Cdc2-Cyclin B Phosphorylates p70 S6 Kinase on Ser\textsuperscript{411} at Mitosis\textsuperscript{*}

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The carboxyl terminus of p70 S6 kinase (p70\textsubscript{6k}) has a set of Ser and Thr residues (Ser\textsuperscript{411}, Ser\textsuperscript{418}, Ser\textsuperscript{421}, and Thr\textsuperscript{429}) phosphorylated \textit{in vivo} by an unidentified kinase(s). These Ser/Thr sites are immediately followed by proline, a motif that is commonly seen in the substrates of cyclin-dependent kinases (Cdk) and mitogen-activated protein kinases. A previous study has shown that Cdc2 (Cdk1) indeed phosphorylates these p70\textsubscript{6k} Ser/Thr residues \textit{in vitro}. Here, we demonstrate that Cdc2-cyclin B complex phosphorylates Ser\textsuperscript{411} in the KIRSPPR sequence, whereas other Cdk-cyclin complexes including those containing Cdk2, Cdk4, or Cdk6 do not. Additionally, Ser\textsuperscript{411} phosphorylation \textit{in vivo} was increased at mitosis in parallel with Cdc2 activation, and it was suppressed by a dominant negative form of Cdc2. These data indicate that p70\textsubscript{6k} is a physiological substrate of Cdc2-cyclin B in mitosis. Since the activity of p70\textsubscript{6k} is low during mitosis, Cdc2-cyclin B may play a role in inactivating p70\textsubscript{6k} during mitosis, where protein synthesis is suppressed.

The p70 S6 kinase (p70\textsubscript{6k}) is a Ser/Thr kinase that is activated by mitogenic stimulation at the G\textsubscript{0}/G\textsubscript{1} transition of the cell cycle in mammalian cells (1–3). It phosphorylates five Ser residues in ribosomal protein S6 in \textit{vivo} (4) and is the major S6 kinase in \textit{vivo} in mammalian cells (5, 6). The role of the p70\textsubscript{6k} activation pathway in cell proliferation is not fully understood; the pathway may regulate translation of mRNAs encoding ribosomal proteins that have a conserved sequence at their 5′-end (pyrimidine tract followed by GC-rich regions) (7–9). Thus, the p70\textsubscript{6k} pathway may up-regulate ribosome biogenesis, which is critical for quiescent cells to enter and proceed through the cell division cycle (10). Indeed, the microinjection of quiescent rat fibroblasts with polyclonal antibodies against Cdk2, Cdk4, or Cdk6 abolishes serum-induced entry into S phase of the cell cycle (11). In addition, inhibition of the kinase pathway by rapamycin either prolongs G\textsubscript{1} progression or blocks the G\textsubscript{1}/S transition of the cycle (10, 12).

p70\textsubscript{6k} has two distinct sets of Ser/Thr residues phosphorylated \textit{in vitro}. One set includes Thr\textsuperscript{429} and Thr\textsuperscript{389}, and Ser\textsuperscript{404} (13, 14), and the other set includes Ser\textsuperscript{411}, Ser\textsuperscript{418}, Ser\textsuperscript{424}, and Thr\textsuperscript{421} at the carboxyl terminus (15). Phosphorylation of the former set, especially Thr\textsuperscript{429} and Thr\textsuperscript{389}, plays a critical role in activation of the kinase, since replacement of these Thr residues by Ala abrogates kinase activity (14, 16, 17). Additionally, phosphorylation of these Thr and Ser residues is sensitive to rapamycin, which inactivates the kinase indirectly (13), and it is believed that phosphatidylinositol 3-kinase and the structurally related enzyme, mTOR (also termed FRAP or RAFT), are involved in the regulation of phosphorylation/dephosphorylation of these sites (16, 18). Recently, it was shown that Akt (also termed protein kinase B) is involved in activation of p70\textsubscript{6k} by phosphatidylinositol 3-kinase (19), but direct regulators of p70\textsubscript{6k} are still not elucidated. In contrast to these phosphorylation sites, the role of phosphorylation at the carboxyl terminus (Ser\textsuperscript{411}, Ser\textsuperscript{418}, Ser\textsuperscript{421}, Thr\textsuperscript{421}) is still obscure. The collection of studies examining mutations in these residues revealed that these phosphorylation sites are not essential for rapamycin-sensitive regulation of the kinase activity (20, 21). Of interest, all of these Ser and Thr are within a conserved sequence (Ser/Pro or Thr/Pro) seen in substrates of cyclin-dependent kinase (Cdk) families or mitogen-activated protein kinase families (22).

