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Characterization of Synthetic Peptide Substrates for p34^{cdc2} Protein Kinase

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Abstract Synthetic peptide substrates for the cell division cycle regulated protein kinase, p34^{cdc2}, have been developed and characterized. These peptides are based on the sequences of two known substrates of the enzyme, Simian Virus 40 Large T antigen and the human cellular recessive oncogene product, p53. The peptide sequences are H-A-D-A-Q-H-A-T-P-P-K-K-K-R-K-V-E-D-P-K-D-F-OH (T antigen) and H-K-R-A-L-P-N-N-T-S-S-S-P-Q-P-K-K-K-P-L-D-G-E-Y-NH_2 (p53), and they have been employed in a rapid assay of phosphorylation in vitro. Both peptides show linear kinetics and an apparent K_m of 74 and 120 μM, respectively, for the purified human enzyme. The T antigen peptide is specifically phosphorylated by p34^{cdc2} and not by seven other protein serine/threonine kinases, chosen because they represent major classes of such enzymes. The peptides have been used in whole cell lysates to detect protein kinase activity, and the cell cycle variation of this activity is comparable to that measured with specific immune and affinity complexes of p34^{cdc2}. In addition, the peptide phosphorylation detected in mitotic cells is depleted by affinity adsorption of p34^{cdc2} using either antibodies to p34^{cdc2} or by immobilized p13, a p34^{cdc2}-binding protein. Purification of peptide kinase activity from mitotic HeLa cells yields an enzyme indistinguishable from p34^{cdc2}. These peptides should be useful in the investigation of p34^{cdc2} protein kinases and their regulation throughout the cell division cycle.

Key words: phosphorylation, linear kinetics, T antigen peptide, whole cell lysates, mitotic cells

Synthetic peptide substrates for protein kinases have made an important contribution to the progress of research in protein phosphorylation [1]. Such peptides permit the rapid quantitation of specific protein kinase activities in crude extracts of cells or tissues for the analysis of the involvement of protein kinases in cell physiology [2]. In this paper, we report the development and characterization of synthetic peptide substrates for the cell division cycle-regulated protein kinase, p34^{cdc2} (reviewed in 3). This enzyme is a protein serine/threonine kinase that plays a central role in the induction of mitosis in mammalian cells [4–6] and in the promotion of amphibian oocyte maturation [7–9]. Several cellular and viral proteins have been identified as substrates for p34^{cdc2} [summarized in 10], including i) nuclear proteins, cyclins [11], lamin B [12–14], histone H1 [15], and nucleolin [16]; and ii) oncoproteins, SV40 Large T antigen [17], p53 [18], c-abl [19], and pp60$^{c\text{-}src}$ [20]. The concensus sequence for the phosphorylation site appears to be *S/TP*$(X)_nZ$, where X is a polar residue, Z is a basic residue (lysine or arginine), and n = 1 to 3 [10]. One of the obstacles to research on p34^{cdc2} has been the assay, which usually involves specific precipitation of the enzyme from the sample using an antibody or an immobilized p34^{cdc2} binding protein, followed by phosphorylation of histone H1, polyacrylamide gel electrophoresis, autoradiography, and scintillation counting [21]. A further complication is the observation that p34^{cdc2} binds to a variety of proteins that appear to regulate its activity [3,22]. The association of p34^{cdc2} with these regulatory proteins, such as cyclins, changes during the cell division cycle [11,22]. In addition, other regulatory events, such as the tyrosine phosphorylation of p34^{cdc2} itself, also vary during the cell division cycle, affecting enzymatic activity [23,24]. Thus, measurements of activity using precipitation methods are subject to limitations based on the availability of and modifications to the binding sites of those molecules in the precip-

Abbreviations used: Tris, tris(hydroxymethyl)aminomethane; ATP, adenosine 5'-triphosphate; EDTA, ethylenediamine tetraacetic acid; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; HPLC, high performance liquid chromatography; DTT, dithiothreitol.

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itate and may not reflect the actual population of kinase molecules in the cell extract. The use of synthetic peptide substrates for $p34^{cdc2}$ provides a rapid technique for measurement of soluble enzyme activity in unfractionated extracts, and these peptides can be used to monitor the purification of the enzyme from cultured cells without the use of immune recognition or affinity precipitation of the protein.

