

## Demonstration of Cyclin-dependent Kinase Inhibitory Serine/Threonine Kinase in Bovine Thymus\*

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**A synthetic peptide corresponding in sequence to residues 6–20 of p34<sup>cdc2</sup>, cdc2(6–20), and a substitution analogue, cdc2(6–20)F15K19, which contains Thr-14 as the only phosphorylation target were used as substrates to identify a novel protein kinase in bovine thymus cytosol. The kinase catalyzed the phosphorylation of Thr-14 in both peptides and was purified extensively on the basis of its peptide phosphorylation activity. Upon SDS-polyacrylamide gel electrophoresis analyses, the purified samples consistently displayed a prominent 43-kDa protein band which could undergo *in gel* autophosphorylation, thus suggesting that this band represented the kinase protein. The suggestion was supported further by the observation that both cdc2(6–20) peptide phosphorylation and the autophosphorylation reaction of the 43-kDa protein were inhibited by millimolar concentrations of cAMP. The kinase was found to inactivate Cdc2/cyclin B, Cdk2/cyclin A, and neuronal Cdc2-like kinase (Nck), a heterodimer of Cdk5 and neuronal Cdk5 activator (Nck5a), under phosphorylation conditions. The phosphorylation of Nck by the purified thymus kinase occurred on Cdk5. The monomeric form of Cdk5 was also phosphorylated by the kinase. Phosphoamino acid and phosphopeptide analysis of the phosphorylated Nck revealed that Thr-14 of Cdk5 was the sole site of protein phosphorylation. The results suggest that this thymus kinase is a novel Cdk inhibitory protein kinase, distinct from the recently cloned dual functional and membrane-associated Cdc2 inhibitory kinase, Myt1 (Mueller, P. R., Coleman, T. R., Kumagai, A., and Durphy, W. G. (1995) *Science* 270, 86–90).**

Animal cells contain a family of Cdc2 homologous proteins which are involved in the regulation of cell cycle progression (1–5). These proteins, whose activities depend on their association with cyclins, are called cyclin-dependent kinases, Cdks (6). In addition to depending on cyclins for activity, Cdks are regulated by protein phosphorylation mechanisms involving a network of specific protein kinases and protein phosphatases. For example, Cdc2 kinase is negatively regulated during S and G<sub>2</sub> phases by phosphorylation on a specific tyrosine, Tyr-15,

and a threonine, Thr-14 residue (7–10). Since these residues are located in the nucleotide binding loop, one of the most conserved regions of Cdc2 family proteins, these phosphorylations may represent a general regulatory mechanism for Cdc2-like kinases. The kinase catalyzing the phosphorylation of Tyr-15 of Cdc2 has been identified as related to the protein product of the yeast cell cycle regulatory gene *wee1* (11–13). On the other hand, the kinase that catalyzes the phosphorylation of Cdc2 at Thr-14 has not been identified positively. A membrane fraction purified from *Xenopus* eggs and HeLa cells was shown to promote the phosphorylation of Cdc2 on both Tyr-15 and Thr-14 (14, 15). The protein kinase responsible for such a dual specific phosphorylation event has been cloned recently (16). The protein kinase, Myt1, contains a putative transmembrane segment and is located exclusively in a membrane fraction of cells. On the other hand, no Thr-14 kinase activity has been reported in a cytosolic fraction of cells where a major population of Cdc2 is located (17, 18).

Not all the Cdks are cell cycle regulators. Neuronal Cdc2-like kinase (Nck), a heterodimer of Cdk5 and a regulatory protein which is expressed specifically in neurons of the central nervous system, is a prime example of a Cdc2-like kinase not involved in cell cycle control (19, 20). As a functionally unique Cdc2-like kinase, Nck also displays unique molecular and regulatory properties that distinguish it from the cell cycle regulatory Cdc2-like kinases (20). The regulatory subunit of the enzyme, neuronal Cdk5 activator (Nck5a), performs a cyclin-like function but shows only marginal amino acid sequence similarity to cyclins (19–21). While most well characterized Cdks depend on an activating kinase, Cdk activating kinase (22–24), in addition to cyclin, for kinase activity, the activation of Cdk5 by neuronal Cdk5 activator is independent of Cdk activating kinase (20, 25).

The present study demonstrates the existence of a protein kinase in bovine thymus cytosol that induces the inactivation of Cdc2/cyclin B kinase, Cdk2/cyclin A kinase, and Nck. Characterization of the kinase-catalyzed phosphorylation of Nck indicates that the kinase, designated the Cdk T14 kinase, phosphorylates Cdk5 at the Thr-14 residue. The results suggest that this kinase may be the Cdk inhibitory protein Ser/Thr kinase that has been proposed to play cell cycle regulatory roles. In addition, the present study suggests that Nck, like cell cycle regulatory Cdc2-like kinases, may be regulated by the inhibitory protein Ser/Thr phosphorylation mechanism.

### MATERIALS AND METHODS

**Purification of the Cdk T14 Kinase**—All of the purification procedures were carried out at 4 °C. Bovine thymus (800 g) was homogenized with 1.5 liters of buffer A (25 mM HEPES-NaOH, pH 7.0, 1 mM EDTA,

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1 mM DTT,<sup>1</sup> 0.3 mg/ml benzamidine, 1  $\mu$ g/ml leupeptin, 2  $\mu$ g/ml anti-pain, 0.2 mM phenylmethylsulfonyl fluoride) containing 0.1 mg/ml soybean trypsin inhibitor. The homogenate was centrifuged at  $10,000 \times g$  for 30 min, and the supernatant, collected through glass wool, was centrifuged further at  $100,000 \times g$  for 40 min. The  $100,000 \times g$  supernatant was applied to a 400-ml DEAE-Sepharose CL-6B column pre-equilibrated with buffer A. The column was washed extensively with buffer A containing 0.08 M NaCl, followed by elution with 0.08–0.45 M/3-liter linear NaCl gradient. The kinase-containing fractions were pooled and loaded onto a 100-ml hydroxylapatite column pre-equilibrated with buffer A. After washing with buffer A, the column was eluted by 0–0.2 M/liter linear potassium phosphate gradient in buffer A. The pooled kinase fractions (350 ml) were diluted to 1 liter with buffer A, and the hydroxylapatite step was repeated with a smaller column (50 ml) and a smoother potassium phosphate gradient (0–0.17 M/500 ml). The kinase fraction was loaded onto a 15-ml phenyl-Sepharose CL-4B column pre-equilibrated with buffer A. The column was washed with buffer A and then eluted by 0–65%/250-ml linear ethylene glycol gradient using a peristaltic pump (1 ml/min). The kinase-containing fractions eluted at the ethylene glycol concentration of 35–50% were pooled and subjected to FPLC Mono Q chromatography with a 0.15–0.35 M/30-ml linear NaCl gradient elution. To apply to the FPLC Superose-12 gel filtration column (100 ml), the kinase sample (5 ml) from Mono Q column was concentrated with Centricon 30 (Amicon) to 1.5 ml. The column was pre-equilibrated and run with buffer A containing 0.15 M NaCl. The pooled kinase fraction was finally rechromatographed on the FPLC Mono Q column.