A previous study demonstrated that Cdc2 phosphorylated these Ser/Thr residues \textit{in vitro} when purified p70\textsubscript{6k} was used as a substrate, while the effect of mitogen-activated protein kinase was less clear (23). Indeed, of these Ser/Thr phosphorylation sites, Ser\textsuperscript{411} is within a perfect consensus sequence (K/R-S-P/R-P/R/K/H) as a substrate for Cdc2 (24). However, Cdc2, which associates with either cyclin A or cyclin B, is activated exclusively in S to G2/M phases of the cell cycle (25), whereas the identified regulations of p70\textsubscript{6k} activity occur in earlier phases of the cell cycle (G\textsubscript{1}/G\textsubscript{0} to G\textsubscript{2}/M), as described above. Therefore, it was postulated that a Cdc2 homolog that is activated at an earlier phase of the cell cycle, such as Cdk2, Cdk4, or Cdk6, might phosphorylate p70\textsubscript{6k} \textit{in vivo}, or alternatively that phosphorylation of p70\textsubscript{6k} by Cdc2 was solely \textit{in vitro}. Here, we demonstrate that Cdc2-cyclin B has the highest activity among Cdk family members for phosphorylation of the carboxyl terminus of p70\textsubscript{6k} \textit{in vitro}, and moreover Cdc2 phosphorylates the carboxyl terminus of p70\textsubscript{6k} during mitosis \textit{in vivo}.

**EXPERIMENTAL PROCEDURES**

Reagents—Phorbol 12,13-dibutyrate (PDBu, Sigma) and calcium ionophore, ionomycin (Calbiochem, San Diego, CA) were dissolved in dimethyl sulfoxide (Me\textsubscript{2}SO). Rapamycin was obtained from the Drug Synthesis and Chemistry Branch, NCI, and dissolved in ethanol to give a 1 mg/ml stock solution. Aphidicolin (Sigma) was prepared as a 5 mg/ml stock solution in ethanol. Nocodazole (methyl-[5-2-thienylcarboxy]-1H-benzimidazol-2-yl)carbamate, Sigma) was prepared as a 1 mg/ml stock solution in Me\textsubscript{2}SO. Antibodies used in the study were as

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1 The abbreviations used are: Cdk, cyclin-dependent kinase; PDBu, phorbol 12,13-dibutyrate; HA, hemagglutinin; Cdc, cell division cycle; PMSF, phenylmethylsulfonyl fluoride; TLE/TLC, two-dimensional thin layer electrophoresis/chromatography.
follows: anti-Cdc2, rabbit polyclonal antibodies raised against human Cdc2 (kindly provided by David Beach); anti-Cdk4, rabbit polyclonal antibodies raised against an epitope 283–298 of human Cdk4 (sc-163, Santa Cruz Biotechnology, Santa Cruz, CA); anti-Cdk4, rabbit polyclonal antibodies raised against an epitope 282–303 of human Cdk4 (sc-127, Santa Cruz Biotechnology); anti-cyclin A, rabbit polyclonal antibodies raised against human cyclin A (kindly provided by Jonathan Pines); anti-cyclin B, mouse monoclonal IgGl recognizing an epitope 1–21 of human cyclin B1 (GNS-1, Pharmingen, San Diego, CA). Protein G-Sepharose and protein A-Sepharose (Pharmacia, Piscataway, NJ) were used for the isolation of recombinant proteins. Antibodies raised against the COOH terminus (amino acids 479–502, NSG-SYKQAPFPMISKRPEHLRMNL) of p70s6k (anti-p70s6kCT antibody) or raised against the NH2 terminus (amino acids 1–21, PVKKQAFPMISKRPEHLRMNL) of p70s6k (anti-p70s6kN antibody) were raised in rabbits. Anti-phospho-Ser411 antibody was raised in mice with a synthetic phospho-Ser411 peptide. Specific reactive bands were detected using rabbit anti-mouse IgG conjugated to alkaline phosphatase. Development was performed using NBT and BCIP. Preparations of Recombinant p70s6k—Recombinant p70s6k (approximately 100 ng of the protein) purified by TALON beads [p70s6k cDNA fragments prepared previously (17), were introduced into era frugiperda expression of the proteins. Mononuclear cell suspensions were prepared by Ficoll-Hypaque gradient centrifugation, and T cells were obtained by E-rosette enrichment as described (10). Cells were cultured in RPMI 1640 (Life Technologies, Inc.) inactivated fetal bovine serum (HyClone, Logan, UT), 2 mM L-glutamine (Life Technologies, Inc.), 100 units/ml penicillin, and 100 µg/ml streptomycin (Life Technologies, Inc.). COS7 cells were cultured in Dulbecco’s modified Eagle’s medium (Life Technologies, Inc.) supplemented with 10% (v/v) fetal bovine serum, 100 units/ml penicillin, and 100 µg/ml streptomycin. S9 (Spodoptera frugiperda) cells were maintained at 30 °C in serum-free SF900 II SFM medium (Life Technologies, Inc.) supplemented with 50 units/ml penicillin and 50 µg/ml streptomycin.