MATERIALS AND METHODS

Peptide Synthesis

The peptides were synthesized by solid phase methods [25] on *p*-methyl-benzylhydrylamine polystyrene resin using preformed, symmetric anhydrides and hydroxybenzotriazole-activated esters of N-α-Boc protected amino acids on an Applied Biosystems, Inc. Model 430A automated peptide synthesizer. Couplings were done in dimethylformamide and dichloromethane as solvents, and unreacted peptide was capped with acetic anhydride. The side chain-protected amino acids were serine-O-benzyl, threonine-O-benzyl, glutamyl-γ-O-benzyl ester, aspartyl-β-O-cyclohexyl ester, N-π-benzyloxymethyl-histidine, N-ε-chloro-benzyloxycarbonyl lysine, Ng-Tosyl-arginine, and O-2-bromobenzyloxycarbonyl tyrosine. The peptides were deprotected and cleaved from the resin with liquid HF at −10°C for 2 h in the presence of 5% (v/v) anisole and 5% (v/v) dimethyl sulfide. The peptide was precipitated with ethyl ether and solubilized in 0.1% (w/v) aqueous trifluoroacetic acid. The solution was subjected to HPLC using a Waters Delta Prep 3000 instrument on a column (4.9 × 30 cm) of 300 Å, C_{18} silica (Waters) and eluted with 0.1% (w/v) trifluoroacetic acid and a linear gradient of acetonitrile (Burdick and Jackson). The peptide was further purified by HPLC on a column (2.2 × 25 cm) of silica using C_{18}-bonded, 300 Å pore size silica, 10 μm in diameter (Vydac, The Separations Group, Hesperia, CA). The structure of the final peptide was verified by amino acid analysis, automated sequence analysis, plasma desorption mass spectrometry, and analytical microbore HPLC as described [26].

Protein Kinase Assays

The measurement of protein kinase activity was done using a modification [26] of the methods that utilize phosphocellulose paper (Whatman P81) to immobilize the peptide [27,28]. The reactions for $p34^{cdc2}$ kinase activity were carried out in an assay mixture containing $p34^{cdc2}$ buffer (50 mM Tris-HCl, pH 8.0; 10 mM $MgCl_2$; 1 mM dithiothreitol; 1 mM EGTA), 5 μl ATP ([γ-^{32}P]ATP, 0.6 mM, 500–1,000 cpm/pmol), 5 μl substrate peptide (stock solution, 6 mM in buffer), and 0.5 μl enzyme, in a total volume of 30 μl. The final concentrations of the reactants were 0.1 mM ATP and 1 mM peptide substrate. The mixture was incubated at 30°C for 10 min, and the reaction was stopped by the addition of trichloroacetic acid to a final concentration of 10% (w/v). Following centrifugation to remove precipitated protein, aliquots (10 μl) of the supernatant were applied to a strip of phosphocellulose paper. The paper was washed four times for 5 min each in 2 L of 100 mM phosphoric acid, dried, and the radioactivity was measured by scintillation counting in water. All other protein kinase assays were done as described [26,28, 29,38,43]. Casein kinase I and casein kinase II were isolated from bovine liver and assayed using dephosphorylated casein exactly as described [26,29]. The cAMP-dependent protein kinase catalytic subunit (Sigma Chemical Co., St. Louis, MO) was assayed using Kemptide [2] as previously described [27] for 5 min with a final concentration of 0.2 mM ATP. Glycogen synthase kinase 3 (a gift of Balwant Khatra, Long Beach, CA) was assayed for 30 min at 30°C in a mixture containing buffer (10 mM β-glycerophosphate, pH 7.8; 1 mM EGTA, and 5 mM magnesium acetate), 0.5 μM cAMP-dependent protein kinase inhibitor peptide (Sigma Chemical Co., St. Louis, MO), 200 ng glycogen synthase kinase 3, 0.1 mM [γ^{32}P]ATP (1,700 cpm/pmol), and either 2 mM peptide or 0.43 mg/ml glycogen synthase as substrate. Phosphorylase b kinase (Sigma Chemical Co., St. Louis, MO) was assayed as described [28]. Calmodulin kinase II (a gift of Marita King, Columbus, OH) was assayed in CaMK buffer (50 mM Hepes, pH 7.5; 10 mM magnesium acetate; 10 μg/ml calmodulin; and 1 mM $CaCl_2$) with 0.1 mM [γ^{32}P]ATP 350 cpm/pmol, 0.25 μg calmodulin kinase II, and either 20 μM syntide II [43] or 2 mM peptide. Protein kinase C from rabbit brain (a gift of Susan Jaken, Lake Placid, NY) was assayed in PK-C buffer (20 mM Tris-HCl, pH 7.4; 7.5 mM $MgCl_2$; 1 mM EGTA; 1.5 mM $CaCl_2$; and 100 μg/ml phosphatidyl serine), 0.125 mM [γ^{32}P]ATP (600 cpm/pmol) 500 ng protein kinase C, and 0.6 mg/ml histone III-S (Sigma Chemical Co., St. Louis, MO) or 2 mM peptide for 30 min at 30°C [38].

Purification of p34^{cdc2}

The enzyme was purified from nocodazole-treated HeLa cells according to the method of Brizuela et al. [21], with the following modifications. The initial extraction of cells was done in hypotonic buffer (10 mM sodium phosphate, pH 7.0, 10 mM NaF, 5 mM $MgCl_2$, 1 mM EDTA, 10 mM β-glycerophosphate, 1 mM EGTA, containing the protease inhibitors 50 mg/L phenylmethylsulfonylfluoride, 1 mg/L leupeptin, 10 mg/L soybean trypsin inhibitor, 1 mg/L aprotinin, and 10 mg/L tosyl-phenylchloromethylketone [21,22] using 20 strokes of a tight-fitting Dounce homogenizer. The buffer was adjusted to 0.5 M NaCl, followed by five more strokes of the homogenizer, a procedure that gives more efficient overall recovery of activity (J. Bischoff and D. Beach, personal communication). All other procedures were as described, but we used low pressure columns (1 × 5 cm) of S-Sepharose and Q-Sepharose (Pharmacia, Piscataway, NJ) in place of Mono-S and Mono-Q FPLC columns. Protein concentrations were determined by quantitative dye-binding using bovine serum albumin as standard [29]. Protein concentrations below 1 μg were determined by a colloidal gold assay [30] using a commercial kit (Integrated Separation Systems).