**In Vitro Reconstitution of Nck and Cdk2/Cyclin A**—Reconstitution of active Nck from the bacterially expressed GST-Cdk5 and GST-Nck5a (neuronal Cdk5 activator) was described previously (20, 25). For the reconstitution of an active Cdk2 kinase, a bacterially expressed GST-Cdk2 and protein A-poly(His) fused cyclin A (pA-His-cyclin A) were used. The GST-Cdk2 expressed in bacteria strain BL21 was treated with thrombin (30 units/mg). GST and undigested protein were removed by GSH-agarose beads. The 0.9- $\mu$ g aliquot of thrombin-treated Cdk2 was mixed with 3.5  $\mu$ g of pA-His-cyclin A (1:1.3 molar ratio) and incubated in kinase buffer (see below) and 0.5 mM ATP at 30 °C for 30 min. After 30 min, an aliquot of partially purified cyclin-dependent kinase activating kinase was added and incubated at room temperature for another 30 min. Activated Cdk2/pA-His-cyclin A was purified with nickel-nitrilotriacetic acid agarose (Qiagen).

**Kinase Assay and Cdk Inactivation Assay**—The Cdk T14 kinase was assayed routinely using either cdc2(6–20) or peptide analogue cdc2(6–20)F15K19. The 30- $\mu$ l reaction mixture comprised of kinase buffer (20 mM MOPS-NaOH, pH 7.4, 10 mM MgCl<sub>2</sub>, 10 mM  $\beta$ -glycerophosphate, 2 mM sodium fluoride), 0.1  $\mu$ M microcystin LR, 0.1 mM [ $\gamma$ -<sup>32</sup>P]ATP, 0.5 mM cdc2(6–20) synthetic peptide substrate, and the kinase sample. A nucleotide was included in the reaction mixture when its effect was tested. The kinase reaction was started by the addition of the enzyme sample and carried out for 30 min at 30 °C. The reaction was terminated by the addition of 5.4  $\mu$ l of 50% trichloroacetic acid. To the mixture was added 9.6  $\mu$ l of 0.1 g/ml bovine serum albumin as a carrier for protein precipitation. The mixture was centrifuged at 12,000 rpm for 5 min at 4 °C, and 30  $\mu$ l of the supernatant was analyzed for phosphate incorporation using phosphocellulose paper as described (26). In a typical assay, <10% of the phosphate group was transferred to a peptide substrate.

For the Cdk inactivation assay, the reaction mixture containing kinase buffer, 0.1 mM [ $\gamma$ -<sup>32</sup>P]ATP, reconstituted Cdk2/pA-His-cyclin A, Nck, or Cdc2/cyclin B purified from sea star oocyte and the appropriate amount of the Cdk T14 kinase was incubated for an appropriate time period (described in the figure legends) at 30 °C. The Cdk kinase assay was started by addition of the histone H1(9–18) peptide (90  $\mu$ M). Final reaction volume was 30  $\mu$ l (50  $\mu$ l in the case for Cdc2 due to low kinase concentration of the original sample of Cdc2 kinase). The control reaction was carried out under the identical conditions except that the T14 kinase was not added.

**In Gel Autophosphorylation of the Cdk T14 Kinase**—Purified Cdk T14 kinase was applied to 10% SDS-PAGE. SDS sample was not boiled. The gel was then washed with 20% 2-propanol, and 50 mM HEPES, pH 7.4 (30 min  $\times$  2), 50 mM HEPES, pH 7.4, 5 mM 2-mercaptoethanol (30 min  $\times$  2). The gel was soaked in 6 M urea, 50 mM HEPES, pH 7.4, 5 mM 2-mercaptoethanol for 1 h with constant agitation to denature the

protein. To renature the protein, the gel was incubated at 4 °C successively with 50 mM HEPES, pH 7.4, 5 mM 2-mercaptoethanol, 0.05% Tween 20 containing 3 M, 1.5 M, and 0.75 M urea (30 min for each) and the same buffer without urea (1 h  $\times$  4). After renaturation, the gel was equilibrated at 4 °C for 30 min in 50 mM HEPES, pH 7.4, 10 mM MgCl<sub>2</sub>, 2 mM MnCl<sub>2</sub>. Then the gel was incubated at 30 °C for 1 h in 20 ml of the same buffer supplemented with 50  $\mu$ Ci of [ $\gamma$ -<sup>32</sup>P]ATP. When the effect of a nucleotide on the autophosphorylation was examined, the gel was incubated at room temperature for another 15 min in the kinase buffer containing a 1.5 mM concentration of the nucleotide before addition of ATP to initiate the kinase reaction. The phosphorylation reaction was terminated by immersing the gel in 5% trichloroacetic acid, 1% sodium pyrophosphate. The gel was washed with the same solution several times to remove the background radioactivity. The proteins were visualized by Coomassie staining, and autophosphorylated proteins were detected by autoradiography.

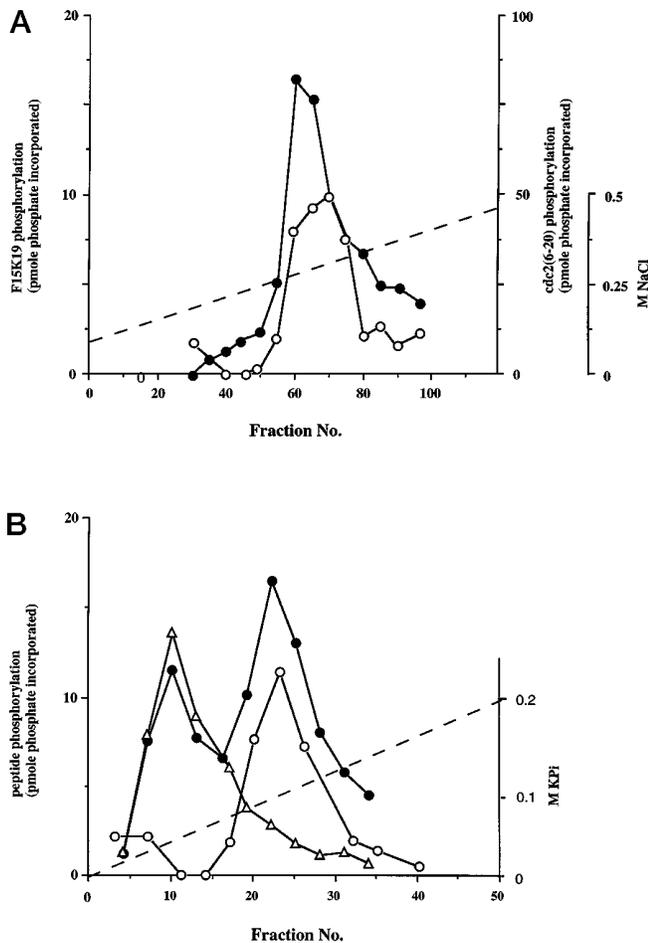
**Phosphorylation of Cdk5**—GST-Cdk5 (1  $\mu$ g), the same amount of GST-Cdk5 complexed with GST-Nck5a or GST-Nck5a alone, was incubated for 30 min at 30 °C in 10  $\mu$ l containing kinase buffer and 0.4 mM ATP. Then, the mixture of 1  $\mu$ l of 20 mg/ml bovine serum albumin, 2.5  $\mu$ l of [ $\gamma$ -<sup>32</sup>P]ATP (25  $\mu$ Ci), 3  $\mu$ l of 10  $\times$  kinase buffer, and 2.5  $\mu$ l of water was added to the mixture, followed by the addition of 21  $\mu$ l of T14 kinase (total volume, 40  $\mu$ l). The mixture was incubated further at 30 °C for 90 min. After incubation, 20  $\mu$ l of 10 mM ATP, 20  $\mu$ l of 0.1 g/ml bovine serum albumin, 900  $\mu$ l of PBS containing 1 mM DTT, and 20  $\mu$ l of GSH-agarose beads (1:1 slurry in PBS, Sigma) were added and mixed for 30 min. The beads were washed extensively with PBS containing 1 mM DTT and PBS containing 1 mM DTT, 0.35 M NaCl, 1% Triton X-100. The proteins bound to beads were eluted with 10 mM reduced glutathione in 50 mM Tris-HCl, pH 8.0, and subjected to 7.5% SDS-PAGE. GST-Cdk5 was visualized by Western blot using  $\alpha$ -Cdk5 polyclonal antibody, and phosphoprotein was detected by autoradiography.