Preparation of Recombinant p70s6k—Recombinant p70s6k (p70WT) was made using a baculovirus expression system (BAC-TO-TO baculovirus expression system, Life Technologies, Inc.). Briefly, HA-tagged wild type p70s6k cDNA fragments prepared previously (17), were introduced into pFasticBacHTb donor plasmid using the unique SauI site of the vector. Then, the recombinant vector was transformed into DH10BAC competent cells, which contain the bacmid with a mini-attTn7 target site and the helper plasmid for efficient transposition. The p70WT recombinant bacmids identified by disruption of the loc2a gene, were then transfected into S9 cells. Similarly, the recombinant bacmid containing HA-tagged-mutant p70s6k (T229A or A237T) was produced and transfected into S9 cells. The recombinant p70WT was recovered by a metal affinity resin (TALON, CLONTECH, San Diego, CA), and confirmed in immunoblot analysis by reactivity with several different antibodies raised against p70s6k peptides. The recombinant wild type p70s6k (recWT-p70) had a high kinase activity for phosphorylation of ribosomal S6 peptide as described previously (29). In contrast, recT229A-p70 had little or no activity, and recA237T-p70 had approximately one-fifth of the activity compared with the wild type. The recombinant viruses were amplified by serial infection and the supernatants were used for further expression of the proteins.

In Vitro Phosphorylation of Recombinant p70s6k—Recombinant p70s6k (approximately 100 ng of the protein) purified by TALON beads were washed once with a kinase buffer (50 mM Tris-HCl, pH 7.4, 10 mM MgCl2, 1 mM dithiothreitol), and the beads were suspended in 50 µl of the kinase buffer containing 100 µM unlabeled ATP, 200 µC/mM [γ-32P]ATP, and 1 µl of a purified Xenopus Cdc2-cyclin B or Cdk2-cyclin E complex (activities: ~1 pmol/µl/min to transfer phosphate to whole histone H1 peptide). The reaction was allowed to proceed for 30 min at 30 °C. As a control reaction, histone H1 (1 µg) was added in 50 µl of reaction mixture instead of rec-p70 resin. The reaction was then completed by adding a protein loading buffer. After boiling for 5 min, labeled proteins were separated by 10% SDS-polyacrylamide gel. Phosphopeptide Mapping—Recombinant p70s6k was cut out from SDS-polyacrylamide gel, and homogenized in 50 mM ammonium bicarbonate (pH 7.4) containing 1% SDS and β-mercaptoethanol. Extracted proteins were precipitated by trichloroacetic acid in the presence of 10 µg of a carrier protein (RNase), washed with ethanol/ethyl ether (1:1), and treated with hydroxyl peroxide/formic acid (1:10) solution. After drying, proteins were subjected to serial chymotrypsin and trypsin digestion. Samples were then dissolved in an electrophoresis buffer (2.25% formic acid, 7.75% acetic acid), loaded to TLC-cellulose plates (Merek5716 cellulose, 20 × 20 cm, EM Science), and separated by electrophoresis at 1200 V for 30 min. The peptides were further separated by ascending chromatography for 16 h using a buffer containing 1% acetic acid, 0.1% TFA, and 25% acetonitrile. The phosphopeptides were visualized by autoradiography. In some experiments, the Ser411 peptide phosphorylated in vitro was purified using C-18 Sep-Pak cartridge (Millipore, Milford, MA), digested by chymotrypsin/trypsin and subjected to thin layer electrophoresis/chromatography as described above.