Gel Electrophoresis and Immunoblots

Gel electrophoresis was performed on 10% (w/v) polyacrylamide gels using the buffer system of Laemmli [31]. Proteins were transferred electrophoretically to nitrocellulose filters (Bio-Rad, Richmond, CA), and immunoblots were performed as described [32] using the rabbit antiserum G6 [22] to p34^{cdc2} (directed against a carboxy-terminal eptiope of the human protein) as primary antibody, and [^{125}I]-goat anti-rabbit immunoglobulin F(ab)$_2$ as secondary antibody (New England Nuclear, Boston, MA). The gels were stained with Coomassie blue, dried, and exposed to film (Kodak X-OMat). Silver staining of gels was done as described [33] using a commercial reagent kit (BioRad, Richmond, CA).

Elutriation of Cells

Exponentially growing HeLa cells grown in suspension cultures were separated by centrifugal elutriation and analyzed by flow cytometry exactly as described [22,34]. The proportions of G_1, S, and G_2/M phase cells were determined by DNA content after flow cytometry of propidium iodide-stained samples of fractions from the elutriator. The proportions of cells in the fractions used were G_1 fraction, 78% G_1, 8% S, 11% G_2/M; S fraction, 35% G_1, 27% S, 35% G_2/M; and G_2/M fraction, 11% G_1, 16% S, 67% G_2/M. Cell lysates for assay were prepared by homogenization of cells in 0.5 volumes (0.5 mL buffer/mL packed cells) of lysis buffer (20 mM Tris-HCl pH 8.0, 137 mM NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 10% (v/v) glycerol, 1% (v/v) Nonidet P-40, 0.4 mM sodium orthovanadate, 10 mM EDTA, and 1 mM phenylmethylsulfonylfluoride), incubated on ice for 30 min, centrifuged at 15,000g for 10 min at 4°C, and the supernatant collected [22].

Immunoprecipitation

The immunoprecipitation of p34^{cdc2} from lysates of HeLa cells were done as described [21,22,32] using a rabbit polyclonal antibody directed against a synthetic peptide representing the carboxy-terminal six amino acid residues of human p34^{cdc2} [22]. All lysates were preadsorbed twice with 0.5 ml of 10% (v/v) fixed and killed S. aureus (Zysorbin), followed by incubation with the primary antibody for 60 min at 4°C. Precipitation of the antibody-antigen complex was accomplished with Protein A-Sepharose (Pharmacia, Piscataway, NJ). In experiments using C160 antibody (a mouse monoclonal antibody to p60), the lysate containing primary antibody was further incubated with a rabbit anti-mouse immunoglobulin prior to adsorption with Protein A-Sepharose [35]. Affinity adsorption of lysates to p13-Sepharose was done as described [34] without pre-adsorption.

RESULTS

Synthetic peptide substrates for p34^{cdc2} were designed based on the sequences of Simian Virus 40 Large T antigen (LTag) and the recessive oncogene p53 for which we previously characterized the sites of phosphorylation by p34^{cdc2} [17,18]. The sequence of the LTag peptide was H-A-D-A-Q-H-A-T-P-P-K-K-K-R-K-V-E-D-P-K-D-F-OH (CSH103) and the sequence for the p53 peptide was H-K-R-A-L-P-N-N-T-S-S-S-P-Q-P-K-K-K-P-L-D-G-E-Y-NH_2 (CSH133). The peptide CSH103 has alanine residues in positions 3 and 6 that correspond to serines at positions 120 and 123 in the intact protein. The alanine residue at position 6 in the peptide was introduced to avoid phosphorylation at multiple sites by p34^{cdc2}. The alanine residue at position 3 was introduced to eliminate the possibility that the peptide would

