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 p34cdc2, 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 Cdk2/cyclin B, Cdk2/cyclin A, and neuronal Cdk2-like kinase (Nclk), a heterodimer of Cdk5 and neuronal Cdk5 activator (Nck5a), under phosphorylation conditions. The phosphorylation of Nclk 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 Nclk revealed that Thr-14 of Cdk5 was the sole site of protein phosphorylation. The results suggest that this thymus kinase is a novel Cdk inhibitor protein kinase, distinct from the recently cloned dual functional and membrane-associated Cdk2 inhibitory kinase, Myt1 (Mueller, P. R., Coleman, T. R., Kumagai, A., and Murphy, 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, Cdk5 (6). In addition to depending on cyclins for activity, Cdk5s 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 G2 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 Cdk2 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 Cdk2 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 Cdk5s are cell cycle regulators. Neuronal Cdc2-like kinase (Nclk), 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, Ndk 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 Cdk5 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 Ndk. Characterization of the kinase-catalyzed phosphorylation of Ndk 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 Nclk, 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, 20 mM dithiothreitol, 0.01% soybean trypsin inhibitor, and 10 mM NaF).
1 mg DTT, 0.3 mg/ml benzamidine, 1 mg/ml leupeptin, 2 mg/ml anti-
pain, 0.2 mg/ml phenylmethylsulfonyl fluoride containing 0.1 mg/ml soy-
bean trypsin inhibitor. The homogenate was centrifuged at 10,000 \times g for
30 min, and the supernatant, collected thorough glass wool, was
centrifuged further at 100,000 \times g for 40 min. The 100,000 \times g super-
natant 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/30-linear NaCl gradient. The kinase-containing fractions were
pooled and loaded onto a 100-ml hydroxylapatite column pre-equili-
brated with buffer A. After washing with buffer A, the column was eluted by 0–0.2 M/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-linear ethylene glycol gra-
dient using a peristaltic pump (1 ml/min). The kinase-containing frac-
tions 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-linear NaCl gradient elution. To apply to the FPLC Super-
sieve-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 sample was equilibrated and run with buffer A containing 0.25
M NaCl. The pooled kinase fraction was finally rechromatographed on
the FPLC Mono Q column.

In Vitro Reconstitution of Ndk and Cdk2 Cyclin A—Reconstitution
of active Ndk from the bacterially expressed GST-Ndk5 and GST-
Ndk5a (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
GST-Cdk5 complexed with GST-Nck5a or GST-Nck5a alone, was incu-
bated for 30 min at 30°C in 10 ml containing kinase buffer and 0.4 mM
ATP. Then, the mixture of 1 ml of 20 mg/ml bovine serum albumin, 2.5
M of [γ-32P]ATP (25 ml), 3 ml of kinase buffer, and 2.5 ml of water
was added to the mixture, followed by the addition of 21 ml of T14
kinase (total volume, 40 ml). The mixture was incubated further at
30°C for 90 min. After incubation, 20 ml of 10 mM ATP, 20 ml of 0.1 mM
bovine serum albumin, 0.1 ml of GST-agarose beads (1:1 slurry in PBS, Sigma) were added and mixed
for 30 min. The beads was washed extensively with PBS containing 1
ml DT and PBS containing 1 ml DTT, 0.35 M NaCl, 1% Triton X-100.

The proteins bound to beads were eluted with 10 mM reduced glutath-
one (50 ml) Tris-HCl, pH 8.0, and subjected to 7.5% SDS-PAGE.
GST-Cdk5 was visualized by Western blot using α-Cdk5 polyclonal
antibody, and phosphoprotein was detected by autoradiography.