**Phosphoamino Acid and Phosphopeptide Analysis**—For phosphoamino acid analysis, phosphorylated GST-Cdk5 was sliced from the blot (polyvinylidene difluoride) and hydrolyzed in 6 N HCl for 2 h at 110 °C. The solution containing phosphoamino acid was dried to remove HCl and dissolved in 10  $\mu$ l of phosphoamino acid standard solution containing phosphoserine, phosphothreonine, and phosphotyrosine. The sample was subjected to thin layer electrophoresis (TLE) at pH 3.5, and radioactive phosphoamino acid was detected by autoradiography (27). For phosphotryptic mapping, the dried SDS-gel piece containing phosphorylated GST-Cdk5 was swollen in 150–250  $\mu$ l of 0.1 M ammonium bicarbonate, 0.1 mM CaCl<sub>2</sub> containing 1.5  $\mu$ g of *N*-tosyl-L-phenylalanyl chloromethyl ketone-treated trypsin and incubated at 37 °C for 24 h. The supernatant was lyophilized, and recovered phosphotryptic peptide was analyzed by two-dimensional electrophoresis/chromatography (28). To prepare Thr-14-phosphorylated peptide standard for both phosphoamino acid analysis and phosphotryptic mapping, the reaction mixture similar to the routine kinase assay mixture containing cdk5(6–20) synthetic peptide instead of the Cdc2 peptide was applied to a Sep-Pak C18 column (Millipore) pre-equilibrated with 0.1% trifluoroacetic acid. After washing the column with 0.1% trifluoroacetic acid, phosphopeptide was recovered with acetonitrile, 0.1% trifluoroacetic acid. The solvent was removed by a Speed Vac, and the peptide was dissolved in water for further analysis.

## RESULTS

**Demonstration of the Cdk T14 Kinase**—In earlier studies, we showed that a synthetic peptide derived from the nucleotide binding loop of p34<sup>cdc2</sup> with the amino acid sequence of KVEKIGEGTYGVVYK, cdc2(6–20), was a specific and efficient substrate of Src family kinases (29, 30). Others have found that this peptide is also a substrate of the bacterially expressed 49-kDa form of human Wee1 tyrosine kinase (13). As Src family kinases are membrane-bound and expected to be removed by high speed centrifugation, the possibility of using this peptide as a substrate of Wee1 activity in cytosol was explored. DEAE-Sepharose chromatography of a high speed centrifugation supernatant of bovine thymus extract gave rise to a single peak of cdc2(6–20) peptide kinase activity (Fig. 1A). To test whether the peptide kinase activity is from a protein tyrosine kinase, the same column fractions were assayed for kinase activity using the Tyr-negative analogue of cdc2(6–20), cdc(6–20)F15K19, which was not expected to be phosphorylated by Wee1. Surprisingly, a single peak of kinase activity was also

<sup>1</sup> The abbreviations used are: DTT, dithiothreitol; GST, glutathione S-transferase; PBS, phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis; FPLC, fast protein liquid chromatography; MOPS, 4-morpholinepropanesulfonic acid.



**FIG. 1. Demonstration of the *cdc2* N-terminal peptide kinase activities in bovine thymus.** *A*, the 100,000  $\times$  *g* cytosolic fraction prepared from 500 g of bovine thymus was chromatographed on a 400-ml DEAE-Sepharose CL-6B column as described under "Materials and Methods." The 15- $\mu$ l aliquots of column fractions were assayed for *cdc2*(6-20) (●) and *cdc2*(6-20)F15K19 (○) phosphorylation activity. ---, NaCl gradient. *B*, pooled *cdc2*(6-20)F15K19 peptide kinase fraction of DEAE-Sepharose CL-6B column was applied to an 80-ml hydroxylapatite column. After extensive wash with buffer A, the column was eluted with 0–0.2 M/800-ml linear potassium phosphate gradient. The 12- $\mu$ l aliquots of column fractions were assayed for *cdc2*(6-20) (●), *cdc2*(6-20)F15K19 (circlo), or *cdc2*(6-20)A14 ( $\Delta$ ) phosphorylation activity. ---, potassium phosphate gradient.

detected toward this peptide analogue (Fig. 1A). The kinase activity peaks detected by using the two peptides did not coincide but overlapped with each other to a large extent. A simple interpretation of these results is that there are two kinases capable of using *cdc2*(6-20) as substrate, and one of these can also phosphorylate *cdc2*(6-20)F15K19.

