A Synthetic Peptide Derived from p34<sup>cdc2</sup> Is a Specific and Efficient Substrate of src-Family Tyrosine Kinases*

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Heung-Chin Cheng‡‡, Hajime Nishio‡‡, Osamu Hatase‡, Stephen Ralph**, and Jerry H. Wang‡‡‡

From the ‡Medical Research Council Group in Signal Transduction, Department of Medical Biochemistry, Faculty of Medicine, University of Calgary, Calgary, Alberta, T2N 4N1, Canada; the **Department of Physiology, Kagawa Medical School, Isehobe, Miki-cho, Kita, Kagawa, 761-07, Japan; and the ‡‡Melbourne Tumour Biology Branch, Ludwig Institute for Cancer Research, P. O. Royal Melbourne Hospital, Parkville, Victoria 3050, Australia

A peptide derived from p34<sup>cdc2</sup>, cdc2(6-20)NH<sub>2</sub> with the amino acid sequence of KVEKIGETGYVVK-amide, was found to be a specific and efficient substrate for a pp60<sup>src</sup>-related protein tyrosine kinase from bovine spleen (STK). Glu-12 and Thr-14 were identified to be substrate specificity determinants in this peptide (Cheng, H.-C., Litwin, C. M. E., Hwang, D. M., and Wang, J. H. (1991) J. Biol. Chem. 266, 17919–17925). In this study, we demonstrated the presence of the cdc2 kinase family of the mammalian cell. The cdc2 kinase family includes members of the src-family of tyrosine kinases but none of the other non-receptor-linked and membrane-associated src-family. There are at least identified src-related tyrosine kinases, they are src, yes, fyn, lyn, lck, hck, fyn, and blk protooncogene products (1, 2). All members of the src-family tyrosine kinases have molecular mass in the range of 55 to 60 kDa and myristoylated glycine residues at the amino termini. With the exception of the variable regions of about 80 amino acid residues located at the amino-terminal portion, they are highly homologous over most of the protein structures (3). Among the various regions in the homologous structures of the src-family tyrosine kinases, the autophosphorylation site is one of the regions showing the highest significant activity toward cdc2(6-20)NH<sub>2</sub>, they were substrate specificity determinants.

A large number of cellular protein tyrosine kinase genes have been cloned and sequenced. These kinases may be placed into three general classes: (i) the membrane receptor protein tyrosine kinases including among many of its members the extensively characterized EGF1 and insulin receptors; (ii) the non-receptor-linked and cytosolic protein tyrosine kinases with Ab1 and Fes protooncogene products as the representative members; and (iii) the non-receptor-linked and membrane-associated src-family. There are at least identified src-related tyrosine kinases, they are src, yes, fyn, lyn, lck, hck, fyn, and blk protooncogene products (1, 2). All members of the src-family tyrosine kinases have molecular mass in the range of 55 to 60 kDa and myristoylated glycine residues at the amino termini. With the exception of the variable regions of about 80 amino acid residues located at the amino-terminal portion, they are highly homologous over most of the protein structures (3). Among the various regions in the homologous structures of the src-family tyrosine kinases, the autophosphorylation site is one of the regions showing the highest significant activity toward cdc2(6-20)NH<sub>2</sub>, they were substrate specificity determinants.

1 The abbreviations used are: EGF, epidermal growth factor; p34<sup>cdc2</sup>, cell division cycle gene product; EGF-R, epidermal growth factor receptor; pp60<sup>src</sup>, the 60-kDa cellular homolog of the transforming protein of the Rous sarcoma virus; lck, the protooncogene encoding the T cell protein tyrosine kinase, p56<sup>pp60</sup>src; STK, the pp60<sup>src</sup>-related protein tyrosine kinase isolated from bovine spleen (8); c-src, cellular homolog of oncogene product from Gardner-Rasheed feline sarcoma virus (1); lyn and fyn are members of the src-family of tyrosine kinases and are expressed predominantly or uniquely in cells of hemopoietic origin; p46<sup>LYK</sup>, the 43-kDa oncogene product of Abelson murine leukemia virus (v-abl); Fes, Gardner-Arnstein and Snyder-Thelen feline sarcoma viruses; rask(436-468), a synthetic peptide with a sequence of YVYKETIGVYSVCKRCVHK derived from a segment in the putative ATP-binding site of mouse ribosomal S6 kinase I (8, 19); cdc2(6-20)NH<sub>2</sub>, a synthetic peptide with a sequence of KVEKIGETGYVVK-amide derived from the segment containing the regulatory tyrosine residue in p34<sup>cdc2</sup>[Val<sup>14</sup>], cdc2(6-20)NH<sub>2</sub>[Ser<sup>14</sup>], cdc2(6-20)NH<sub>2</sub>[Val<sup>14</sup>Ser<sup>14</sup>], cdc2(6-20)NH<sub>2</sub>[Val<sup>14</sup>Ser<sup>14</sup>Val<sup>14</sup>Ser<sup>14</sup>], a random copolymer of glutamate and tyrosine with a glutamate to tyrosine molar ratio of 4:1 (5); α-src(416) antibody, an antibody raised against a synthetic peptide derived from the consensus autophosphorylation site of pp60<sup>src</sup>-related protein tyrosine kinases (10); poly(Glu/Tyr), a random copolymer of glutamate and tyrosine with a glutamate to tyrosine molar ratio of 4:1 (5); α-src(416) antibody, an antibody raised against a synthetic peptide derived from the consensus autophosphorylation site of pp60<sup>src</sup>-related protein tyrosine kinases (7); TK-I, TK-II, TK-III, and TK-IV, the first, second, third, and fourth protein tyrosine kinase peaks eluted from the column in hydroxylapatite column chromatography of membrane extract of bovine thymus, TK-I and TK-II were subsequently identified as lck and fyn protein kinases; FPLC, fast protein liquid chromatography; mAb, monoclonal antibody; Heps, 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid; SDS, sodium dodecyl sulfate.

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†† Recipient of a postdoctoral scholarship from the Japanese Ministry of Education.

‡‡ Alberta Heritage Foundation for Medical Research Scientist. To whom correspondence and reprint requests should be addressed.
degree of sequence homology (1).