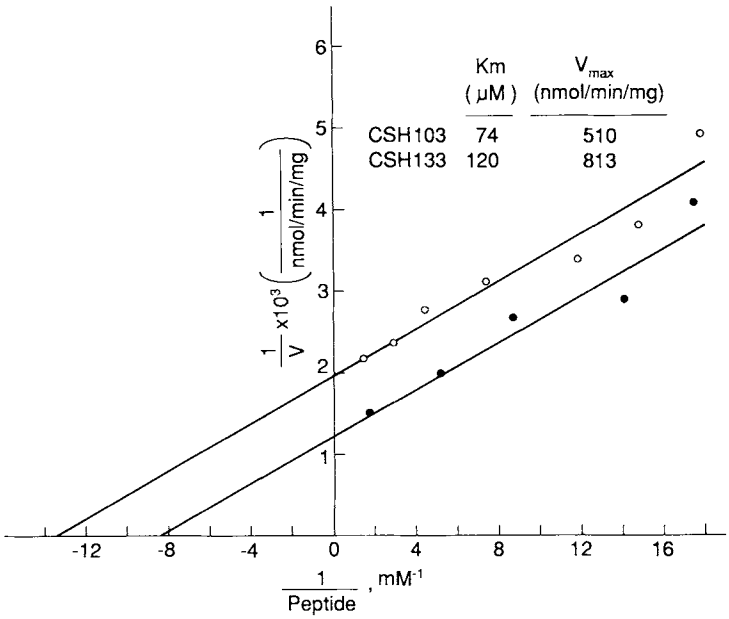

Fig. 1. Lineweaver-Burk plot of peptide phosphorylation. Various amounts of peptides CSH103 (○—○) and CSH133 (●—●) were phosphorylated for 10 min at 30°C with $p34^{cdc2}$ isolated from mitotic HeLa cells (nocodazole-treated) as the active complex with cyclin B [18]. The K_m and V_{max} values calculated for each peptide are given in the inset.

be a substrate for a protein serine kinase with another specificity. In fact, serine 120 of LTag has been implicated as a potential substrate for a double-stranded DNA-dependent protein kinase (C. W. Anderson, personal communication). Peptide CSH133 represents the unadulterated p53 sequence, in which serine 11 is the exclusive $p34^{cdc2}$ phosphorylation site [18]. Both peptides have stretches of basic amino acids that are part of the native sequence, are proximal to the phosphorylation site, and allow the peptides to bind to phosphocellulose paper. This ability permits the design of a rapid and simple assay according to those previously described for other protein kinases [26–28]. In addition, the peptides utilized here have been characterized chemically and physically using mass spectrometry, to avoid contaminating adducts that are not detectable by amino acid analysis or protein sequencing. For both peptides, the measured mass agreed with the predicted, isotopically averaged mass to >99.9%, and there was no evidence of anisole adducts or dehydration products. Our earlier studies with synthetic substrates for casein kinase II [26] have indicated that a number of chemical artifacts of the *synthesis* can alter the phosphorylation of the peptide substrate. Therefore, we used peptides purified to apparent chemical homogeneity to test their biological specificity as substrates for $p34^{cdc2}$.

Using purified HeLa cell $p34^{cdc2}$ in solution, both peptides showed linear incorporation of phosphate for 15 minutes, and the enzyme showed K_ms for peptides CSH103 and CSH133 of 74 and 120 μM, respectively. The corresponding V_{max} values of 510 (CSH103) and 813 (CSH133) nmol/min/mg enzyme were also determined (Fig. 1). The specificity of the peptides was addressed by measuring the ability of eight different purified protein serine/threonine kinases to phosphorylate the peptides under conditions that are optimal for each enzyme. As shown in Table I, casein kinases I and II, protein kinase A, glycogen synthase kinase 3, and skeletal muscle phosphorylase b kinase did not incorporate phosphate into the peptides under optimal conditions for each of the enzymes. However, two Ca^{2+}-requiring enzymes, protein kinase C and calmodulin kinase II, incorporated small amounts of phosphate into the peptides under optimal buffer conditions for those enzymes. When the peptides were assayed in the optimal $p34^{cdc2}$ buffer containing the calcium chelator, EGTA, no detectable incorporation of phosphate was observed for CSH103 (Table I). Similarly, peptide CSH133 was not phosphorylated by calmodulin kinase II in $p34^{cdc2}$ buffer. How-

TABLE I. Phosphorylation of Synthetic Peptides by Protein Kinases

Enzyme	Control substrate	Phosphate incorporated (pmol/10 min)		
		Control substrate	Peptide CSH103	Peptide CSH133
HeLa p34^{cdc2}	Histone H1	43	27.9	17.8
Casein kinase I	Casein	480	<1	<1
Casein kinase II	Casein	88	<1	<1
cAMP-dependent protein kinase	Kemptide	204	<1	<1
Glycogen synthase kinase 3	Glycogen Synthase	103	<1	<1
Phosphorylase b kinase	Phosphorylase b	545	<1	<1
Protein kinase C				
in PK-C buffer[a]		254	1.4	17.1
in p34^{cdc2} buffer[b]	Histone IIIS	251	<1	8.4
Calmodulin Kinase II				
in CaMK buffer[c]		821	1.3	7.3
in p34^{cdc2} buffer[b]	Syntide II	27	<1	<1

[a]The PK-C buffer contained 20 mM Tris-HCl, pH 7.4; 7.5 mM $MgCl_2$; 1 mM EGTA; 1.5 mM $CaCl_2$; 100 μg/ml phosphatidyl serine.
[b]The p34^{cdc2} buffer contained 50 mM Tris-HCl, pH 8.0; 10 mM $MgCl_2$; 1 mM DTT; 1mM EGTA.
[c]The CaMK buffer contained 50 mM Hepes, pH 7.5; 10 mM magnesium acetate; 10 μg/ml calmodulin; 1 mM $CaCl_2$.