To test such a suggestion, fractions from the DEAE-Sepharose column containing the overlapping kinase activities were pooled and further analyzed. The pooled sample was fractionated on a hydroxylapatite column, and column fractions were analyzed for kinase activities using three different peptides: *cdc2*(6-20), *cdc2*(6-20)F15K19, and *cdc2*(6-20)A14, a substitution peptide with Thr-14 replaced by alanine. Fig. 1B shows that the pooled DEAE-Sepharose sample gave rise to two peaks of *cdc2*(6-20) peptide kinase activity on the hydroxylapatite column, and that only the second peak contained *cdc2*(6-20)F15K19 phosphorylation activity. On the other hand, only the first peak was capable of phosphorylating *cdc2*(6-20)A14. The observation that the first activity peak did not phosphorylate *cdc2*(6-20)F15K19 suggests that the kinase in the frac-

tions was a tyrosine-specific rather than a dual specificity protein kinase. Similarly, the failure of the kinase of the second hydroxylapatite peak to phosphorylate *cdc2*(6-20)A14 suggested that the second peak contained a Thr-specific rather than a dual specificity kinase. An analogous peptide derived from the amino acid sequence residues 6 to 20 of Cdk5, *cdk5*(6-20) (KLEKIGEGTYGTVFK), was also tested and found to be an efficient substrate for the kinases of both peaks (not shown). The second hydroxylapatite kinase activity peak may represent the putative Cdk inhibitory protein Ser/Thr kinase since all peptides containing a threonine residue corresponding to Thr-14 could serve as efficient substrates, whereas the *cdc2*(6-20) peptide analogue with Thr-14 substituted by alanine was not a substrate. This kinase is designated as the Cdk T14 kinase in this paper.

As there are hundreds of protein Ser/Thr kinases in a typical tissue or cell extract, the possibility that *cdc2*(6-20)F15K19 peptide was phosphorylated by a kinase unrelated to the Cdk T14 kinase had to be examined critically. One criterion for establishing the identity of Thr-14 kinase is that the kinase has to display the ability to inhibit active Cdk kinases. To address this question, a sample of 100,000  $\times$  *g* fraction from 500 g of bovine thymus was processed through successive column chromatography steps including DEAE-Sepharose, hydroxylapatite, and phenyl-Sepharose columns as described in the legend of Fig. 2. Fractions containing *cdc2*(6-20)F15K19 peptide kinase activity from the phenyl-Sepharose column were pooled and applied to a FPLC Mono Q column. Column fractions from the Mono Q column were then analyzed for both the peptide kinase activity and the ability to inhibit a reconstituted Cdk2/protein A-poly(His)-cyclin A (pA-His-cyclin A) kinase. Fig. 2A shows that the Mono Q chromatography profile of the peptide kinase activity correlated closely with the Cdk2/pA-His-cyclin A kinase inhibitory activity, suggesting that the two activities were derived from the same kinase.

In addition, the inhibitory activity of the Cdk T14 kinase was tested on two other members of the Cdc2-like kinase family: sea star oocytes Cdc2/cyclin B kinase (from Upstate Biological Inc.) and a reconstituted neuronal Cdc2-like kinase (Nclk) comprised of GST-Cdk5 and neuronal GST-Nck5a (neuronal Cdk5 activator). Fig. 2, B and C, shows the time courses and the dose dependence of the inhibition of Cdc2/cyclin B and Nclk by the Cdk T14 kinase, respectively. Almost complete inhibition could be achieved by using high concentrations of the Cdk T14 kinase or by a moderate concentration of the kinase with a long incubation time. These results, therefore, further support the suggestion that the *cdc2*(6-20)F15K19 kinase activity represents the putative protein Ser/Thr kinase that inhibits Cdk kinases by phosphorylating the Thr-14 residue in Cdk.

**Purification of the Cdk T14 Kinase**—A purification procedure was developed to purify the Cdk T14 kinase from bovine thymus cytosol. The purification procedure is described in detail under "Materials and Methods." The enzyme was monitored during the purification by its kinase activity toward 0.5 mM *cdc2*(6-20)F15K19 (Table I). Any of the Tyr kinase activity was eliminated by the phenyl-Sepharose column step, as the Thr kinase activity bound to the column at low salt conditions while the Tyr kinase activity did not (not shown). Typically, 0.04–0.05 mg of protein could be recovered from the second Mono Q FPLC column, the final stage of purification, from about 800 g of bovine thymus as the starting material. The overall yield and fold of the enzyme purification are difficult to estimate since the kinase activity in crude thymus extract is undetectable (Table I). The lack of demonstrable kinase activity in the thymus extract was not because the enzyme was too dilute in the sample. The total volume of the thymus extract and that of the