While the structural characterization of the various protein tyrosine kinases has been extensive, progress in the elucidation of catalytic properties of these enzymes is limited. In particular, substrate specificities of protein tyrosine kinases are poorly defined. Biochemical studies of protein tyrosine kinases have frequently used the so-called general tyrosine kinase substrates such as angiotensin and the poly(Glu/Tyr) copolymer (4, 5). The implication of such a practice is that tyrosine kinases are highly promiscuous in terms of their in vitro substrate specificity (6).

We have recently purified a pp60" src-related protein tyrosine kinase from bovine spleen (STK) to apparent homogeneity (7). On the basis of immunological characterization of the present study, STK appears to be the bovine homolog of pp60" (8), a B-cell enriched src-family protein tyrosine kinase. One unique property of the bovine pp60" is that it showed an unusually high activity toward a synthetic peptide, cdc2(6-20)NH2, derived from p34co, the protein serine/threonine kinase that plays a key role in the control of cell division (8). The activity of cdc2(6-20)NH2 has been shown to be regulated by phosphorylation-dephosphorylation at a tyrosine residue, Tyr-15, in a cell cycle-dependent manner (9). Significantly, the phosphorylation of an extension analog of cdc2(6-20)NH2 peptide by pp60" also occurs at Tyr-15 (8). The specificity of cdc2(6-20)NH2 as a spleen tyrosine kinase substrate has been further defined by using the synthetic peptide approach. A highly homologous peptide derived from Ss ribosomal protein kinase (19), rsk(436-456), was shown to be a very poor substrate of pp60". An analog of cdc2(6-20)NH2 with both Glu-12 and Thr-14 substituted by the corresponding amino acid residues of rsk(436-456) was found to be as poor a substrate as rsk(436-456) for pp60" (8).

The present study was initiated to examine whether the high efficiency and specificity of cdc2(6-20)NH2 peptide phosphorylation was a property unique to pp60". The evidence suggests that this property is not unique to pp60" but, instead, is shared by src-family tyrosine kinases. This property can therefore be used to differentiate src-family tyrosine kinases from other protein tyrosine kinases. In addition, we have demonstrated and fractionated multiple protein tyrosine kinases of distinct substrate specificities in the membrane extract of bovine thymus. Among the thymus membrane protein tyrosine kinases, two were demonstrated to be immunologically indistinguishable from the protein products of the protooncogenes, lck and fyn.

**Experimental Procedures**

**Materials**—The pp60"-related spleen tyrosine kinase and the partially purified epidermal growth factor receptor were prepared as described previously (7). The purified pp60" from human platelets (10) and the recombinant p43" containing the catalytic domain of the v-abl oncogene-encoded protein (11) were obtained from Oncogene Science (Manhasset, NY). RR-SRC was a synthetic peptide derived from the autophosphorylation site of pp60" (12), and it was obtained from Peninsula Laboratories Inc. Poly(Glu/Tyr), a random copolymer of glutamic acid and tyrosine with the glutamate to tyrosine molar ratio of 4:1 (5), was from Sigma. The cdc2(6-20)NH2 peptide and its substitution analogs as well as rsk(436-456) were synthesized as previously described (8). The a-lck antibody was a kind gift of Dr. Andre Veillette of McGill Cancer Center, McGill University. It was raised against a synthetic peptide with a sequence corresponding to amino acid residues 39-64 of the murine lck sequence (13). The peptide sequence is not shared by other protein tyrosine kinases. The specificity of the antibody was tested previously by the ability of 1 μg/ml of the synthetic peptide to block recognition of the pp60" by immunoprecipitation and immunoblotting (13). The preparation and characterization of the a-src(416) antibody directed against a synthetic peptide with sequence corresponding to the consensus autophosphorylation site of src-family tyrosine kinases were described in our previous report (7). The a-src(416) antibody was shown to cross-react specifically with the spleen tyrosine kinase and pp60" and failed to interact with EGF-receptor and several serine/threonine protein kinases including cAMP-dependent protein kinase, myosin light chain kinase, and casein kinases I and II (7). The a-gef antibody against a synthetic peptide derived from the amino-terminal 2-25 residues of the c-gef protein was a kind gift of Dr. Andrew Laudano of the University of New Hampshire (21). The monoclonal a-pp60" antibody (mAb 327) was kindly provided by Dr. Donald Fujii of the University of Kentucky; its characterization and reactivity were described previously by Lipsich et al. (22). The specific a-lyn antibody was raised by immunizing rabbits with a glutathione-S-transferase/lyn fusion protein which contained amino acids 7 to 430 of murine pp60" (27). The specificity of the a-lyn antibody has been tested using translated lyn, lck, and ltk kinases produced with the insect cell lyse tyrosine kinase, and the a-pp60"NH2 peptide was immuno precipitated the lyn kinase (data not shown). The monospecific a-fyn antibody was from Upstate Biotechnology, Inc. (New York), and it was raised against a synthetic peptide with a sequence corresponding to residues 35-51 of human lyn; this sequence is unique to lyn only and does not exist in any other tyrosine kinases (28). All other antibodies were either obtained from commercial sources or were described as in our previous reports (7, 8). The FPLC Mono Q ion exchange column (1 ml size) was from Pharmacia LKB Biotechnology Inc. Pansorbin was from Boehringer Mannheim Canada.

**Tyrosine Kinase Assays**—The enzyme assays were carried out at 30 °C for 15 to 20 min in a 100-μl volume containing the assay buffer (50 mM Tris-HCl, pH 7.0, 1 mM dithiothreitol, 2 mM MgCl2, 0.2 mg/ml Na3VO4, 75 μM p-nitrophenyl phosphate, 100 μM γ-[32P]ATP and 300 μM peptide substrate or 0.5 mg/ml poly(Glu/Tyr). For the blank, the reaction mixture containing no peptide substrate or no poly(Glu/Tyr) was processed identically. The reaction was terminated as described by Cheng et al. The peptide substrate and Cobrin antibody (8) for poly(Glu/Tyr). The enzyme activity was expressed as pico moles of PO4 incorporated into the substrate per min. The EGF receptor kinase activity was assayed as detailed by Litwin et al. (7). For kinetic analyses of the src-family tyrosine kinases, the assay was carried out in a volume of 50 μl containing the assay buffer, 100 μM γ-[32P]ATP, an aliquot of the lysate tyrosine kinase, and cdc2(6-20)NH2 peptide at concentrations of 37.5 μM, 50 μM, 75 μM, 150 μM, or 300 μM. In all assays, less than 3% of the substrates were consumed in the reaction.

