50-60)	3.56	0.111	5.7		NP	
[MeVal^{56}]Vim(50-60)	2.46	0.0534	4.0		NP	
[MeLeu^{56}]Vim(50-60)	5.70	0.118	3.8		NP	
[Arg^{58}]Vim(50-60)	0.0994	0.397	100	1.43	0.524	100
[Aze^{56}, Arg^{58}]Vim(50-60)	0.357	0.285	20.0	5.80	0.161	7.6
[Pip^{56}, Arg^{58}]Vim(50-60)	0.174	0.250	36.1	2.80	0.212	20.7
[Sar^{56}, Arg^{58}]Vim(50-60)	4.01	0.235	1.5	41.9	0.218	1.4
[MeAla^{56}, Arg^{58}]Vim(50-60)	2.11	0.384	4.6	26.5	0.150	1.5
[MeVal^{56}, Arg^{58}]Vim(50-60)	0.70	0.0730	2.6		NP	
[MeLeu^{56}, Arg^{58}]Vim(50-60)	0.951	0.0882	2.3		NP	

NP, not phosphorylated.

K_m of 0.886 mM and a V_{max} of 0.481 μmol/min/mg, or a K_m of 0.099 mM and a V_{max} of 0.397 μmol/min/mg, respectively (Table I). cdk5 from porcine brains phosphorylated [Arg^{58}]Vim(50-60) with a K_m of 1.43 mM and a V_{max} of 0.524 μmol/min/mg, but failed to phosphorylate Vim(50-60) (Table I). The sites phosphorylated in the peptides were assigned to be Ser-55 by amino acid sequencing, as described in "EXPERIMENTAL PROCEDURES" (data not shown).

Effect of Proline-Substitution on Phosphorylation by cdc2 Kinase—The replacement of Pro-56 in the peptide Vim(50-60) or [Arg^{58}]Vim(50-60) by the *N*-methylamino acids increased the K_m values 3- to 6-fold or 7- to 40-fold, respectively (Table I). The extent in increase of K_m values depended on size of the side chains of the *N*-methylamino acids introduced. Of these *N*-methylamino acids, MeVal with the side chain similar to proline was favored. Thus, opening the ring structure of proline significantly increased K_m values. These results suggest that the ring structure of proline is important for recognition of the peptides by cdc2 kinase.

The peptide [Aze^{56}]Vim(50-60) or [Pip^{56}]Vim(50-60), containing Aze or Pip in place of Pro-56, gave a similar K_m value to that obtained with the peptide Vim(50-60) (Table I). Thus, substitution of proline in Vim(50-60) by proline homologs did not affect the K_m value for cdc2 kinase. However, deleterious effects of the two proline homologs on the K_m value were apparent by substitution of proline in [Arg^{58}]Vim(50-60) which contains the basic residue on the carboxyl-terminal side of the site. The peptide [Pip^{56}, Arg^{58}]Vim(50-60) or [Aze^{56}, Arg^{58}]Vim(50-60) gave a 2- or 4-fold higher K_m value compared to that obtained with [Arg^{58}]Vim(50-60), respectively (Table I). Thus, the ring size of proline was significant for recognizing cdc2 kinase.

The replacement of Pro-56 in peptides Vim(50-60) and [Arg^{58}]Vim(50-60) by the *N*-methylamino acids or the proline homologs decreased the V_{max} values severalfolds (Table I). Significant deleterious effects were observed with MeVal and MeLeu. Apparently, the proline homologs were preferable to *N*-methylamino acids. Thus, the pyrrolidine ring of proline was required to maintain a high V_{max} value for cdc2 kinase.

Effect of Proline-Substitution on Phosphorylation by cdk5—Of the peptides synthesized in this study, [Arg^{58}]Vim(50-60) and its analogs containing Aze, Pip, Sar or MeAla in place of Pro-56 were effectively phosphorylated by cdk5 (Table I). Although the K_m values for cdk5 were one order of magnitude higher than those for cdc2 kinase, the effect of replacement of Pro-56 by Aze, Pip, Sar, or MeAla on the K_m value parallels qualitatively that found in phosphorylation of the same peptides by cdc2 kinase (Table I). These results suggest that cdk5 recognized peptides with a proline specificity similar to that of cdc2 kinase.