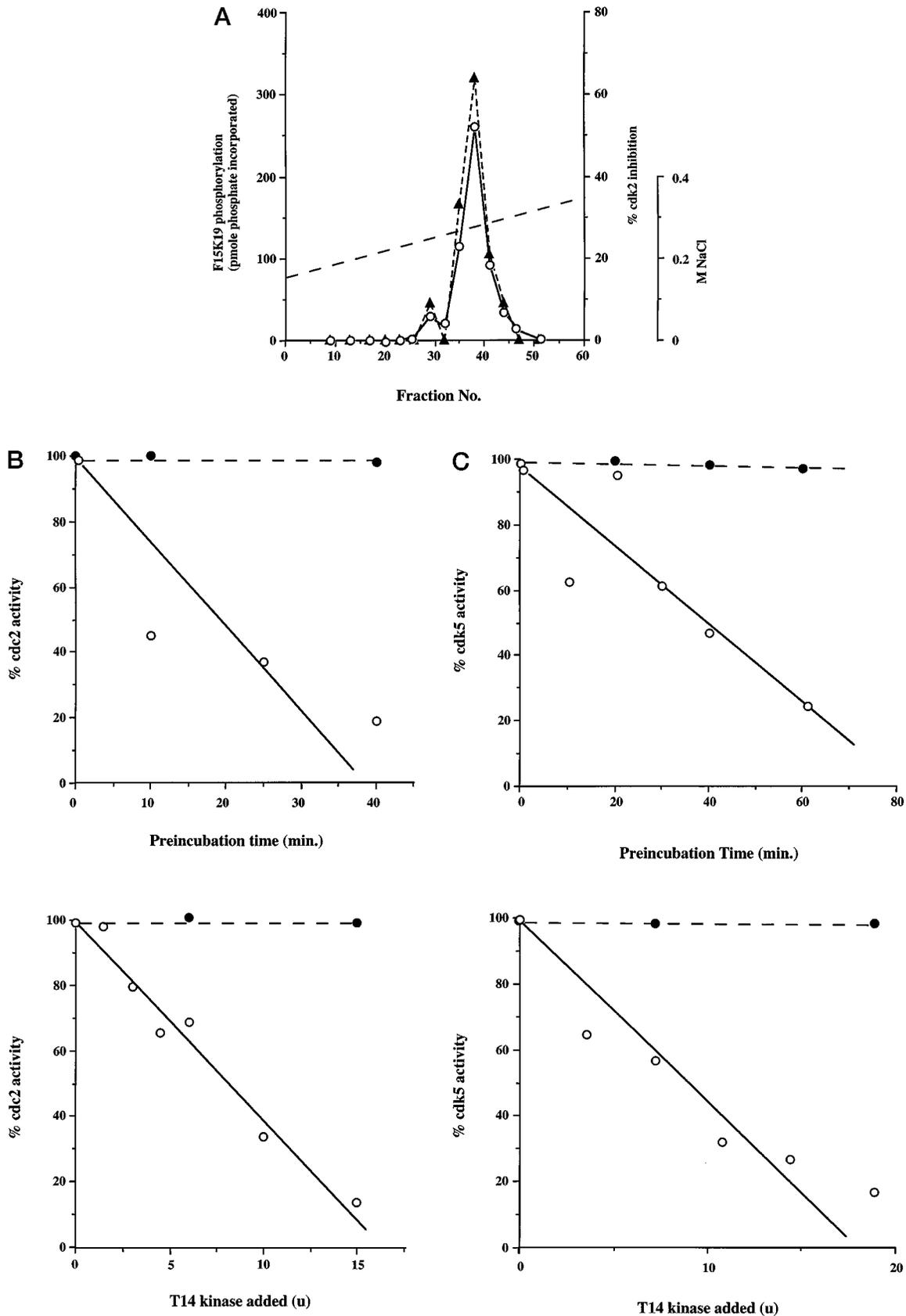


FIG. 2. **Inactivation of Cdk kinases by the Cdk T14 kinase.** A, cytosolic fraction from 500 g of bovine thymus was processed through DEAE-Sepharose and hydroxylapatite column chromatography as shown in Fig. 1. The *cdc2(6-20)F15K19* kinase activity emerged from the hydroxylapatite column was pooled and applied onto a 10-ml phenyl-Sepharose CL-4B column pre-equilibrated with buffer A. The column was washed with the same buffer, and the Thr kinase was eluted with 0–65% 160-ml linear ethylene glycol gradient. Post-phenyl-Sepharose kinase sample was subjected to FPLC Mono Q chromatography. The 6- $\mu$ l aliquots of column fractions were assayed for *cdc2(6-20)F15K19* kinase activity ( $\circ$ ). For the Cdk2/pA-His-cyclin A kinase inactivation assay ( $\blacktriangle$ ), the 6.5- $\mu$ l aliquots of fractions were incubated with 23 units (1 unit = 1 pmol of phosphate transferred/min) of Cdk2/pA-His-cyclin A in 30  $\mu$ l under phosphorylation conditions for 40 min, followed by a 30-min histone H1 peptide

TABLE I  
Purification of the Cdk T14 kinase from bovine thymus cytosol

Purification step	Total protein	Total activity	Specific activity <sup>a</sup>	Yield <sup>b</sup>	Purification <sup>c</sup>
	mg	nmol/min	nmol/min/mg	%	-fold
Crude extract	8148	ND <sup>d</sup>			
DEAE-Sepharose	2210	181	0.08	100	1
Hydroxylapatite	860	280	0.33	155	4
Hydroxylapatite	125	124	0.99	68	12
Phenyl-Sepharose	4.34	37	8.47	20	106
Mono Q	1.3	12	9.2	6.6	115
Superose 12	0.18 <sup>e</sup>	4.7	26	2.6	325
Mono Q	0.045 <sup>e</sup>	5.4	120	3	1500

<sup>a</sup> Specific activity at 0.5 mM cdc2(6–20)F15K19.

<sup>b</sup> Yield of the Cdk T14 kinase is expressed as a percentage of the DEAE-Sepharose pool (100%).

<sup>c</sup> Purification from DEAE-Sepharose pool.

<sup>d</sup> Not detected.

<sup>e</sup> The amount of protein was densitometrically determined.

DEAE-Sepharose fraction where the kinase activity could readily be determined, were about the same.

The enzyme sample emerged from the second FPLC Mono Q column; the last step of purification was highly purified but not yet homogeneous. SDS-PAGE analysis of the column fractions consistently revealed a protein band of apparent molecular mass of 43 kDa which represented the major protein component of the kinase fractions. Fig. 3A shows the protein elution profile and the peptide kinase activity profile as well as Nck inhibitory activity of a typical Mono Q column chromatography. As same as the result in Fig. 2A, both activities comigrated perfectly. An SDS-PAGE analysis of the protein patterns of the column fractions as revealed by silver stain is shown in Fig. 3B. Note the presence of the prominent 43-kDa protein band (indicated by an *asterisk*) whose staining intensity correlated with the peptide kinase activity and Nck inhibitory activity.

To determine the protein concentration of the purified kinase, a sample was subjected to SDS-PAGE, and the gel was stained by Coomassie Brilliant Blue and then analyzed by densitometric measurement. The intensity of the densitometric tracing of the sample was then compared to a protein concentration calibration curve using bovine serum albumin. Using the protein concentration so determined and the kinase activity determined at 0.1 mM ATP and 0.5 mM cdc2(6–20)F15K19, the specific activity of the purified kinase was determined as 0.12  $\mu\text{mol}/\text{min}/\text{mg}$  (Table I). In addition, the densitometric analysis of the purified sample indicated that 50% of total protein of the sample was represented by the 43-kDa protein. The specific activity was, therefore, calculated as 0.24  $\mu\text{mol}/\text{min}/\text{mg}$  if only the 43 kDa was considered. The enzyme was also characterized in terms of its  $K_m$  and  $V_{\text{max}}$  values using the peptide substrate. At the ATP concentration of 0.1 mM, the  $K_m$  of the peptide and  $V_{\text{max}}$  of the enzyme were found to be 0.58 mM and 0.37  $\mu\text{mol}/\text{min}/\text{mg}$ , respectively. The  $K_m$  for ATP was determined at 0.5 mM cdc2(6–20)F15K19 to be 80  $\mu\text{M}$ .

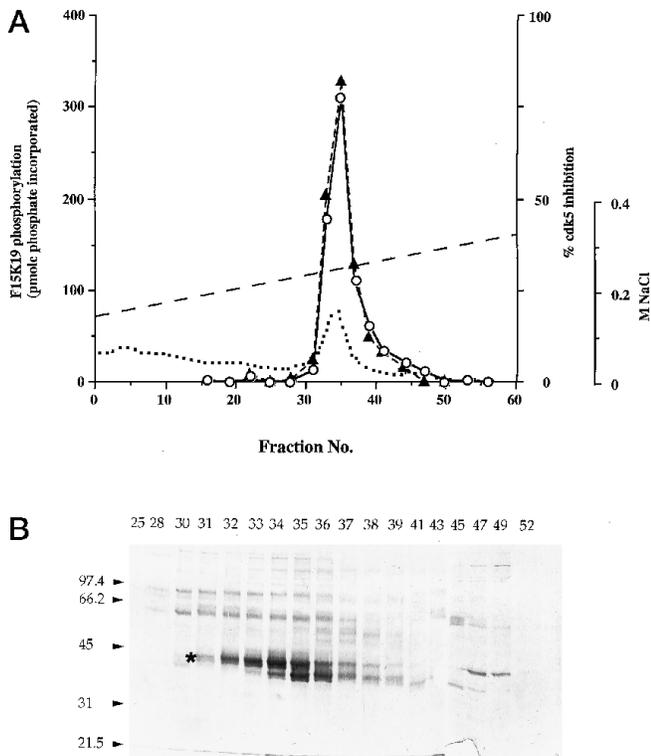
**Correlation of the Cdk T14 Kinase Activity to the Autophosphorylation of the 43-kDa Kinase Protein**—To test whether the 43-kDa protein was indeed the protein kinase, the protein components in a purified kinase sample were separated by SDS-PAGE, and the ability of the individual separated proteins to undergo *in gel* autophosphorylation reaction was then determined. Fig. 4 shows the results of such an experiment. The autoradiogram of a gel displayed a prominent band of apparent

molecular mass of 43 kDa (*right panel*), corresponding to the major protein band (*left panel*). A low intensity radioactive band with an apparent molecular mass of 59 kDa was also seen on the autoradiogram. The 59-kDa protein, however, was not reproducibly observed in purified enzyme samples.