**Protein Concentration Determination**—The relative protein concentration of individual column fractions was measured by Bradford's assay (14) and expressed as absorbance at 595 nm. For crude membrane extracts, the presence of 5% Nonidet P-40 interfered with the Bradford assay significantly; therefore, a modified Lowry assay was used (16, 17).

**Immunoblot Analyses**—Immunoblot analyses were performed according to the method of Towbin et al. (20) using alkaline phosphatase-linked second antibody and p-nitro blue tetrazolium chloride/5-bromo-4-chloro-3-indolyl phosphate as colorimetric substrate.

**Extraction of Protein Tyrosine Kinases from Membrane Fractions of Bovine Tissues**—Fresh bovine tissues including brain, thymus, lung, spleen, liver, and kidney were obtained from a local slaughterhouse and transported to the laboratory on ice. All procedures were carried out at 4 °C unless otherwise indicated. After removing excess fat and membranes, the tissues were ground in a meat grinder and then further homogenized in the homogenization buffer (200 ml of the homogenization buffer per 100 g wet weight of the tissue). The homogenization buffer contained 50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 2 mM MgCl2, 0.3 mM phenylmethylsulfonyl fluoride, 0.2 mg/ml benzamidine, and 0.1 mg/ml soybean trypsin inhibitor. The homogenate was centrifuged at 2000 × g for 10 min to remove undissrupted cells and nuclei. The supernatant was then centrifuged at 100,000 × g for 45 min. The pellets of the 100,000 × g spines were each resuspended in the homogenization buffer and recentrifuged. For extraction of the membrane-bound protein tyrosine kinases, the washed membranes were resuspended in each resuspension buffer (200 ml per 100 g wet weight of the original tissue) and were centrifuged at 50,000 × g for 1 h. Following extraction, the suspension was clarified by centrifugation at 100,000 × g for 1 h. The supernatant, containing the membrane-bound protein tyrosine kinases of the tissues, were assayed for protein.
tyrosine kinase activities using cdc2(6-20)NH2, [Val\textsuperscript{16}]angiotensin II, and poly(Glu/Tyr) as substrates.

Analysis of cdc2(6-20)NH2 Phosphorylated by Tissue Extracts—Since cdc2(6-20)NH2 contains a threonine in addition to 2 tyrosine residues, it was necessary to examine whether cdc2(6-20)NH2 was phosphorylated by tyrosine kinases rather than serine/threonine protein kinases in tissue extracts. After the phosphorylation reaction, proteins in the reaction mixture were precipitated by the addition of trichloroacetic acid to a final concentration of 10%. After centrifugation at 10,000 × g for 15 min, the supernatant containing the phosphorylated cdc2(6-20)NH2 was saved. The phosphorylated cdc2(6-20)NH2 was then isolated by the C\textsubscript{18} Sep-pak cartridge as described by Cheng et al. (8). Phosphoamino acid analysis of the purified phospho-cdc2(6-20)NH2 was performed according to the method of Hunter and Sefton (18).

Hydroxylapatite Column Chromatography of Membrane Extract of Bovine Thymus—Membrane extract of 100 g of bovine thymus was applied to a 100-ml hydroxylapatite column pre-equilibrated with buffer A (25 mM Heps, pH 7.0, 10% (v/v) glycerol, 1 mM dithiobiotil, 1 mM EDTA, 0.1% Nonidet P-40, 0.3 mM phenylmethylsulfonyl fluoride, 0.2 mg/ml benzamidine, 0.1 mg/ml soybean trypsin inhibitor). The column was washed with 200 ml of buffer A and then eluted with a linear gradient of 0.0-0.3 M potassium phosphate in buffer A at pH 7.0 (total volume of the gradient was 800 ml). Fractions of 8-ml size were collected. The fractions were assayed for protein tyrosine kinase activities using cdc2(6-20)NH2 and poly(Glu/Tyr) as substrates and monitored for immunoreactivity toward the α-src(416), α-lek, α-p60\textsuperscript{src} (mAb 327), and anti-c-fgr antibodies.

Immunoprecipitation of pp55\textsuperscript{src} Using a-α-fyn Antibody and Assay for cdc2(6-20)NH2 Tyrosine Kinase Activity in the α-α-fyn Immunoprecipitate—Membrane extract prepared from 500 g of bovine thymus was first chromatographed on a 500-ml DEAE-Sepharose column. Fractions with cdc2(6-20)NH2 and poly(Glu/Tyr) tyrosine kinase activity were pooled and chromatographed on a hydroxylapatite column as described above except that a 300-ml size hydroxylapatite column was used, and the total volume of the gradient was 2 liters. Fractions of 22-ml size were collected. The fractions were assayed for tyrosine kinase activity using cdc2(6-20)NH2 and poly(Glu/Tyr) as substrates. The elution profiles of the four thymus tyrosine kinases were shown in Fig. 2. The elution profiles of the four thymus tyrosine kinases were shown in Fig. 2. The immunoprecipitates of each fraction were washed with 6 M urea in 20 ml of buffer A, and the cdc2(6-20)NH2 peptide tyrosine kinase activity associated with the α-α-fyn immunoprecipitate was monitored by the tyrosine kinase assay procedure described in this section.

FPLC Mono Q Ion Exchange Column Chromatography of TK-I, TK-II, TK-III, and TK-IV—TK-I and TK-II partially purified by rechromatography on the FPLC hydroxylapatite column as well as the peak activity fractions of TK-III and TK-IV in the first hydroxylapatite column (Fig. 2) were collected and dialyzed against 4 liters of buffer A. The dialyzed samples were applied to a 1-ml FPLC Mono Q ion exchange column pre-equilibrated with buffer A, washed with 5 ml of buffer A, and then eluted with a linear gradient of 0.0-1.5 M NaCl in buffer A at a flow rate of 0.75 ml/min (total volume of the gradient was 30 ml). Fractions of 0.75 ml were collected. The fractions were assayed for tyrosine kinase activity as well as immunoreactivities toward the α-src(416) and α-lek antibodies.