Phosphoamino Acid and Phosphopeptide Analysis—For phos-
phoamino acid analysis, phosphorylated GST-Cdk5 was sliced from the
blot (polyvinylidene difluoride) and hydrolyzed in 6 M HCl for 2 h at
110°C. The solution containing phosphoamino acid was dried to remove
HCl and dissolved in 10 μl of phosphoamino acid standard solution
containing 2% 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 μl of 0.1 mM
ammonium bicarbonate, 0.1 mM CaCl2 containing 1.5 μg of N-tosyl-
phenylalanyl chloromethyl ketone-treated trypsin and incubated at 37°C
for 24 h. The supernatant was lyophilized, and recovered phosphopeptide
was analyzed by two-dimensional electrophoresis/chromatogra-
phy (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 cdk5c6-
20F15K19 was incubated for 30 min at 30°C. The gel was washed with
150 μl of 1 M NaCl, 1% Triton X-100, 0.1% triton X-100, and 0.1% trit-
fluoroacetic 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 p34cdc2 with the amino acid sequence of KVEK1GEGTYGVVY, 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

1 The abbreviations used are: DTT, dithiothreitol; GST, glutathione
transferase; PBS, phosphate-buffered saline; PAGE, polyacrylamide
gel electrophoresis; FPLC, fast protein liquid chromatography; MOPS,
4-morpholinopropanesulfonic acid.
 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-Sephrose column containing the overlapping kinase activities were pooled and further analyzed. The pooled sample was fractionated on a hydroxyapatite 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-Sephrose sample gave rise to two peaks of cdc2(6–20) peptide kinase activity on the hydroxyapatite 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 fractions was a tyrosine-specific rather than a dual specificity protein kinase. Similarly, the failure of the kinase of the second hydroxyapatite peak to phosphorylate cdc2(6–20)A14 suggested that the second peak contained aThr-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) (KLEKIGEYGTGTFK), was also tested and found to be an efficient substrate for the kinases of both peaks (not shown).

The second hydroxyapatite 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 g fraction from 500 g of bovine thymus was processed through successive column chromatography steps including DEAE-Sephrose, hydroxyapatite, 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 an 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 Cdk5 (Ndk) 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 Ndk 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 Cdk5.

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
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-ml aliquots of column fractions were assayed for cdc2(6–20)F15K19 kinase activity (E). For the Cdk2/pA-His-cyclin A kinase inactivation assay (A), the 6.5-μl aliquots of fractions were incubated with 23 units (1 unit = 1 pmol of phosphate transferred/min) of Cdk2/pA-His-cyclin A in 30 μl under phosphorylation conditions for 40 min, followed by a 30-min histone H1 peptide
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 µmol/min/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 µmol/min/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 µmol/min/mg, respectively. The $K_m$ for ATP was determined at 0.5 mM cdc2(6–20)F15K19 to be 80 µM.

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 activity 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. 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 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 Cdk T14 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-

### Table I

| Purification step | Total protein | Total activity | Specific activity | Yield | Purification |
|------------------|--------------|---------------|------------------|-------|--------------|
| Crude extract    | 8148         | ND            | 0.00             | 100   | 1            |
| DEAE-Sepharose   | 2210         | 381           | 0.08             | 155   | 4            |
| Hydroxylapatite  | 860          | 288           | 0.33             | 68    | 12           |
| Hydroxylapatite  | 125          | 124           | 0.99             | 106   |              |
| Phenyl-Sepharose | 4.34         | 37            | 8.47             | 6.6   | 115          |
| Mono Q           | 1.3          | 12            | 9.2              | 2.6   | 325          |
| Superose 12      | 0.18         | 4.7           | 26               | 120   | 3            |
| Mono Q           | 0.045        | 5.4           | 8.0              | 1500  |              |

*Specific activity at 0.5 mM cdc2(6–20)F15K19.