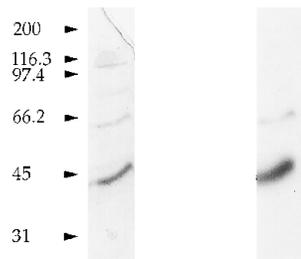
Although the experimental result of Fig. 4 revealed the 43-kDa protein as a protein kinase, it did not necessarily indicate that the enzyme was the kinase responsible for the phosphorylation of cdc2(6–20)F15K19 peptide. During the study of regulatory properties of the purified thymus kinase, we found that relatively high concentrations (millimolar concentrations) of cAMP could cause marked inhibition of the kinase-catalyzed phosphorylation of cdc2(6–20)F15K19 peptide. The other nucleotides tested, including AMP, cGMP, and GMP, showed much weaker inhibitory activity toward the peptide phosphorylation activity. A dose-dependent inhibition of the kinase activity by cAMP as well as those by the other nucleotides are shown in Fig. 5A. The inhibition of the peptide phosphorylation activity occurs at nonphysiological concentrations of cAMP, suggesting that the inhibition is not a physiologically relevant phenomenon. However, we have used this cAMP effect to test whether or not the 43-kDa protein is the kinase responsible for the phosphorylation of cdc2(6–20)F15K19 peptide. Thus, a purified sample of the kinase was subjected to SDS-PAGE, and the effects of the various nucleotides on the *in gel* autophosphorylation of the 43-kDa protein were examined. Fig. 5B shows that the autophosphorylation of the 43-kDa protein, like the kinase activity toward cdc2(6–20)F15K19 peptide, was markedly inhibited by cAMP and weakly inhibited by AMP, cGMP, or GMP. The observation strongly suggests that the 43-kDa protein is the protein kinase catalyzing cdc2(6–20) peptide phosphorylation.

**Phosphorylation of Cdk5 on Thr-14 Residue**—The purified Cdk T14 kinase was characterized in more detail in terms of its phosphorylation of protein substrates, using a Nck sample reconstituted with the bacterially expressed GST-Cdk5 fusion protein and GST-Nck5a, as well as the monomeric GST-Cdk5 as the substrates. As shown in Fig. 6, the Cdk T14 kinase-catalyzed phosphorylation of Nck occurred on GST-Cdk5 (*right panel, lanes 1 and 5*). No Cdk5 phosphorylation was detected in control (*right panel, lanes 2 and 4*). The phosphorylation could not be attributed to autophosphorylation, as the GST-Cdk5 sample used in this experiment had been pretreated with non-

kinase assay. — —, NaCl gradient. *B, upper panel*, 1.5 units of sea star Cdc2/cyclin B kinase was preincubated with 15 units of partially purified Cdk T14 kinase (○) or control, a Mono Q fraction without T14 kinase activity (●) under phosphorylation conditions for various time periods indicated, and then assayed (30 min) for kinase activity using histone H1(9–18) peptide. *Lower panel*, Cdc2/cyclin B (1.5 units) was preincubated with various amounts of the Cdk T14 kinase for 60 min, followed by a 30-min histone H1 peptide phosphorylation assay. *C, upper panel*, 23 units of reconstituted Nck was preincubated with 48 units of the Cdk T14 kinase for various time periods indicated, followed by 10-min histone H1 peptide phosphorylation assay. *Lower panel*, reconstituted Nck (23 units) was preincubated with various amounts of T14 kinase for 60 min, followed by a 10-min histone H1 peptide phosphorylation assay.



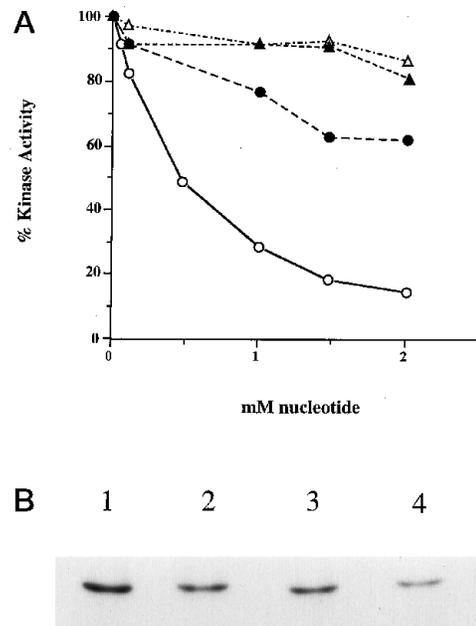
**FIG. 3. Purification of the Cdk T14 kinase.** *A*, post-Superose 12 sample (see "Materials and Methods") of the Cdk T14 kinase was rechromatographed on a FPLC Mono Q column.  $\circ$ , cdc2(6-20)F15K19 phosphorylation by 6  $\mu$ l of column fraction.  $\blacktriangle$ , inactivation of reconstituted Nck (8 units) by 7.5  $\mu$ l of column fraction under the condition described in Fig. 2*A* except that the reaction time of Nck was 20 min.  $\cdots$ , protein profile;  $-\cdots-$ , NaCl gradient. *B*, Mono Q column fractions of *A* (13.5  $\mu$ l) were analyzed by 10% SDS-PAGE/silver staining. The location of the major protein band of 43 kDa is marked by \*.



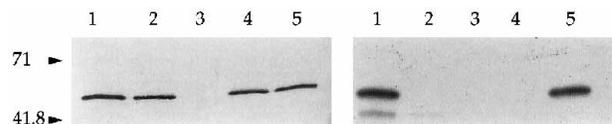
**FIG. 4. In gel autophosphorylation of the Cdk T14 kinase.** Proteins in the purified Cdk T14 kinase sample (48  $\mu$ l) of the Cdk T14 kinase were separated by 10% SDS-PAGE and subjected to an *in gel* phosphorylation reaction (see "Materials and Methods"). *Left*, Coomassie staining of the purified Cdk T14 kinase. *Right*, autoradiogram.

radioactive ATP under autophosphorylation conditions (see "Materials and Methods"). The possibility that the observed phosphorylation arose from a stimulation of the autophosphorylation of Nck reaction by an activator rather than the catalytic action of an exogenous kinase appears to be remote since the bacterially expressed GST-Cdk5 fusion protein which has no kinase activity itself could also be phosphorylated by the Cdk T14 kinase sample (*lane 1*).