RESULTS

Demonstration of cdc2(6-20)NH2 Peptide Tyrosine Kinase Activity in Bovine Tissues—Previously, we demonstrated that cdc2(6-20)NH2 peptide and its truncated analog Cys-cdc2(8-20) were highly efficient substrates of the pp60\textsuperscript{src}-related spleen tyrosine kinase relative to the general tyrosine kinase substrate, [Val\textsuperscript{16}]angiotensin II (7, 8). To determine if this is unique to the spleen tyrosine kinase, we surveyed the tyrosine kinase activity toward cdc2(6-20)NH2, [Val\textsuperscript{16}]angiotensin II, and another general tyrosine kinase substrate poly(Glu/Tyr) in membrane extracts of various bovine tissues. Poly(Glu/Tyr) and [Val\textsuperscript{16}]angiotensin II are considered as general tyrosine kinase substrates due to the lack of serine and threonine residues in their structures (4, 5). The tissues surveyed included brain, thymus, spleen, liver, and kidney. While the protein tyrosine kinase activity of the membrane extracts could be readily determined by using either cdc2(6-20)NH2 or poly(Glu/Tyr), the tyrosine kinase activity toward [Val\textsuperscript{16}]angiotensin II was too low to be accurately determined in some of the tissue membrane extracts. In all cases, the kinase activity toward cdc2(6-20)NH2 was much higher than that toward [Val\textsuperscript{16}]angiotensin II (data not shown). These observations suggested that the high cdc2(6-20)NH2 peptide tyrosine kinase activity was not unique to the tyrosine kinase purified from spleen. Since cdc2(6-20)NH2 peptide contains a threonine residue in addition to tyrosine residues, its phosphorylation by the various tissue extracts was analyzed by isolating the phosphorylated peptide and determining the site of phosphorylation (see "Experimental Procedures"). The results indicated that phosphotyrosine was the only phosphoamino acid residue detected in phospho-cdc2(6-20)NH2 phosphorylated by the various tissue extracts (data not shown).

Separation of Multiple Protein Tyrosine Kinases in Membrane Extract of Bovine Thymus—The relative protein tyrosine kinase activity toward cdc2(6-20)NH2 and poly(Glu/Tyr) in the membrane extracts varied from one tissue to another (Fig. 1). One possible explanation for this observation is that bovine tissues contain multiple forms of protein tyrosine kinases of differential activities toward the two substrates. To test for such a possibility, a number of chromatographic columns including DEAE-cellulose, phenyl-Sepharose, and hydroxylapatite columns were tested for their ability to separate the putative multiple kinases. Thymus membrane extract was selected for such an analysis because of its high content of protein tyrosine kinase activity. Fig. 2 shows that hydroxylapatite column was capable of separating the poly(Glu/Tyr) tyrosine kinase activity of thymus membrane extract into four peaks, only the first two peaks (TK-I and TK-II) showed significantly high kinase activity toward cdc2(6-20)NH2. The column fractions were also analyzed by immunoblot for the presence of pp60\textsuperscript{src}-related protein tyrosine kinases using the anti-pp60\textsuperscript{src}-auto phosphorylation site peptide antibody (α-src(416) antibody). As shown in Fig. 1.
A Peptide Substrate for src-Family Tyrosine Kinases

3, a cluster of 55-kDa immunoreactive protein bands were detected by the antibody, the intensity of the immunostain appeared to correlate with the cdc2(6-20)NH2 peptide kinase activities. In addition, a protein band of 66 kDa was detected by the antibody, but its staining intensity displayed no correlation with protein tyrosine kinase activities. These results suggested that the kinase activity for cdc2(6-20)NH2 may be attributed to the cluster of 55-kDa protein bands which were members of the src-family tyrosine kinases.

The two cdc2(6-20)NH2 peptide tyrosine kinase peaks, TK-I and TK-II, were rechromatographed separately on a FPLC hydroxylapatite column. The two tyrosine kinases were eluted from the FPLC hydroxylapatite column at different phosphate concentrations (data not shown). Thus, TK-I and TK-II were either distinct members of the src-family of tyrosine kinases or stably modified forms of a tyrosine kinase. These two possibilities may be distinguished by using antibodies specific toward individual members of the src-family.

TK-III and TK-IV were further purified by FPLC Mono Q ion exchange chromatography. Each of them was eluted from the column as a single poly(Glu/Tyr) tyrosine kinase activity peak with little or no cdc2(6-20)NH2 peptide kinase activity (data not shown). The column fractions were analyzed by immunoblot using an antibody against the cdc2(6-20)NH2 kinase activity. The immunoblot staining intensity displayed no correlation with protein tyrosine kinase activities. These results suggested that the kinase activity for cdc2(6-20)NH2 may be attributed to the cluster of 55-kDa protein bands which were members of the src-family tyrosine kinases.

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results suggest strongly that TK-I is the bovine lck protein tyrosine kinase. Antibodies specific for individual src-family members have also been used to identify TK-II and the spleen tyrosine kinase. When samples of TK-I, pp60src, STK, TK-I, TK-II, TK-III, and TK-IV were analyzed for reactivity toward the α-fyn antibody, only that of TK-II appeared to contain an intense immunoreactive band of molecular weight of about 55,000 which co-migrated with the α-src(416) immunoreactive band (Fig. 5B). However, all samples contained immunostaining bands of molecular weight 65,000–70,000. These bands appeared to be due to the contaminants in the SDS-gel electrophoresis reagents, since they were also seen in a blank lane that did not contain protein samples. Nevertheless, we carried out immunoprecipitation analysis to further demonstrate specific interaction of the α-fyn antibody with TK-II. As shown in Fig. 5A, even though both TK-I and TK-II displayed significant cdc2(6–20)NH₂ tyrosine kinase activity, only the hydroxylapatite column fractions corresponding to TK-II could display cdc2(6–20)NH₂ tyrosine kinase activity in the α-fyn immunoprecipitate. The specificity of immunoreactivity of TK-II with the α-fyn antibody was further supported by the inability of TK-II to interact with the α-pp60src (mAb 327), α-fgr, α-lyn, and α-lck antibodies. These results strongly suggest that TK-II is the bovine fyn protein kinase.