**Yield of the Cdk T14 kinase is expressed as a percentage of the DEAE-Sepharose pool (100%).

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Cdk Inhibitory Ser/Thr Kinase
radioactive ATP under autophosphorylation conditions (see "Materials and Methods") of the Cdk T14 kinase was rechromatographed on a FPLC Mono Q column. O, cdc2(6–20)F15K19 phosphorylation by 6 µl of column fraction. ▲, inactivation of reconstituted Ndk (8 units) by 7.5 µl of column fraction under the condition described in Fig. 2A except that the reaction time of Ndk was 20 min. ---, protein profile; - - - , NaCl gradient. B, Mono Q column fractions of A (13.5 µl) were analyzed by 10% SDS-PAGE/silver staining. The location of the major protein band of 43 kDa is marked by *.

FIG. 3. Purification of the Cdk T14 kinase. A, post-Superase 12 sample (see "Materials and Methods") of the Cdk T14 kinase was rechromatographed on a FPLC Mono Q column. ▲, cdc2(6–20)F15K19 phosphorylation by 6 µl of column fraction. ▲, inactivation of reconstituted Ndk (8 units) by 7.5 µl of column fraction under the condition described in Fig. 2A except that the reaction time of Ndk was 20 min. ---, protein profile; - - - , NaCl gradient. B, Mono Q column fractions of A (13.5 µl) 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 µl) of the Cdk T14 kinase were separated by 10% SDS-PAGE and subjected to an in gel phosphorylation reaction (see "Materials and Methods"). Left, Coomasie 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 Ndk 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 phosophoamino acid analysis. As shown in Fig. 7A, 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 used in the phosphorylation reaction had no effect (Fig. 6A, 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. 7B). 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-
suggested 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 Cdc2 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.2 In the present instance, however, the enzyme purified

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2 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 phos-
phorylate 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 Ndk is a relatively new addition to the family of Cdc2-like
kinases, its regulatory properties have only begun to be investi-
gated (19–21, 25, 28, 33). On the basis of sequence homology,
it has been suggested that the various regulatory phosphoryla-
tion reactions modulating Cdc2 kinase activity may also be
involved in the regulation of Ndk 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 phosphoryla-
tion (20, 25). This is in contrast to a number of well-docu-
mented 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 Ndk 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 Ndk.