To determine the amino acid residue phosphorylated in the peptide and protein substrates, the Cdk T14 kinase-phosphorylated GST-Cdk5 and the peptide substrates cdc2(6-20) and cdk5(6-20) were subjected to phosphoamino acid analysis. As shown in Fig. 7*A, a-c*, all the substrate tested contained phosphothreonine as the only phosphoamino acid. Addition of sodium orthovanadate which would inhibit protein-tyrosine phosphatases that might have contaminated the kinase sample



**FIG. 5. Inhibition of the Cdk T14 kinase activity by cAMP.** *A*, the purified Cdk T14 kinase activity was assayed for cdc2(6-20)F15K19 in the presence of various concentrations of nucleotide.  $\blacktriangle$ , GMP;  $\triangle$ , cGMP;  $\bullet$ , AMP;  $\circ$ , cAMP. *B*, the 43-kDa protein in the purified Cdk T14 kinase sample was visualized by *in gel* autophosphorylation/autoradiography. *In gel* autophosphorylation reaction was carried out in the presence of nucleotide. *Lane 1*, control; *lane 2*, cGMP; *lane 3*, AMP; *lane 4*, cAMP.



**FIG. 6. Analysis of GST-Cdk5 phosphorylation by the Cdk T14 kinase.** GST-Cdk5, reconstituted Nck, or GST-Nck5a was subjected to phosphorylation reactions in the presence of [ $\gamma$ - $^{32}$ P]ATP with or without the Cdk T14 kinase and then isolated by using GSH-agarose. The isolated protein was applied to 7.5% SDS-PAGE and analyzed by Western blot with  $\alpha$ -Cdk5 antibody (*left*) and autoradiography (*right*). *Lane 1*, the Cdk T14 kinase + GST-Cdk5; *lane 2*, GST-Cdk5 alone; *lane 3*, the Cdk T14 kinase + GST-Nck5a; *lane 4*, reconstituted Nck alone; *lane 5*, the Cdk T14 kinase + reconstituted Nck.

used in the phosphorylation reaction had no effect (Fig. 6*A, d*). The same result was obtained for Cdk T14 kinase-phosphorylated Cdk2 protein (not shown).

To further identify the site of phosphorylation of GST-Cdk5 by the putative T14 kinase, the phosphorylated Nck was subjected to tryptic digestion, and the resulting peptide mixture was analyzed by two-dimensional electrophoresis/chromatography and autoradiography. The phosphopeptide map was then compared to that of the control; the peptide cdk5(6-20) was phosphorylated by the purified kinase and then treated with trypsin. Both the control peptide and the tryptic peptide mixture displayed a single intense spot on the phosphopeptide map (not shown). The phosphopeptide of the sample appeared to have electrophoretic and chromatographic properties identical with the phosphopeptide map of a mixture of the control and the sample (the same amount of radioactivity) gave rise to a single spot (Fig. 7*B*). Together, these results indicate that Cdk5 was phosphorylated by the Cdk T14 kinase specifically on Thr-14.

#### DISCUSSION

In the present work, we have identified in bovine thymus cytosol a novel protein kinase that is capable of *in vitro* inac-

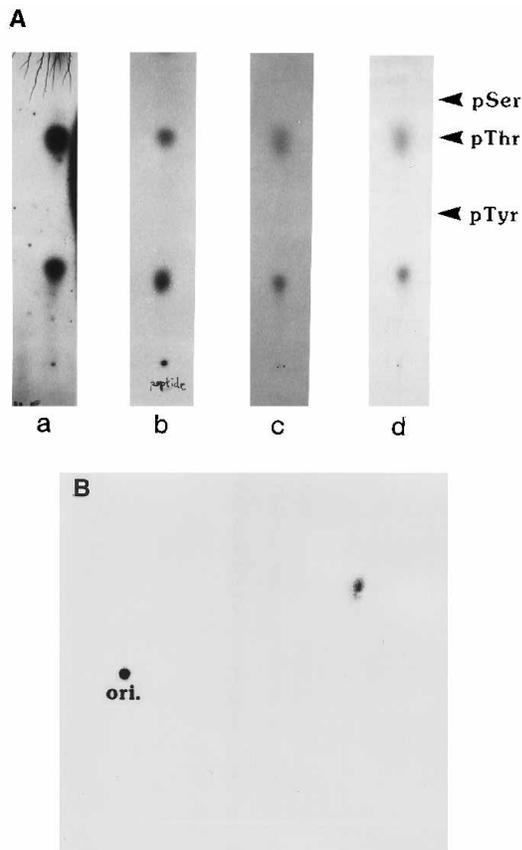


FIG. 7. **Determination of phosphorylation site of Cdk5.** A, phosphoamino acid analysis of Cdk T14 kinase-phosphorylated GST-Cdk5 and peptides. a, Cdk T14 kinase-phosphorylated Cdk5 protein; b, cdc2(6-20); c, cdk5(6-20); d, cdk5(6-20) + 50  $\mu$ M Na<sub>3</sub>VO<sub>4</sub>. B, two-dimensional phosphotryptic peptide map of Cdk5 protein. The tryptic peptide prepared from Cdk T14 kinase-phosphorylated GST-Cdk5 or cdk5(6-20) was standardized by its radioactivity. The mixture of them was then subjected to TLE/TLC.

tivation of a number of Cdc2-like kinases, including Cdc2/cyclin B, Cdk2/cyclin A, and neuronal Cdc2-like kinase (Nck). On the basis of the enzyme specificity toward peptide substrates and chemical characterizations of the enzyme-catalyzed Nck phosphorylation reaction, the novel kinase is suggested to be specific for the negative regulatory threonine residue of Cdks, *i.e.* Thr-14, hence the name Cdk T14 kinase. The cytosolic localization and strict specificity toward Thr-14 clearly distinguish the Cdk T14 kinase identified in this study from the reported dual specificity and membrane-associated kinase (14-16). Since most of the Cdks exist in cell cytosol (17, 18), it is tempting to suggest that the Cdk T14 kinase is the major enzyme responsible for the inhibitory phosphorylation of Cdks on Thr-14. However, additional characterizations of the enzyme, particularly in terms of its activity and regulation in relation to cell cycle progression, have to be carried out to test such a suggestion more rigorously.