TABLE II. Purification of Peptide Kinase Activity From Mitotic HeLa Cells

Procedure	Total protein (mg)	Total activity (Units)[a]	Recovery (%)	Specific activity (Units/mg)	Purification (fold)
100,000g	1,795.7	1,211	14.9	0.67	1.0
$(NH_4)_2SO_4$	981.8	8,107	100	8.26	12.3
DEAE-Cellulose	401.8	5,970	73.6	14.9	22.2
S-Sepharose	38.8	6,036	74.5	155.6	232.2
Casein-Sepharose	0.211	839	10.4	3,976	5,935
Q-Sepharose	0.0074	45	0.56	6,081	9,076

[a]One unit of total enzyme activity is defined as the incorporation of 1.5 nmol phosphate into peptide CSH103 in 10 min at 30°C using [γ-^{32}P] ATP.

ever, under these conditions, protein kinase C phosphorylated peptide CSH133 to approximately 49% of the level of phosphorylation in the optimal protein kinase C buffer (Table I). These results indicate that CSH103 is a specific substrate for p34^{cdc2}, while CSH133 is phosphorylated by both p34^{cdc2} and, to a lesser degree, protein kinase C.

The specificity of the peptide CSH103 was further substantiated by purifying the peptide kinase activity from HeLa cells and showing that the peptide kinase co-purifies at every step with active p34^{cdc2}, assayed by immunoprecipitation with specific antiserum followed by phosphorylation of histone H1. The HeLa cells used were arrested in mitosis with the microtubule-disrupting agent, nocodazole, because mitotic cells have elevated levels of p34^{cdc2} kinase activity and serve as a good source for the enzyme [21,22]. The results of the purification are summarized in Table II. After the initial homogenization in hypotonic and hypertonic buffers, a homgenate supernatant was collected by centrifugation at 100,000g. Activity was further purified by ammonium sulphate precipitation (25–50% saturation). Total activity increased from the homogenate supernatant to the ammonium sulphate fraction, suggesting an inhibitory factor present in the crude extracts. It is likely that this is due to the effects of NaF and β-glycerophosphate in the homogenization buffer, as these are known inhibitors of p34^{cdc2} [21,37]. In each subsequent chromatographic step, fractions were analyzed for kinase activity using peptides CSH103 and CSH133, as well as histone H1. In parallel, each fraction was immunoprecipitated with an anti-

body to a carboxy-terminal region of human $p34^{cdc2}$. Each immune complex was assayed for histone H1 kinase activity. Figure 2 shows the cation exchange, casein-agarose, and anion exchange chromatography steps. In each case, the specific $p34^{cdc2}$ histone H1 kinase activity comigrates with the peptide kinase activity. The final, purified peptide kinase gave a single band migrating with M_r = 34,000 on an immunoblot probed with antibody to human $p34^{cdc2}$ (Fig. 3A). In vitro phosphorylation (Fig. 3B) of the purified peptide kinase also showed proteins with M_r = 60,000–62,000. These probably represent cyclins [11] that are known to be associated with the catalytic unit of $p34^{cdc2}$ [5,21,35,36]. Several higher molecular weight bands are also present with M_r > 100,000. On silver-stained polyacrylamide gels, the major two bands migrate with M_r = 34,000 and 62,000, as do $p34^{cdc2}$ and cyclin B (Fig. 3C). Again several higher molecular weight bands are present with mobilities similar to the substrates seen upon in vitro phosphorylation. Thus, the purified peptide kinase appears to consist of $p34^{cdc2}$, cyclins, and several higher molecular weight proteins, similar to that isolated from mitotic cells as an active enzyme complex [21].

We further tested the peptides for their ability to be phosphorylated by crude lysates of HeLa cells enriched for particular phases in the cell division cycle: G_1, S, and G_2/M. Three populations of HeLa cells were isolated by centrifugal elutriation as previously described [19]. The CSH103 kinase activity increased 3.9-fold between G_1- and S-phase extracts, and 6.5-fold between G_1 and G_2/M cell lysates (Table III). The peptide kinase activity in the G_1 cell extracts was noteworthy on two accounts. First, they displayed higher incorporation of phosphate into CSH133 than CSH103, perhaps reflecting a low level of phosphorylation by protein kinase C, as well as $p34^{cdc2}$. Second, the level of phosphorylation of peptide CSH103 in the G_1 cell extracts was higher than expected, prompting further studies of the phosphorylation of the peptide in specific immune and affinity complexes. The CSH103 phosphorylation in G_1, S, and G_2/M whole cell lysates was compared to values obtained for CSH103 phosphorylation by specific $p34^{cdc2}$ complexes (Table III), including: i) immune complexes using $p34^{cdc2}$ antibody; ii) immune complexes with an antibody to the protein p60, an adenovirus E1A-binding protein that interacts with $p34^{cdc2}$ [35]; and iii) complexes with immobilized $p13^{suc1}$, a protein that binds tightly to $p34^{cdc2}$ [34]. The relative increase in CSH103 phosphorylation between G_1 and G_2/M cell extracts was 5.4 in $p34^{cdc2}$ antibody immune complexes, 5.1 in p60 antibody immune complexes, and 5.4 in p13-Sepharose complexes, compared to the value of 6.5 measured in cell lysates. The relative increase in histone H1 phosphorylation was greater than that for peptide phosphorylation in all cases: 7.0 for $p34^{cdc2}$ antibody immune complexes, 10.2 for p60 antibody immune complexes, and 10.5 for p13-Sepharose complexes. The histone H1 phosphorylation by whole cell lysates was not determined since there are multiple protein kinases that will phosphorylate histone H1 [38].