When column fractions from Mono Q ion exchange column chromatography of STK were analyzed for cdc2(6–20)NH₂ peptide kinase activity and α-lyn immunoreactivity, the α-lyn immunoreactivity was shown to co-elute with cdc2(6–20)NH₂ peptide kinase activity from the column (Fig. 6A). The specificity of the interaction between STK and the α-lyn antibody was supported by the observation that the α-lyn antibody failed to interact with TK-I, TK-II (data not shown), and pp60src (Fig. 6B), thus suggesting that STK is bovine lyn protein kinase. As shown in Fig. 6B, two predominant immunoreactive protein species were detected in both the anti-src(416) and α-lyn immunoblots of the purified STK preparation suggesting that the STK preparation might contain both the 56- and 53-kDa alternatively spliced products of lyn protooncogene (27). Previously, we reported the presence of three anti-src(416) immunoreactive protein species in the purified STK preparation (7). The reason for this discrepancy is not known; possibly it is due to variation among different enzyme preparations.

To facilitate presentation of data and discussion of our results, TK-I, TK-II, and STK were, respectively, referred to as TK-I/lck, TK-II/fyn, and STK/lyn.

Efficient Phosphorylation of cdc2(6–20)NH₂ Is a Property Unique and Common to the src-Family Tyrosine Kinases—To further examine the suggestion that the high efficiency of cdc2(6–20)NH₂ peptide phosphorylation was a unique property shared by members of the src-family tyrosine kinases, we measured the relative rates of phosphorylation of cdc2(6–20)NH₂, [Val⁵]angiotensin II, poly(Glu/Tyr), and RR-SRC catalyzed by several pp60src-related tyrosine kinases including the purified pp60src from human platelets, STK/lyn, TK-I/lck, and TK-II/fyn, as well as several non-src-related tyrosine kinases including TK-III, TK-IV, EGF-receptor kinase, and the recombinant p43abol. The last peptide substrate, RR-SRC, with amino acid sequence RRLIEDAEYQAARG, was derived from the consensus autophosphorylation site sequence of src-family tyrosine kinases (1, 12). The rates of phosphorylation of the various substrates by the individual tyrosine kinases were compared with those of [Val⁵]angiotensin II. As shown in Table 1, all pp60src-related protein tyrosine kinases catalyzed the phosphorylation of cdc2(6–20)NH₂ peptide with rates 40- to 90-fold higher than those of [Val⁵]angiotensin II phosphorylation. The property of phosphorylating cdc2(6–20)NH₂ with high efficiency, however, was not exhibited by other protein tyrosine kinases which were not members of the src-family. The four non-src-related tyrosine kinases examined phosphorylated cdc2(6–20)NH₂ peptide at rates which were 0.5 to 2.5 times those of [Val⁵]angiotensin II phosphorylation (Table I). While all four src-family tyrosine kinases, pp60src, STK/lyn, TK-I/lck, and TK-II/fyn exhibited common substrate specificity properties in showing preference for cdc2(6–20)NH₂ peptide over all other substrates tested, they differed in their individual substrate spec-

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**Fig. 4.** FPLC Mono Q ion exchange column chromatography of TK-I and TK-II. After rechromatography on the FPLC hydroxylapatite column (Fig. 2), the peak activity fractions of TK-I and TK-II were further purified and analyzed by Mono Q ion exchange column chromatography. The column fractions were assayed for cdc2(6–20)NH₂ peptide tyrosine kinase activity as well as the α-src(416) and α-lck immunoreactivities. The numbers on top of the immunoblots indicate the column fractions used in the analyses.
A Peptide Substrate for src-Family Tyrosine Kinases

A

9253

Fraction No.

TK-I

TK-III

TK-IV

pp60

STK

TK-I

TK-II

TK-III

TK-IV

Anti-FYN

Anti-SRC(416)

50kDa

Enzyme Activity in α-fyn immunoprecipitate

pmol/min

pmol/min

Panel A shows the cdc2(6-20)NH2 peptide tyrosine kinase activity profiles. The column fractions were also assayed for poly(Glu/Tyr) tyrosine kinase activity, and the elution profiles of TK-I, TK-II, TK-III, and TK-IV were determined (data not shown). Locations of the peak activity fractions of the four thymus tyrosine kinases were indicated by arrows. Only TK-I and TK-II displayed significant cdc2(6-20)NH2 peptide tyrosine kinase activity. The cdc2(6-20)NH2 peptide tyrosine kinase activity in the α-fyn immunoprecipitates of the hydroxylapatite column fractions was shown, and only α-fyn immunoprecipitates from fractions corresponding to TK-II displayed significant cdc2(6-20)NH2 tyrosine kinase activity.

A

9254

B

protein kinases including TK-I, TK-II, TK-III, TK-IV, pp60SRC", and STK were applied to 10% SDS-polyacrylamide gels. After electrophoresis, the proteins were electrotransferred to Immobilon membranes. The membranes were then probed with the anti-src(416) antibody (1:100 dilution) or the α-fyn antibody (1:100 dilution) as primary antibodies, and the immunoreactive proteins were detected as described under "Experimental Procedures."