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 phos-
phorylation 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 possi-
ability 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 activa-
tion by Cdk activating kinase-catalyzed Cdk phosphorylation
and the inhibitory phosphorylations on Thr-14 and Tyr-15 (re-
viewed in Ref. 31). Thus, the question as to the favored condi-
tions 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 char-
acteristics 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 to-
ward 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 correspond-
ing 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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REFERENCES
1. Draetta, G. (1990) Trends Biochem. Sci. 15, 378–383
2. Murray, A. W., and Kirschner, M. W. (1989) Science 246, 614–621
3. Nurse, P. (1990) Nature 344, 503–508
4. Pine, J., and Hunter, T. (1990) New Biol. 2, 389–401
5. Hunt, T. (1989) Curr. Opin. Cell Biol. 1, 268–274
6. Norbury, C., and Nurse, P. (1992) Annu. Rev. Biochem. 61, 441–470
7. Gould, K., and Nurse, P. (1989) Nature 342, 39–45
8. Krek, W., and Nigg, E. (1991) EMBO J. 10, 305–316
9. Krek, W., and Nigg, E. (1991) EMBO J. 10, 3331–3341
10. Norbury, C., Blow, J., and Nurse, P. (1991) EMBO J. 10, 3321–3329
11. Parker, L. L., Atherton-Fessler, S., and Piwiewska-Worms, H. (1992) Proc. Natl.
Acad. Sci. U. S. A. 89, 2917–2921
12. Igarashi, M., Nagata, A., Jinn, S., Suto, K., and Okayama, H. (1991) Nature
353, 80–83
13. Parker, L. L., and Piwiewska-Worms, H. (1992) Science 257, 1955–1957
14. Kombluth, S., Sebastian, B., Hunter, T., and Newport, J. (1994) Mol. Biol. Cell
5, 273–282
15. Atherton-Fessler, S., Feng, L., Gabrielli, B., Lee, M. S., Peng, C.-Y., and
Piwiewska-Worms, H. (1994) Mol. Biol. Cell 5, 899–901
16. Mueller, P. R., Coleman, T. R., Kumagai, A., and Durphy, W. G. (1995) Science
270, 86–90
17. Rattner, J. B., Lew, J., and Wang, J. H. (1990) Cell. Mol. Cytochemistry 17,
227–235
18. Ooka, K., Hisanaga, S., Okano, T., Tachibana, K., and Kishimoto, T. (1992)
EMBO J. 11, 1763–1772
19. Tsai, L.-H., Delalle, I., Caviness, V. S., Jr., Chae, T., and Harlow, E. (1994)
Nature 371, 419–423
20. Lew, J., Huang, Q.-Q., Qi, Z., Winkfein, R. J., Aebersold, R., Hunt, T., and
Wang, J. H. (1994) Nature 371, 423–426
21. Ishiguro, K., Kobayashi, S., Omori, A., Takamatsu, M., Yonekura, S., Anzai,
H., and Uchida, T. (1994) FEBS Lett. 342, 203–208
22. Solomon, M., Lee, T., and Kirschner, M. W. (1992) Mol. Biol. Cell 3, 13–27
23. Fesquet, D., Labbe, J.-C., Derancourt, J., Capony, J.-P., Galas, S., Girard, F.,
Lorca, T., Shuttleworth, J., Doree, M., and Cavardore, J.-C. (1993) EMBO J.
12, 3111–3121
24. Poon, R. Y. C., Yamashita, K., Adamczewski, J. P., Hunt, T., and Shuttleworth,
J. (1993) EMBO J. 12, 3123–3132
25. Qi, Z., Huang, Q.-Q., Lee, K.-Y., Lew, J., and Wang, J. H. (1995) J. Biol. Chem.
270, 10847–10854
26. Litwin, C. M. E., Cheng, H., and Wang, J. H. (1991) J. Biol. Chem. 266,
2587–2596
27. Hunter, T., and Sefton, B. (1980) Proc. Natl. Acad. Sci. U. S. A. 77, 1311–1315
28. Lew, J., Winkfein, R. J., Paule, H. K., and Wang, J. H. (1992) J. Biol.
Chem. 267, 25922–25926
29. Cheng, H.-C., Nishio, H., Hatase, O., Ralph, S., and Wang, J. H. (1992) J. Biol.
Chem. 267, 9248–9256
30. Cheng, H.-C., Matsuura, I., and Wang, J. H. (1993) Mol. Cell. Biochem. 127–128,
103–112
31. Morgan, D. O. (1995) Nature 374, 131–134
32. Haas, G. J. D., Walworth, N., Carr, A. M., and Gould, K. L. (1995) Mol. Biol.
Cell 6, 371–385
33. Lew, J., Beaudette, K., Litwin, C. M. E., and Wang, J. H. (1992) J. Biol.
Chem. 267, 13383–13390
34. Gu, Y., Rosenblatt, J., and Morgan, D. O. (1992) EMBO J. 11, 3995–4005
35. Sebastian, B., Kakizuka, A., and Hunter, T. (1993) Proc. Natl. Acad. Sci.
U. S. A. 100, 3521–3524
36. Solomon, M. J., Glotzer, M., Lee, T. H., Phillip, M., and Kirschner, M. W.
(1990) Cell 63, 1013–1024
37. Parker, L. L., Atherton-Fessler, S., Lee, M. S., Ogg, S., Falk, J. L., Swenson,
K. I., and Piwiewska-Worms, H. (1991) EMBO J. 10, 1255–1263
38. Meijer, L., Azzi, L., and Wang, J. Y. J. (1991) EMBO J. 10, 1545–1554
39. Watanabe, N., Broome, M., and Hunter, T. (1990) EMBO J. 14, 1787–1891