The protein kinase has been purified extensively, but not yet to a homogeneous state. Analysis of the purified samples by SDS-PAGE consistently showed a 43-kDa protein that represented about 50% of the total proteins on the gel. On the basis of a number of observations, it may be concluded that the 43-kDa protein is the Cdk inhibitory kinase. The protein kinase nature of the 43-kDa band was revealed by the ability of the protein to undergo autophosphorylation on SDS-PAGE gel (Fig. 4). The *in gel* autophosphorylation and protein stain intensity of the 43-kDa band on SDS-PAGE invariably correlated with the protein kinase activity in the Mono Q column eluents,

suggesting strongly that this protein kinase is responsible for the Cdk kinase activity (Fig. 3A). Furthermore, we observed that the kinase was inhibited by cAMP and weakly by AMP, and that the *in gel* autophosphorylation of the 43-kDa protein was inhibited similarly by the nucleotides (Fig. 5).

It should be noticed that the inhibition of the kinase by cAMP requires close to millimolar concentrations of the nucleotide, which are significantly higher than those found in cells. The observation suggests that the inhibition of the kinase by cAMP is not a physiologically relevant effect. On the other hand, the possibility that physiological concentrations of cAMP could affect the kinase under special cellular conditions and/or in concert with other endogenous regulatory factors cannot be excluded. Cdks as well as protein kinases and phosphatases that are involved in regulation of Cdks are all controlled by multiple regulatory factors (for review, see Ref. 31). How these multiple factors interact to modulate each other's effects is far from clear.

The detection of a Thr-14-specific Cdk inhibitory kinase has made it possible to study the molecular mechanism of the interaction of Thr-14 phosphorylation with other regulatory mechanisms that modulate the activities of Cdks. For example, we have shown that Thr-14 phosphorylation of Cdk5 is independent of the binding of Cdk5 to its specific activator. More importantly, the present study has unambiguously established that phosphorylation of Thr-14 alone is sufficient to bring about close-to-complete inhibition of many cdc2-like kinases (Fig. 2, B and C). The observation strongly supports the "double block model" proposed by Krek and Nigg (9). These investigators observed that overexpression of A14F15 double-site Cdc2 mutant in HeLa cells caused severe premature activation whereas overexpression of single-site Cdc2 mutant of either Ala-14 or Phe-15 had only a mild effect on the cell cycle. Thus, they proposed that phosphorylation of either Thr-14 or Tyr-15 of Cdc2 could restrain the premature activation of Cdc2 kinase.

The phosphorylation on Thr-14 of Cdc2 had been thought to be a unique phosphorylation regulatory mechanism in higher eukaryotes since Cdc2 in yeasts was not found to be phosphorylated on Thr-14. However, Haese *et al.* (32) recently showed that under certain conditions, such as overexpression of Wee1 kinase, Thr-14 phosphorylation of Cdc2 could be demonstrated in *S. pombe*. The phosphorylation of Thr-14 in yeasts appears to be dependent on the prior phosphorylation of Tyr-15 and the presence of active Wee1 kinase. Although Mik1 has been suggested to play a redundant role to Wee1 in Tyr-15 phosphorylation in yeasts, overexpression of Mik1 did not result in the phosphorylation of Cdc2 on Thr-14 residue. These results are compatible with the suggestion that Thr-14 phosphorylation in yeasts is catalyzed by Wee1 kinase. In view of the interlocking regulation of cell cycle factors, the possibility that a distinct yeast Thr-14 kinase whose activation is coupled to the activity of Wee1 kinase should also be considered. The identification of the Cdk Thr-14 kinase of the present study in terms of its primary structure may aid in the search for the putative yeast T14 kinase.

The identification and quantification of the Cdk T14 kinase during purification depended on the use of specific peptide substrates: cdc2(6-20) and a Tyr-negative peptide analogue (*i.e.* cdc2(6-20)F15K19). Peptides as modeled kinase substrates should be applied cautiously. We previously showed that Src family kinases catalyzed highly efficient phosphorylation of cdc2(6-20) on Tyr-15 (29, 30), yet they failed to catalyze the phosphorylation of Cdc2 protein to any significant extent.<sup>2</sup> In the present instance, however, the enzyme purified

<sup>2</sup> C. M. E. Litwin, unpublished observation.

on the basis of its peptide phosphorylation activity was found to catalyze the phosphorylation of relevant protein substrates and to elicit the expected effect on their activities. A bacterially expressed truncated form of human Wee1 kinase has been reported to phosphorylate Cdc2 N-terminal peptide (13), but the intact form of Wee1 kinase has not been shown to phosphorylate the peptide. The possibility that the enzyme purified in this study may represent a proteolytically derived form of the kinase has not been ruled out.

As Nck is a relatively new addition to the family of Cdc2-like kinases, its regulatory properties have only begun to be investigated (19–21, 25, 28, 33). On the basis of sequence homology, it has been suggested that the various regulatory phosphorylation reactions modulating Cdc2 kinase activity may also be involved in the regulation of Nck activity, for instance, as is the case for Cdk2 (34, 35). However, we have recently shown that the activation of Cdk5 by its specific activator, neuronal Cdk5 activator (Nck5a), is independent of Cdk5 phosphorylation (20, 25). This is in contrast to a number of well-documented cases of cyclin activation of Cdks where the kinase activation depends on the phosphorylation of the Cdk protein on a specific threonine residue by Cdk activating kinase (22–24). On the other hand, the observation that Nck can be phosphorylated on Thr-14 *in vitro* by the Cdk T14 kinase to result in the enzyme inactivation supports the suggestion that the mechanisms of negative regulatory phosphorylations are conserved in Nck.

A number of studies have suggested that phosphorylation of Cdc2 on tyrosine and threonine residues takes place in a cyclin-dependent manner (36–39). On the other hand, the phosphorylation of Cdk5 by the purified Cdk T14 kinase could occur in the absence of its partner, Nck5a (Fig. 5). While this apparent difference between Cdc2 and Cdk5 phosphorylation may be attributed to the unique structure of Cdk5 and Nck5a, which shows very limited sequence homology to cyclins, the possibility that it is a result of a difference in experimental conditions should not be overlooked (24). The control of Cdk activities involves the interplay of various mechanisms including cyclin activation, inhibition by specific protein inhibitors, the activation by Cdk activating kinase-catalyzed Cdk phosphorylation and the inhibitory phosphorylations on Thr-14 and Tyr-15 (reviewed in Ref. 31). Thus, the question as to the favored conditions of the Cdk phosphorylation by the Cdk T14 kinase has to be addressed by systematic studies of the effect of the various regulatory factors and conditions on the phosphorylation reactions.

The main thrust of the present work is to demonstrate the existence of a novel protein kinase that is capable of *in vitro* inhibition of a number of Cdc2-like kinases. The general characterizations of the enzyme are compatible with the suggestion that this kinase is the physiological kinase for the Cdk Thr-14 phosphorylation. Future work will have to be carried out toward testing this suggestion and elucidating how this enzyme contributes to the regulation of the Cdc2 family kinases in the cells. It should be noticed that the significance of this kinase may be broader than the regulation of Cdk family kinases. We

have carried out gene library search for cdc2(6–20) homologous sequences with serine or threonine at the position corresponding to Thr-14 of Cdc2 and uncovered a large number of protein kinases and other proteins that contain such sequences (results not shown).

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