Fig. 5. Immunological identification of TK-II as p55αααα. A, immunoprecipitation using α-fyn antibody and assay for cdc2(6-20)NH2 peptide kinase activity in the α-fyn immunoprecipitate. Bovine thymus membrane extract was first chromatographed in the DEAE-Sepharose column. Fractions containing tyrosine kinase activity were pooled and further purified by hydroxylapatite column chromatography as described under "Experimental Procedures." The hydroxylapatite column fractions were assayed for tyrosine kinase activity using cdc2(6-20)NH2 peptide and poly(Glu/Tyr) as substrates. Moreover, specific interaction of tyrosine kinases with α-fyn antibody was tested by the immunoprecipitation assay detailed under "Experimental Procedures." Panel A shows the cdc2(6-20)NH2 peptide tyrosine kinase activity profiles. The column fractions were also assayed for poly(Glu/Tyr) tyrosine kinase activity, and the elution profiles of TK-I, TK-II, TK-III, and TK-IV were determined (data not shown). Locations of the peak activity fractions of the four thymus tyrosine kinases were indicated by arrows. Only TK-I and TK-II displayed significant cdc2(6-20)NH2 peptide tyrosine kinase activity. The cdc2(6-20)NH2 peptide tyrosine kinase activity in the α-fyn immunoprecipitates of the hydroxylapatite column fractions was shown, and only α-fyn immunoprecipitates from fractions corresponding to TK-II displayed significant cdc2(6-20)NH2 tyrosine kinase activity. B, protein kinases including TK-I, TK-II, TK-III, TK-IV, pp60SRC", and STK were applied to 10% SDS-polyacrylamide gels. After electrophoresis, the proteins were electrotransferred to Immobilon membranes. The membranes were then probed with the anti-src(416) antibody (1:100 dilution) or the α-fyn antibody (1:100 dilution) as primary antibodies, and the immunoreactive proteins were detected as described under "Experimental Procedures."

ificities in other aspects. For example, markedly different efficiencies in phosphorylating poly(Glu/Tyr) were observed among the four src-family tyrosine kinases; the relative rates of phosphorylating poly(Glu/Tyr) in comparison to those of phosphorylating [Val'langiotensin II ranged from 5- to 53-fold (Table I).

Kinetic analysis of cdc2(6-20)NH2 peptide phosphorylation by all four src-family protein tyrosine kinases was conducted to define the kinetic basis of this efficient phosphorylation. The results revealed that they displayed similar $K_m$ values, ranging from 100 to 500 μM (Table II). These values are comparable to those of the peptides considered as best peptide substrates of protein tyrosine kinases (23-25). Since the preparations of the src-family tyrosine kinases were not of the same purity, the absolute $V_{max}$ values of the various kinases could not be compared. However, when the unit of the protein tyrosine kinase activity was defined as picomoles/min of phosphorylation of [Val'langiotensin II under standard assay conditions as described under “Experimental Procedures,” and the $V_{max}$ of the cdc2(6-20)NH2 phosphorylation reactions was expressed as picomoles/min of phosphorylation of cdc2(6-20)NH2 per unit of angiotensin kinase activity, the four src-related kinases were shown to display similar and high $V_{max}$ values, ranging from approximately 110 to 360 (Table II). Results in Tables I and II suggest that although all the src-family tyrosine kinases showed relatively good affinities toward the cdc2(6-20)NH2 peptide, the high efficiency of cdc2(6-20)NH2 peptide phosphorylation was due mainly to the high catalytic potency toward this peptide.

Glu-12 and Thr-14 of cdc2(6-20)NH2 Contain Substrate Specificity Determinants of src-Family Tyrosine Kinases-In our previous study (8), we showed that a peptide derived from Ss ribosomal protein kinase, rsk(436-456), which was highly homologous to cdc2(6-20)NH2, was poorly phosphorylated by
A Peptide Substrate for src-Family Tyrosine Kinases

the purified spleen tyrosine kinase. Comparison of the amino acid sequences of cdc2(6-20)NH2 (KVEKIGETGYGVKamide) and rsk(436-456) (YVVKETIGVGSYSVCKRCVHK) showed that the two peptides contained different amino acids at several positions. However, the difference in efficiency of phosphorylating the two peptides by STK/lyn could be accounted for by the substitution of Glu-12 and Thr-14 of cdc2(6-20)NH2 with valine and serine, respectively, found in corresponding positions in rsk(436-456) (8). To test if pp60-src-related protein tyrosine kinases recognized common substrate specificity determinants in cdc2(6-20)NH2, the relative rates of phosphorylation of cdc2(6-20)NH2 and its substitution analogs at residues 12 and 14, [Val]cdc2(6-20)NH2, [Ser]cdc2(6-20)NH2, and [Val]2, Ser]cdc2(6-20)NH2, as well as rsk(436-456), by the various pp60-src-related tyrosine kinases were determined. In addition, since cdc2(6-20)NH2 contains 2 tyrosine residues, a substitution analog [Lys]cdc2(6-20)NH2 with Tyr-15 as the only tyrosine residue was tested. Table III showed that [Lys]cdc2(6-20)NH2 was as good a substrate as cdc2(6-20)NH2 for all the src-family tyrosine kinases suggesting that Tyr-15 was the major site in cdc2(6-20)NH2 and its analogs phosphorylated by the src-family tyrosine kinases. Similar to the spleen tyrosine kinase, all the src-family tyrosine kinases phosphorylated rsk(436-456) very poorly relative to cdc2(6-20)NH2. The phosphorylation rates of the double substitution analog, [Val]2, Ser]cdc2(6-20)NH2, were nearly as low as those of rsk(436-456) peptide in all cases (Table III). Thus, Glu-12 and Thr-14 residues of cdc2(6-20)NH2 contained common structural features recognized by all four pp60-src-related protein tyrosine kinases.

**DISCUSSION**

In the present study, we have used multiple artificial protein tyrosine kinase substrates and src-family kinase-specific antibodies to characterize protein tyrosine kinases in mammalian tissues. Although the study is far from being exhaustive and systematic, two main suggestions can be derived from the present results. The observations that a number of src-family protein tyrosine kinases share the property of specific and highly efficient phosphorylation of cdc2(6-20)NH2 whereas the protein kinases not belonging to the src-family do not display such a property and that, for all the src-family tyrosine kinases, substituting Glu-12 and Thr-14 of cdc2(6-20)NH2, respectively, by valine and serine abolishes the ability of the peptide to serve as an efficient substrate provide the basis for the first suggestion: specific and efficient phosphorylation of cdc2(6-20)NH2 peptide is common and unique to src-family