Using extracts of G_2/M cells, we measured the ability of the carboxy-terminal antibody to $p34^{cdc2}$ and p13-Sepharose to deplete the peptide kinase activity. After three sequential precipitations of the extracts, 90% of the CSH103 peptide kinase activity was removed by p13-Sepharose, and 55% of the activity was removed by the antibody. Sequential precipitation of extracts with $p34^{cdc2}$ antibody followed by p13-Sepharose depleted 85% of the CSH103 kinase activity. This study indicated that the epitope recognized by the carboxy-terminal antibody to human $p34^{cdc2}$ was immunoreactive for the majority (55%) of the peptide kinase activity, that nearly all (90%) of the activity had the ability to bind to p13, a $p34^{cdc2}$-binding protein [5,21,34], and that there was 10–15% of the activity that did not react with these reagents.

DISCUSSION

The protein kinase $p34^{cdc2}$ appears to play a central role in regulation of the cell division cycle, acting both in the induction of mitosis and in the onset of S phase [3,18]. The ability to rapidly and accurately measure this protein kinase activity is fundamental to biochemical and cell biological investigations of the cell division cycle. To this end, we have developed synthetic peptide substrates for the enzyme and fully characterized their specificity. One of the peptides (CSH103), based on the Simian Virus 40 large T antigen sequence, is specific for $p34^{cdc2}$ over seven other classes of protein serine/threonine kinases involved in signal transduction pathways. The peptide CSH133, based on the p53 sequence, shows a low level of cross reactivity as a substrate with protein kinase C. In both cases, the peptides are preferable to histone H1, a common