These replacements, however, affected the V_{max} value for cdk5 in a different manner from that found in phosphorylation by cdc2 kinase. Replacement of Pro-56 in [Arg^{58}]Vim(50-60) by *N*-methylamino acids or proline homologs led to more degenerative effects on the V_{max} value for cdk5 than for cdc2 kinase (Table I). Replacement of proline by Aze decreased the V_{max} for cdk5 to 31%, while the same replacement decreased the V_{max} for cdc2 kinase to 72%. As a result, the relative ratio of V_{max}/K_m of [Aze^{56}, Arg^{58}]Vim(50-60) over [Arg^{58}]Vim(50-60) for cdk5 was only 7.6%, while that of the same peptide for cdc2 kinase was 20%. Thus, in terms of maintaining a high phosphorylation rate, cdk5 showed a stronger preference for proline over other amino acids, when compared to the preference exhibited by cdc2 kinase.

Molecular Structures of Peptides—To evaluate significance of proline for steric structures at the phosphorylation site, MD and MM simulations were performed for a tetrapeptide, acetylserylprolylglycylarginylmethane (Ac-Ser-Pro-Gly-Arg-CH_3), corresponding to the sequence of peptide [Arg^{58}]Vim(50-60), as described in "EXPERIMENTAL PROCEDURES." Of the two hundred structures generated, the predominant structure was a β-turn ($Pt=97\%$) with a hydrogen bond between the carbonyl group of Ser at the i-th position and the amide NH of Arg at the $i+3$-th position (Fig. 2). Proline at the $i+1$-th position formed a *trans* peptide bond (*36, 37*) with the preceding Ser residue. These results agreed with the prediction by Chou and Fasman (*36*), in which proline is known as the most frequently occurring residue in β-turns, especially at the $i+1$-th position.

MD and MM simulations were performed also for Ac-Ser-*X*-Gly-Arg-CH_3 in which *X* is Pip, MeLeu, or Sar. The molecule containing Pip formed a β-turn with a little lower probability ($Pt=84\%$), when compared to the proline-containing molecule. Strain at the ring structure of the proline homologs might reduce the probability of a β-turn. On the other hand, the molecule containing Sar or MeLeu formed a β-turn at the Ser-*X*-Gly-Arg sequence with an apparently low probability ($Pt=27\%$ for Sar and $Pt=64\%$ for MeLeu). Various extended structures or turn structures having hydrogen bonds between the acetyl group and the amide NH of Gly were observed. Thus, although *N*-methylamino acids can sometime replace a proline residue in turn structures (*38*), Sar or MeLeu in this study

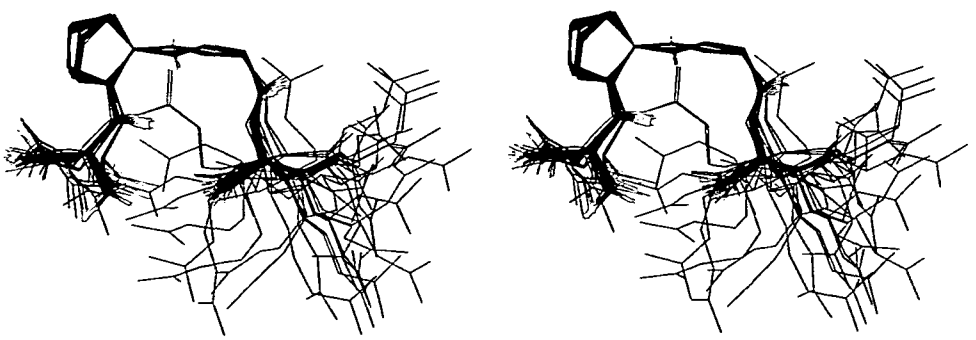

Fig. 2. **β-turn structures of Ac-Ser-Pro-Gly-Arg-CH_3.** Of two hundred structures obtained by MD and MM simulations for the model peptide, 39 structures with low energy were superposed. A hydrogen bond is formed between the carbonyl group of Ser at the i-th position and the amide NH of Arg at the $i+3$-th position. carbon (dark gray); nitrogen (black); oxygen (gray).