<table>
<thead>
<tr>
<th></th>
<th>src-Family tyrosine kinases</th>
<th>Non-src-related tyrosine kinases</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pp60-src</td>
<td>pp56-src</td>
</tr>
<tr>
<td>[Val]Angiotensin II</td>
<td>0.17 (1)</td>
<td>0.29 (1)</td>
</tr>
<tr>
<td>RR-SRC</td>
<td>0.44 (2.6)</td>
<td>2.62 (9.0)</td>
</tr>
<tr>
<td>cdc2(6-20)NH2</td>
<td>7.02 (41.3)</td>
<td>18.75 (64)</td>
</tr>
<tr>
<td>Poly(Glu/Tyr)</td>
<td>2.4 (14.1)</td>
<td>1.5 (5.2)</td>
</tr>
</tbody>
</table>

**Table I**

Relative rates of phosphorylation of cdc2(6-20)NH2, [Val]Angiotensin II, RR-SRC peptide, and poly(Glu/Tyr) by pp60-src-related and non-src-related protein tyrosine kinases

The kinase reaction was performed as described under "Experimental Procedures." The final concentration of each peptide was 300 μM. The final concentration of poly(Glu/Tyr) was 0.5 mg/ml. The enzyme activity was measured as the initial velocity of the phosphorylation reaction, and it was expressed as picomoles of P-O incorporated into the substrate peptides or poly(Glu/Tyr) per min. The numbers in parentheses indicate the phosphorylation rates relative to the rate of [Val]angiotensin II phosphorylation by each enzyme.

**Fig. 6. Immunological identification of the spleen tyrosine kinase (STK) as pp56-src.** A, Mono Q ion exchange column chromatography of the partially purified STK. After purification by DEAE-Sepharose, hydroxylapatite, phenyl-Sepharose, S-200 gel filtration, and Red A-agarose columns (7), the partially purified spleen tyrosine kinase was further chromatographed on a Mono Q ion exchange column with a linear gradient of 0-1 M NaCl in Buffer A (total volume = 20 ml). Fractions of 250 μl each were collected. The fractions were assayed for cdc2(6-20)NH2 tyrosine kinase activity and α-lyn immunoreactivity. B, aliquots of pp56-src and STK were applied to 10% SDS-polyacrylamide gels. After electrophoresis, the proteins were electrotransferred to Immobilon membranes. The membranes were probed with the anti-α-lyn(416) antibody (1:100 dilution) or the α-lyn antibody (1:200 dilution) as primary antibody, and the immunoreactive proteins on the blots were detected as described under "Experimental Procedures."
protein tyrosine kinases. The second suggestion that mammalian tissues contain multiple protein tyrosine kinases with differential substrate specificities is based on the observation that tissue extracts show distinct relative tyrosine kinase activities toward different substrates and the successful fractionation of multiple protein tyrosine kinases in thymus membrane extract. In particular, the first suggestion deals with the substrate specificity which is an important and fundamental aspect of protein tyrosine kinases. To our knowledge, this is the first example that a family of protein tyrosine kinases are defined by their substrate specificities.

Only four src-family kinases and four other protein tyrosine kinases were examined in the present study. The categorization of some of these protein tyrosine kinases was based primarily on their immunoreactivities toward the anti-src autophosphorylation site antibody (α-src(416)). Thus, the suggestion that the specific and efficient phosphorylation of cdc2(6–20)NH₂ is a common and unique property of src-family tyrosine kinases should be considered as tentative. On the other hand, the observation that during the fractionation of the thymus membrane extract, high cdc2(6–20)NH₂ peptide kinase activity invariably co-migrated with α-src(416) antibody immunoreactivity, whereas tyrosine kinase peaks lacking high cdc2(6–20)NH₂ peptide kinase activity showed no α-src(416) immunoreactivity provides strong support for the suggestion. In any case, the suggestion should be used as a working hypothesis. We propose that other members of the src-family of tyrosine kinases and other non-src-related tyrosine kinases be examined for their ability to carry out specific and efficient phosphorylation of cdc2(6–20)NH₂ peptide. A peptide kit consisting of cdc2(6–20)NH₂ and its analogs, [Val¹']cdc2(6–20)NH₂, [Ser¹']cdc2(6–20)NH₂, and [Val¹²,Ser¹⁴]cdc2(6–20)NH₂ can be used as the test substrates, and the results will either substantiate or falsify the hypothesis. If the hypothesis can be substantiated, the peptide kit will become a useful diagnostic tool for identifying additional src-family protein tyrosine kinases.

In addition to shedding light on the catalytic properties of src-family tyrosine kinases, the cdc2(6–20)NH₂ peptide and the peptide kit may have a number of practical applications. Further study of the molecular basis of the cdc2(6–20)NH₂ peptide phosphorylation by src-family kinases may provide a basis for the development of specific inhibitors for these kinases. The peptide kit may be used to establish specific assays for src-family protein tyrosine kinases in crude cell lysates or other biological samples at relatively crude states. The feasibility of establishing such an assay is supported by the observation that cdc2(6–20)NH₂ kinase activity could readily be assayed in membrane extracts of various tissues, and the phosphorylation occurred predominantly, if not exclusively, at Tyr-15. Such an assay system is expected to greatly facilitate the cell biological study of the regulation and the functions of src-family protein kinases.

Although tyrosine phosphorylation of p34cdc2 is widely accepted as an important regulatory mechanism in the control of cell cycle progression, the identity of the protein tyrosine kinase that catalyzes this reaction is not established. The previous observations that the src-related spleen tyrosine kinase catalyzes the efficient and specific phosphorylation of Tyr-15 in cdc2(6–20)NH₂ (7, 8) and that this phosphorylation is critically dependent on the presence of specific amino acid residues (8) led us to suggest that the spleen tyrosine kinase or a src-family tyrosine kinase may be responsible for catalyzing tyrosine phosphorylation of p34cdc2. The suggestion that cdc2(6–20)NH₂ peptide kinase activity can be a measure of p34cdc2 tyrosine kinases is compatible with the observation that tissues such as spleen and thymus containing abundant rapidly proliferating cells displayed a high level of cdc2(6–20)NH₂ peptide kinase activity. Although the suggestive in-