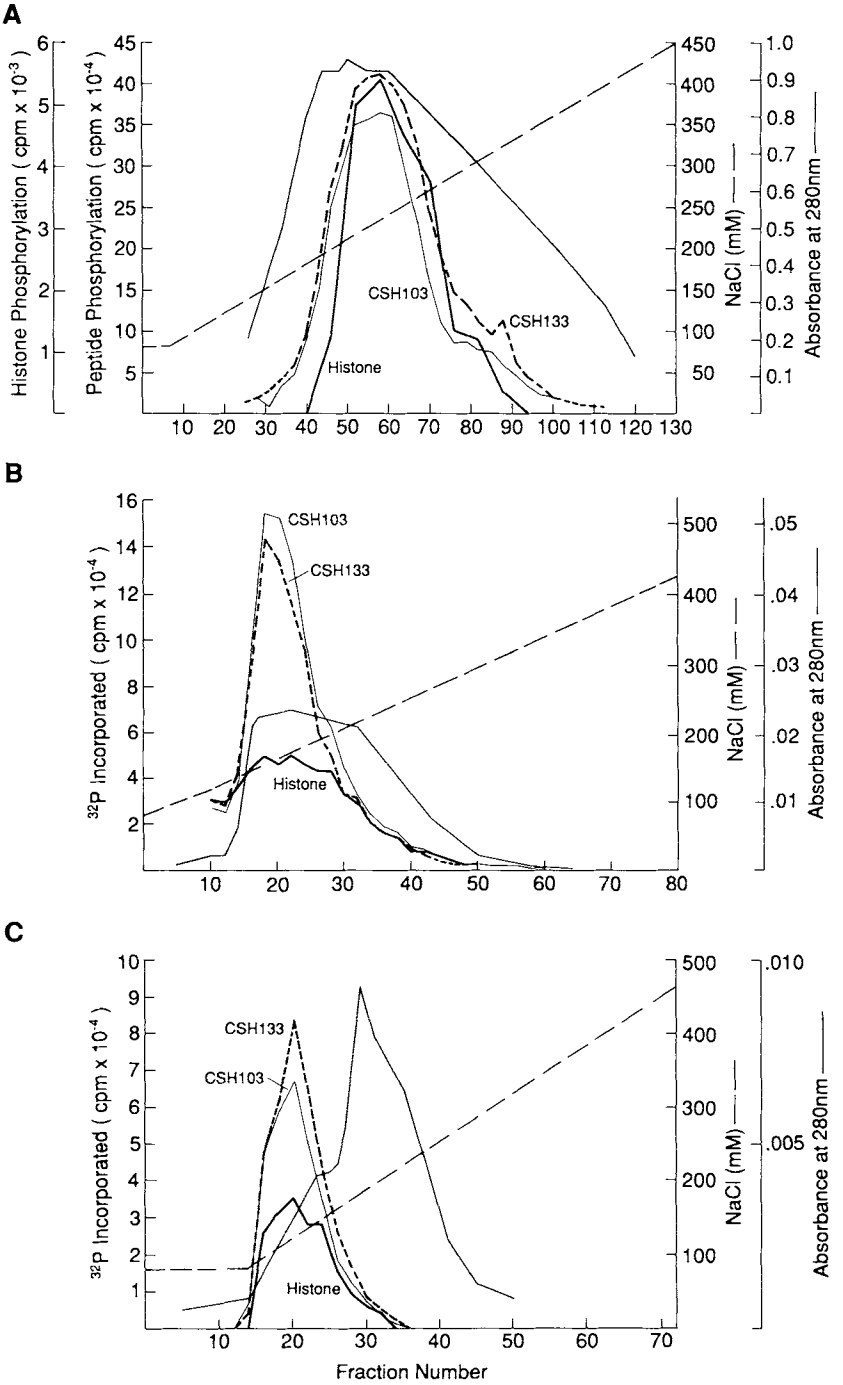

Fig. 2. Chromatographic purification of peptide kinase from mitotic HeLa cells. **A:** Cation exchange chromatography on S-Sepharose; **B:** Affinity-based adsorption chromatography on casein-Sepharose; **C:** Anion exchange on Q-Sepharose. For each column, fractions were monitored for conductivity and absorbance at 280 nm, and the phosphorylation of peptides CSH103 and CSH133 were measured. In addition, the phosphorylation of histone H1 by each fraction and by an immunoprecipitation of each fraction using antibodies to human p34^{cdc2} was also measured. The immunoprecipitated histone H1 kinase activity is shown in panel A and the total histone H1 kinase activity is shown for panels B and C.

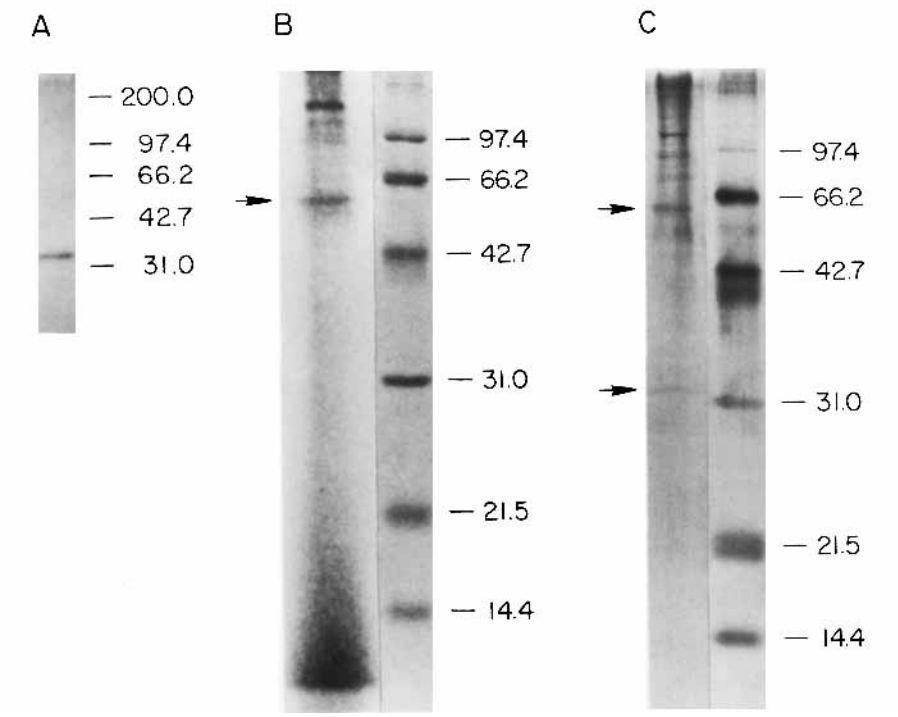

Fig. 3. Characterization of peptide kinase purified from mitotic HeLa cells. **A:** Immunoblot of purified peptide kinase using antibodies to human $p34^{cdc2}$. The peptide kinase was subjected to electrophoresis on 10% (w/v) polyacrylamide gels in the presence of sodium dodecyl sulfate, and the proteins were electrophoretically transferred to nitrocellulose membranes, incubated with antibody directed against the carboxy-terminal epitope of human $p34^{cdc2}$, and subsequently incubated with [^{125}I]-goat anti-rabbit immunoglobulin as described [32]. An autoradiogram of the nitrocellulose filter is shown, and the molecular weights (in kilodaltons) of standard protein standards are shown. **B:** Autophosphorylation of purified peptide kinase. The peptide kinase was phosphorylated for 60 min at 30°C using [γ-^{32}P] ATP (3,000 Ci/mmol) and subjected to electrophoresis on 12.5% polyacrylamide gels [31]. An autoradiogram of the gel is shown with protein standards stained with Commassie blue. The molecular weight (in kilodaltons) of the markers are shown and arrows indicate the band with M_r = 60-62 kD. **C:** Silver stain of peptide kinase. Electrophoresis was done as in B and the gel was stained with silver as described [33]. Protein molecular weight markers are shown as in B, and arrows indicate bands at M_r = 34 kD and 60–62 kD.