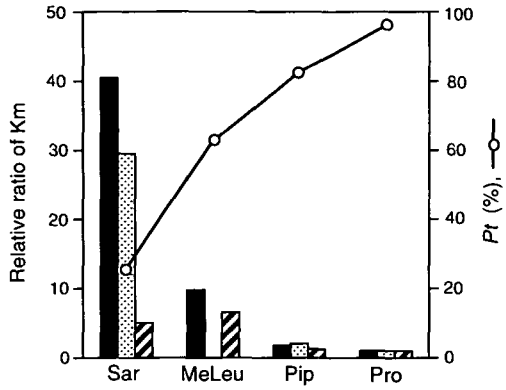

Fig. 3. **Correlation between K_m value and probability of β-turn (*Pt*) at the Ser-*X*-Gly-Arg sequence.** Relative ratio of K_m value was obtained by dividing K_m value of each peptide by that of the proline-containing peptide for cdc2 kinase or cdk5. *Pt*(%) was determined as described in "EXPERIMENTAL PROCEDURES." Phosphorylation of [X^{56}, Arg^{58}]Vim(50-60) by cdc2 kinase (solid bars); phosphorylation of [X^{56}, Arg^{58}]Vim(50-60) by cdk5 (stippled bars); phosphorylation of [X^{56}]Vim(50-60) by cdc2 kinase (hatched bars). *X*; Sar, MeLeu, Pip, or Pro.

apparently decreased the probability of forming the β-turn. These results would be mainly due to the fact that *N*-methylamino acids lack a ring structure like that of proline. Thus, the pyrrolidine ring of proline was optimal for inducing and stabilizing a β-turn at the Ser-*X*-Gly-Arg sequence.

We found that, in proportion to increase in the probability of β-turn formation, the K_m values of the peptides for cdc2 kinase or cdk5 decreased (Fig. 3). The relative activities of the peptides might be related to how well each peptide could assume a β-turn at the phosphorylation site. It was, however, unknown whether the peptides bound to the catalytic site of the enzymes exactly form the turn structure. The implication of the inverse correlation observed here remained to be further clarified. It might be also necessary to take into account the effects of other structural factors including side chain structures on phosphorylation to rationalize the kinetic data.

DISCUSSION

Using synthetic peptides containing various *N*-methylamino acids or proline homologs, we demonstrated here that the pyrrolidine ring of proline adjacent to the phosphorylation site is important for cdc2 kinase or cdk5 phosphorylation. The alteration in the ring size or especially the opening the ring structure of proline caused deleterious effects on both K_m and V_{max} values for the enzymes (Table I). MD simulations indicated that the pyrrolidine ring of proline is optimal to stabilize a β-turn at the phosphorylation site and that the probability of the turn formation might be related to substrate recognition by the enzymes (Fig. 3).

The favorable role of β-turn conformation on protein kinase phosphorylation has been proposed from the secondary structures predicted at the phosphorylation sites in various proteins (*39*). While turn structures might be preserved in protein molecules, short peptides including the ones used in this study, in general, have little stable conformation in solution. The turn structures of peptides might be stabilized as a preferable structure upon binding to the catalytic site of the enzymes. On the other hand, the crystal structure of cAMP-dependent protein kinase with bound pseudosubstrate revealed that the peptide is accommodated in an extended conformation into the catalytic site (*29*). It still remains to be determined experimentally if the peptides in the bound state to the catalytic site of cdc2 kinase or cdk5 form the turn structure. However, it is evident from the kinetic data and MD simulations that the ring structure of proline, which restricts the ϕ angle at $-60°$, is critical for substrate recognition by cdc2 kinase or cdk5. The restriction of the ϕ angle limits the steric structures at least at the region from the α-carbon of phosphorylatable serine to the carbonyl carbon of proline in the peptide (Fig. 2), even if *cis/trans* isomerization (*36, 37*) occurs. The fixed steric structure at the Ser-Pro region results in the high probability of the β-turn formation as estimated here, and might be a core structure involved in substrate recognition by cdc2 kinase or cdk5.