### Table II

**Kinetic parameters of cdc2(6–20)NH₂ phosphorylation by src-family tyrosine kinases**

<table>
<thead>
<tr>
<th>Enzymes</th>
<th>( K_m )</th>
<th>( V_{max} )</th>
<th>pmol PPO₄/min/unit of kinase activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>pp60&lt;sup&gt;s&lt;/sup&gt;</td>
<td>101.6</td>
<td>357.1</td>
<td></td>
</tr>
<tr>
<td>pp60&lt;sup&gt;src&lt;/sup&gt;</td>
<td>277.7</td>
<td>126.6</td>
<td></td>
</tr>
<tr>
<td>p66&lt;sup&gt;s&lt;/sup&gt;</td>
<td>133.3</td>
<td>113.5</td>
<td></td>
</tr>
<tr>
<td>p55&lt;sup&gt;s&lt;/sup&gt;</td>
<td>487</td>
<td>231.5</td>
<td></td>
</tr>
</tbody>
</table>

*The velocity of the phosphorylation reaction was expressed as picomoles of phosphate incorporated into cdc2(6–20)NH₂ per min per unit of kinase activity, the unit of kinase activity was defined as the amount of kinase catalyzing the transfer of 1 pmol of PPO₄ to [Val¹'] angiotensin II/min. The angiotensin kinase activity was determined by assays with 300 μM [Val¹']angiotensin II under conditions described under "Experimental Procedures."*

### Table III

**Rates of phosphorylation of various substitution analogs of cdc2(6–20)NH₂ by pp60<sub>s</sub>-related tyrosine kinases**

The kinase reaction was performed as detailed under "Experimental Procedures." The final concentration of each peptide was 300 μM. The enzyme activity was measured as the initial velocity of the phosphorylation reaction, and it was expressed as picomoles of PO₄ incorporated into the substrate peptide per min. The numbers in parentheses indicate the phosphorylation rates relative to the rate of cdc2(6–20)NH₂ phosphorylation by each enzyme.

<table>
<thead>
<tr>
<th>Substrates</th>
<th>pp60&lt;sup&gt;s&lt;/sup&gt;</th>
<th>pp60&lt;sup&gt;src&lt;/sup&gt;</th>
<th>p66&lt;sup&gt;src&lt;/sup&gt;</th>
<th>p55&lt;sup&gt;s&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>cdc2(6–20)NH₂</td>
<td>5.4 (1)</td>
<td>63 (1)</td>
<td>124 (1)</td>
<td></td>
</tr>
<tr>
<td>[Val¹']cdc2(6–20)NH₂</td>
<td>1.8 (0.3)</td>
<td>33.2 (0.6)</td>
<td>47 (0.38)</td>
<td></td>
</tr>
<tr>
<td>[Ser¹']cdc2(6–20)NH₂</td>
<td>1.1 (0.2)</td>
<td>12.1 (0.2)</td>
<td>11.7 (0.09)</td>
<td></td>
</tr>
<tr>
<td>[Val¹²,Ser¹⁴]cdc2(6–20)NH₂</td>
<td>0.3 (0.05)</td>
<td>2.6 (0.04)</td>
<td>2.3 (0.02)</td>
<td></td>
</tr>
<tr>
<td>[Ly²³]cdc2(6–20)NH₂</td>
<td>6.2 (1.15)</td>
<td>54.2 (0.86)</td>
<td>109.6 (0.88)</td>
<td></td>
</tr>
<tr>
<td>ras(436–456)</td>
<td>0.3 (0.05)</td>
<td>0.5 (0.08)</td>
<td>1.2 (0.01)</td>
<td></td>
</tr>
</tbody>
</table>
volvement of a src-family tyrosine kinase in tyrosine phosphorylation of p34<sup>cdc2</sup> is far from certain, it will indeed be a surprise if the enzyme that catalyzes the phosphorylation of p34<sup>cdc2</sup> at Tyr-15 does not show a specific and high enzyme activity toward Tyr-15 of cdc2(6-20)NH<sub>2</sub> peptide. In this respect, it should be noted again that only the tyrosine kinases which show immunoreactivity toward the anti-src autophosphorylation site antibody (α-src(416)) possessed good activity toward the cdc2(6-20)NH<sub>2</sub> peptide. Clearly, further test for the suggestion that members of src-family kinases phosphorylate p34<sup>cdc2</sup> at Tyr-15 during cell cycle has to be carried out. We propose that one useful experiment is examining the effects of mutation of Glu-12 and Thr-14 of p34<sup>cdc2</sup> to valine and serine, respectively, on the phosphorylation of p34<sup>cdc2</sup> and possible phenotypic changes exhibited by yeast cells expressing such a mutant p34<sup>cdc2</sup>.

The cdk2 kinase, a p34<sup>cdc2</sup>-like protein kinase involved in controlling the G<sub>1</sub> to S phase transition of eukaryotic cells, was recently cloned and sequenced (29, 30). The cdc2(6-20) sequence is present in the amino-terminal portions of both the p34<sup>cdc2</sup> and the cdk2 kinase. It is not known if cdk2 kinase activity is also regulated by phosphorylation of Tyr-15. By the same aforementioned segment regarding the relationship between p34<sup>cdc2</sup> and src-related tyrosine kinases, cdk2 kinase will be a potential in vivo substrate of src-related tyrosine kinases.

Although the demonstration and fractionation of multiple protein tyrosine kinases displaying differential substrate specificity in thymus membrane extract is not the major emphasis of the present study, the results have highlighted the complexities of cellular protein tyrosine kinases in terms of both their molecular entities and substrate specificities. The various thymus protein tyrosine kinases as well as other protein tyrosine kinases examined in the present study displayed different relative activities toward the set of substrate tested. This observation, along with the finding that src-family tyrosine kinases share similar substrate specificity properties, have effectively argued against the commonly held notion that protein tyrosine kinases do not have strict in vitro substrate specificities. While cellular regulating mechanisms such as cellular compartmentalization do play important roles in dictating substrate specificities of protein tyrosine kinases, the intrinsic enzymatic properties of individual protein tyrosine kinases are equally important considerations. Results from the present study suggest that a combination of protein fractionation, immunological analyses, and the enzymological characterization using multiple tyrosine kinase substrates is among the best approaches in sorting out the complexities of cellular tyrosine kinases.

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