TABLE III. Peptide Kinase Activity in Elutriated HeLa Cell Extracts

Sample	Cell cycle stage	Phosphate incorporated (pmol/10 min/10^7 cells) CSH103	CSH133	Histone H1
Cell lysate	G_1	138	204	nd[b]
	S	536	586	nd
	G_2/M	890 [6.5][a]	822 [4.0]	nd
Immunoprecipitate	G_1	29.0	13.2	36.9
$p34^{cdc2}$ Antibody	S	117	87.4	169
	G_2/M	155 [5.4]	155 [11.7]	258 [7.0]
Immunoprecipitate	G_1	0.19	0.15	0.09
p60 Antibody	S	0.65	0.55	0.50
	G_2/M	0.96 [5.1]	0.72 [4.8]	0.92 [10.2]
Precipitate with	G_1	120	59.3	55.3
p13-Sepharose	S	493	407	370
	G_2/M	646 [5.4]	552 [9.3]	578 [10.5]

[a]The value shown in brackets is the relative increase in phosphate incorporation, calculated as the ratio between the values for the G_2/M and G_1 cell extracts.
[b]Not determined.

substrate for p34^{cdc2}, but which is also a good substrate for protein kinase C and several other cellular kinases [38]. Purification of the peptide kinase activity from HeLa cells arrested in mitosis yields an enzyme indistinguishable from p34^{cdc2} by electrophoretic mobility and reactivity with a specific, site-directed antibody. The peptide kinase does not appear to contain an immunoreactive catalytic subunit different than p34^{cdc2}, based on immunoblots. However, there are multiple high molecular weight proteins that copurify with the p34^{cdc2} protein, as have been seen in other purification schemes [21].

Several synthetic peptide substrates for p34^{cdc2} had been previously reported, based on the p53 protein [18,44], nucleolin [16], and nuclear lamin [14]. In each case, the peptides were shown either to be substrates for p34^{cdc2} or to inhibit the enzyme, but in no case was the specificity of the peptide phosphorylation documented. The peptides have multiple serine [18] or threonine [14] residues that could be potential phosphorylation sites for other kinases. In fact, as shown here, the p53 peptide, CSH133, did serve as substrate for protein kinase C as well as p34^{cdc2} [18,44]. We suggest, therefore, that synthetic peptides used for protein phosphorylation research be characterized for specificity among known protein kinases to avoid ambiguities in the interpretation of experiments in biological systems.

The use of peptides also permits rapid assay of whole cell lysates, yielding results similar to those seen using specific precipitation methods followed by assay with histone H1 or with the peptides. For the p60 immune complexes and the p13-Sepharose complexes, the relative increase between G_1 and G_2/M was 10.2 and 10.5 for histone H1 compared to 5.1 and 5.4 for CSH103. Examination of the results for the G_1 extracts reveals that the phosphorylation of peptide CSH103 was twice that of histone H1, while the G_2/M stage activities were essentially the same. Therefore, the difference between the 10-fold relative increase in histone H1 phosphorylation and the 5-fold relative increase for CSH103 phosphorylation can be accounted for by twice as much peptide kinase activity in G_1 compared to histone H1 kinase activity, rather than any differences in G_2/M levels. In other words, using peptide CSH103 as a substrate reveals more enzyme activity in G_1 than does using histone H1. This is not the case for the immune complexes with p34^{cdc2} antibody, in which the relative increases were more similar to each other, 5.4 for CSH103 and 7.0 for histone H1. These results demonstrate that CSH103 can be used for assay of whole cell lysates, as well as for specific immune or affinity complexes of p34^{cdc2}, and that the peptide may detect more activity in the G_1 phase of the cell division cycle.

Most protein kinases are members of subfamilies of enzymes [39], and it is common that isozymes of protein kinases occur, even within the same cell type [40,41]. The possibility remains that enzymes very closely related to p34^{cdc2} could phosphorylate the peptides developed in this study. Such is the case for casein kinase II, in which both the α and α' forms of the catalytic subunit utilize the specific, acidic substrate peptide [28]. Protein kinases utilizing basic substrates, such as the cAMP-dependent protein kinase [40,41] and protein kinase C [42], also have isoforms that phosphorylate their respective synthetic peptide substrates.

Several of our experiments support the idea that other isoforms of p34^{cdc2} exist in HeLa cells. First, as discussed above, the relative increase of peptide CSH103 kinase activity between extracts of cells G_1 and G_2/M is lower than that for histone H1 kinase activity, particularly for precipitation with p13-Sepharose and with antibody to p60. This suggests that the peptide can detect an active kinase in G_1 that does not phosphorylate histone H1. Purification of such a G_1 form of p34^{cdc2} will be facilitated by the rapid peptide assay and may contribute to our knowledge of the role of p34^{cdc2} in the G_1-S transition. Second, the depletion experiments indicate that nearly all of the peptide CSH103 activity can be depleted from extracts of G_2/M cells by p13-Sepharose, but that the p34^{cdc2} antibody depleted only 55% of the activity. One explanation for this observation is that the carboxy-terminal epitope on a population of p34^{cdc2} molecules is masked by an interaction with a binding protein, and, therefore, is not available for reaction with the antibody. Such a complex must still interact with p13, since immobilized p13 depleted the bulk of the activity remaining after depletion by precipitation with the antibody. Alternatively, there could exist another enzyme that is very similar to p34^{cdc2} in substrate specificity, binding to p13, and chromatographic behavior, but containing an altered carboxy-terminus. In this respect, the peptide substrates will provide a useful tool to purify and characterize closely related forms of p34^{cdc2}.

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