The pyrrolidine ring of proline might be suitable to locate properly side chains of amino acids which would interact with the catalytic site of cdc2 kinase or cdk5. The hydroxyl group of Ser neighboring with proline would be close enough for a direct transfer of the γ-phosphate from MgATP. The basic group of Arg/Lys at the third residue on the carboxyl-terminal side of the site would undergo ion pairing with carboxyl groups of acidic residues in the catalytic site. As observed here and previously (*24*), the basic residue enhanced the peptide recognition by cdc2 kinase or cdk5. The maximum enhancement was observed for the peptide containing proline, while moderate or small enhancements were seen for peptides containing proline homologs or *N*-methylamino acids, respectively (Table I). It might be plausible that definite interactions between the peptides and the catalytic site occurs cooperatively by the steric structure induced by proline and by ion pairing by the basic residue on the carboxyl-terminal side of the site. This view is consistent with that the sequence Ser/Thr-Pro-*Z*-Arg/Lys has been found in many of protein substrates for cdc2 kinase and cdk5 (*12, 18, 27, 28*). It remains to be determined whether or not the kinases prefer equally arginine and lysine as a basic residue.

Proline might be involved in a hydrophobic interaction with the P+1 pocket in the catalytic site of cdc2 kinase, since the peptides containing MeVal or MeLeu rather than Sar or MeAla gave relatively low K_m values (Table I). This view accounts for previous findings that peptides in which proline was replaced by a hydrophobic amino acid were more suitable substrates for cdc2 kinase than were peptides in which proline was replaced by a hydrophilic amino acid (*24*). Zhang *et al.* also reported that residues with properly long hydrophobic side chains but not short or hydrophilic ones, introduced in place of proline, gave low but detectable phosphorylation by cdc2 kinase (*25*). Thus, hydrophobicity of the pyrrolidine ring of proline might enhance the site recognition by the enzyme.

We demonstrated here the usefulness of synthetic peptides containing unusual amino acids and MD simulations to probe the substrate specificity of cdc2 kinase or cdk5. We speculate that the pyrrolidine ring of proline adjacent to the site has multiple roles in the substrate-enzyme interaction as mentioned above. An explanation,

however, for the specific requirement of proline to maintain a high V_{max} value for cdc2 kinase or especially for cdk5 remains to be determined. The results obtained here provide useful information for rational design and synthesis of conformationally restricted substrates or inhibitors, in order to distinguish proline-directed protein kinases and to understand their physiological roles.

We are grateful to K. Yamaguchi of Kyushu Institute of Technology for measurement of mass spectra, to Prof. N. Nishino of Kyushu Institute of Technology and M. Ohara for pertinent comments and discussions.

REFERENCES

1. Norbury, C. and Nurse, P. (1992) Animal cell cycles and their control. *Annu. Rev. Biochem.* **61**, 441-470
2. Sherr, C.J. (1994) G1 phase progression: cycling on cue. *Cell* **79**, 551-555
3. King, R.W., Jackson, P.K., and Kirschner, M.W. (1994) Mitosis in transition. *Cell* **79**, 563-571
4. Morgan, D.O. (1995) Principles of cdk regulation. *Nature* **374**, 131-134
5. Tassan, J.P., Jaquenoud, M., Leopold, P., Schultz, S.J., and Nigg, E.A. (1995) Identification of human cyclin-dependent kinase 8, a putative protein kinase partner for cyclin C. *Proc. Natl. Acad. Sci. USA* **92**, 8871-8875
6. Sherr, C.J. (1993) Mammalian G_1 cyclins. *Cell* **73**, 1059-1065
7. Hunter, T. and Pines, J. (1994) Cyclins and cancer II: cyclin D and cdk inhibitors come of age. *Cell* **79**, 573-582
8. Heichman, K.A. and Roberts, J.M. (1994) Rules to replicate by. *Cell* **79**, 557-562
9. Lew, J., Beaudette, K., Litwin, C.M.E., and Wang, J.H. (1992) Purification and characterization of a novel proline-directed protein kinase from bovine brain. *J. Biol. Chem.* **267**, 13383-13390
10. Ishiguro, K., Takamatsu, M., Tomizawa, K., Omori, A., Takahashi, M., Arioka, M., Uchida, T., and Imahori, K. (1992) Tau protein kinase I converts normal tau protein into A68-like component of paired helical filaments. *J. Biol. Chem.* **267**, 10897-10901
11. Hellmich, M.R., Pant, H.C., Wada, E., and Battey, J.F. (1992) Neuronal cdc2-like kinase: a cdc2-related protein kinase with predominantly neuronal expression. *Proc. Natl. Acad. Sci. USA* **89**, 10867-10871
12. Lew, J., Winkfein, R.J., Paudel, H.K., and Wang, J.H. (1992) Brain proline-directed protein kinase is a neurofilament kinase which displays high sequence homology to $p34^{cdc2}$. *J. Biol. Chem.* **267**, 25922-25926
13. Kobayashi, S., Ishiguro, K., Omori, A., Takamatsu, M., Arioka, M., Imahori, K., and Uchida, T. (1993) A cdc2-related kinase PSSALRE/cdk5 is homologous with the 30 kDa subunit of tau protein kinase II, a proline-directed protein kinase associated with microtubule. *FEBS Lett.* **335**, 171-175
14. Tsai, L.-H., Delalle, I., Caviness, Jr. V.S., Chae, T., and Harlow, E. (1994) p35 is a neural-specific regulatory subunit of cyclin-dependent kinase 5. *Nature* **371**, 419-423
15. Lew, J., Huang, Q.-Q., Qi, Z., Winkfein, R.J., Aebersold, R., Hunt, T., and Wang, J.H. (1994) A brain-specific activator of cyclin-dependent kinase 5. *Nature* **371**, 423-426
16. Uchida, T., Ishiguro, K., Ohnuma, J., Takamatsu, M., Yonekura, S., and Imahori, K. (1994) Precursor of cdk5 activator, the 23 kDa subunit of tau protein kinase II: its sequence and developmental changes in brain. *FEBS Lett.* **355**, 35-40
17. Lew, J. and Wang, J.H. (1995) Neuronal cdc2-like kinase. *Trends Biochem. Sci.* **20**, 33-37
18. Moreno, S. and Nurse, P. (1990) Substrates for $p34^{cdc2}$: in vivo veritas? *Cell* **61**, 549-551
19. Chou, Y.-H., Bischoff, J.R., Beach, D., and Goldman, R.D. (1990) Intermediate filament reorganization during mitosis is mediated by $p34^{cdc2}$ phosphorylation of vimentin. *Cell* **62**, 1063-1071
20. Chou, Y.-H., Ngai, K.-L., and Goldman, R. (1991) The regulation of intermediate filament reorganization in mitosis. *J. Biol. Chem.* **266**, 7325-7328
21. Kusubata, M., Tokui, T., Matsuoka, Y., Okumura, E., Tachibana, K., Hisanaga, S., Kishimoto, T., Yasuda, H., Kamijo, M., Ohba, Y., Tsujimura, K., Yatani, R., and Inagaki, M. (1992) $p13^{suc1}$ suppresses the catalytic function of $p34^{cdc2}$ kinase for intermediate filament proteins, *in vitro*. *J. Biol. Chem.* **267**, 20937-20942
22. Tsujimura, K., Ogawara, M., Takeuchi, Y., Imajoh-Ohmi, S., Ha, M.H., and Inagaki, M. (1994) Visualization and function of vimentin phosphorylation by cdc2 kinase during mitosis. *J. Biol. Chem.* **269**, 31097-31106
23. Marin, O., Meggio, F., Draetta, G., and Pinna, L.A. (1992) The consensus sequences for cdc2 kinase and for casein kinase-2 are mutually incompatible. *FEBS Lett.* **301**, 111-114
24. Ando, S., Tsujimura, K., Matsuoka, Y., Tokui, T., Hisanaga, S., Okumura, E., Uchiyama, M., Kishimoto, T., Yasuda, H., and Inagaki, M. (1993) Phosphorylation of synthetic vimentin peptides by cdc2 kinase. *Biochem. Biophys. Res. Commun.* **195**, 837-843
25. Zhang, J., Sancez, R.J., Wang, S., Guarnaccia, C., Tossi, A., Zahariev, S., and Pongor, S. (1994) Substrate specificity of CDC2 kinase from human HeLa cells as determined with synthetic peptides and molecular modeling. *Arch. Biochem. Biophys.* **315**, 415-424
26. Songyang, Z., Blechner, S., Hoagland, N., Hoekstra, M.F., Piwnica-Worms, H., and Cantley, L.C. (1994) Use of an oriented peptide library to determine the optimal substrates of protein kinases. *Current Biol.* **4**, 973-982
27. Miyasaka, H., Okabe, S., Ishiguro, K., Uchida, T., and Hirokawa, N. (1993) Interaction of the tail domain of high molecular weight subunits of neurofilaments with the COOH-terminal region of tubulin and its regulation by tau protein kinase II. *J. Biol. Chem.* **268**, 22695-22702
28. Hisanaga, S., Ishiguro, K., Uchida, T., Okumura, E., Okano, T., and Kishimoto, T. (1993) Tau protein kinase II has a similar characteristic to cdc2 kinase for phosphorylating neurofilament protein. *J. Biol. Chem.* **268**, 15056-15060
29. Knighton, D.R., Zheng, J., Ten Eyck, L.F., Ashford, V.A., Xuong, N.-H., Taylor, S.S., and Sowadski, J.M. (1991) Crystal structure of the catalytic subunit of cyclic adenosine monophosphate-dependent protein kinase. *Science* **253**, 407-420
30. Hisanaga, S., Uchiyama, M., Hosoi, T., Yamada, K., Honma, N., Ishiguro, K., Uchida, T., Dahl, D., Ohsumi, K., and Kishimoto, T. (1995) Porcine brain neurofilament-H tail domain kinase: its identification as cdk5/p26 complex and comparison with cdc2/cyclin B kinase. *Cell Motil. Cytoskel.* **31**, 283-297
31. Bradford, M.M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**, 248-254
32. Kemp, B.E., Benjamini, E., and Krebs, E.G. (1976) Synthetic hexapeptide substrates and inhibitors of 3′:5′-cyclic AMP-dependent protein kinase. *Proc. Natl. Acad. Sci. USA* **73**, 1038-1042
33. Meyer, H.E., Hoffmann-Posorske, E., Korte, H., and Heilmeyer, Jr. L.M.G. (1986) Sequence analysis of phosphoserine-containing peptides. *FEBS Lett.* **204**, 61-66
34. Ando, S., Tanabe, K., Gonda, Y., Sato, C., and Inagaki, M. (1989) Domain- and sequence-specific phosphorylation of vimentin induces disassembly of the filament structure. *Biochemistry* **28**, 2974-2979
35. Lewis, P.N., Momany, F.A., and Scheraga, H.A. (1971) Folding of polypeptide chains in proteins: a proposed mechanism for folding. *Proc. Natl. Acad. Sci. USA* **68**, 2293-2297
36. Chou, P.Y. and Fasman, G.D. (1977) β-Turns in proteins. *J. Mol. Biol.* **115**, 135-175
37. Madison, V. and Schellman, J. (1970) Location of proline derivatives in conformational space. I. Conformational calculations; optical activity and NMR experiments. *Biopolymers* **9**, 511-567
38. Freidinger, R.M., Perlow, D.S., Randall, W.C., Saperstein, R., Arison, B.H., and Veber, D.F. (1984) Conformational modifications of cyclic hexapeptide somatostatin analogs. *Int. J. Pept. Protein Res.* **23**, 142-150
39. Small, D., Chou, P.Y., and Fasman, G.D. (1977) Occurrence of phosphorylated residues in predicted β-turns: implications for β-turn participation in control mechanisms. *Biochem. Biophys. Res. Commun.* **79**, 341-346