Immunoprecipitation—Specific activities of Cdk-cyclin complexes were determined by incorporation into small peptides. For immune complex assay, cells (5 × 106) were washed with phosphate-buffered saline and lysed at 4 °C in 1 ml of lysis buffer (50 mM Tris-HCl, pH 7.4, 1 mM EDTA, 25 mM NaCl, 40 µg/ml PMSF, 50 µg/ml aprotin, 50 µM leupeptin, and 0.1% Nonidet P-40). For Cdk4 and Cdk6 activity, cells were lysed in a buffer containing 50 mM HEPES, pH 7.5, 1 mM EDTA, 2.5 mM EDTA, 150 mM NaCl, 1 mM dithiothreitol, 40 µg/ml PMSF, 50 µg/ml aprotin, 50 µM leupeptin, 10 mM sodium orthovanadate, 10 mM β-glycerophosphate, and 0.1% (v/v) Tween 20. The extract (500 µl) was incubated for 1 h at 4 °C with anti-Cdc2, Cdk2, Cdk4, Cdk6, cyclin A, or cyclin B antibody. The immune complex was absorbed to Protein G-coupled Sepharose beads for 1 h. Alternatively, the extract was incubated with anti-p70s6kCT antibody, and then washed three times with the lysis buffer, and once with kinase buffer (50 mM HEPES, pH 7.2, 10 mM MgCl2, 5 mM MnCl2, 1 mM dithiothreitol for Cdk4 and Cdk6 assay; 50 mM Tris-HCl, pH 7.4, 10 mM MgCl2, 1 mM dithiothreitol for the rest). Following the final wash, the immune complexes were suspended in 50 µl of the kinase buffer containing 100 µM unlabeled ATP; 200 µCi/ml [γ-32P]ATP, 10 mM MgCl2. The reaction was allowed to proceed for 15 min at 30 °C and terminated by the addition of 10 µl of 500 mM EDTA. Following a brief centrifugation, the supernatant (20 µl) was applied to phosphocellulose paper and radioactivity was determined using a liquid scintillation counter. For the purified kinase complexes, Xenopus Cdc2-cyclin B complex or Xenopus Cdk2-cyclin E complex were used instead of immune complexes. Kinetic constants were determined using 10–6 to 10–8 M peptide. The activity of p70s6k was measured as described previously (12).

Radiolabeling of Cells and Immunoprecipitation—Cells were placed in phosphate-free medium containing 10% dialyzed serum and [32P]orthophosphate at 150 µCi/ml (ICN). After a 3-h incubation, the radiolabeled cells were lysed in a buffer containing 25 mM Tris-HCl, pH 7.4, 50 mM NaCl, 0.5% sodium deoxycholate, 2% Nonidet P-40, 0.2% SDS, 1 mg/ml BSA, 1 mg/ml aprotinin, 50 µg/ml leupeptin, 100 µg/ml PMSF. The lysates were then separated by 7.5% SDS-polyacrylamide gel, and radiolabeled proteins were visualized by autoradiography.

Cell—Human peripheral blood cells were obtained by leukopheresis of blood from healthy donors. Mononuclear cell suspensions were prepared by P-factor-Hypaque gradient centrifugation, and T cells were obtained by E-rosette enrichment as described (10). Cells were cultured in RPMI 1640 (Life Technologies, Inc.) inactivated fetal bovine serum (HyClone, Logan, UT), 2 mM L-glutamine (Life Technologies, Inc.), 100 units/ml penicillin, and 100 µg/ml streptomycin (Life Technologies, Inc.). COS7 cells were cultured in Dulbecco’s modified Eagle’s medium (Life Technologies, Inc.) supplemented with 10% (v/v) fetal bovine serum, 100 units/ml penicillin, and 100 µg/ml streptomycin. S9 (Spodoptera frugiperda) cells were maintained at 30 °C in serum-free SF900 II SFM medium (Life Technologies, Inc.) supplemented with 50 units/ml penicillin and 50 µg/ml streptomycin.
RESULTS

Cdc2-Cyclin B but Not Cdk2-Cyclin E Phosphorylates Recombinant p70<sup>65k</sup> on Ser<sup>411</sup> in Vitro—Although Cdc2 was demonstrated to phosphorylate the carboxyl terminus of p70<sup>65k</sup> in vitro in a previous study (23), the specificity for Cdc2 and the physiological relevance of the event has not been clarified. In an attempt to explore the specificity of the event among Cdk family members, we initially compared Cdc2 and Cdk2 in their abilities to phosphorylate recombinant p70<sup>s6k</sup>. Cdc2 is the closest kinase to Cdc2 by homology in amino acid sequence and also in terms of substrate specificity (31). Cdc2 also has established roles in the G1 phases of the cell cycle, when major regulation of p70<sup>65k</sup> is known to occur. Xenopus Cdc2-cyclin B and Cdk2-cyclin E complexes were purified as described previously (28). For substrates, either wild type p70<sup>s6k</sup> (recWT-p70), the kinase-dead T229A mutant (recT229A-p70) or a deletion mutant recT229A-p70 was weakly phosphorylated without addition of Cdc2-cyclin B or Cdk2-cyclin E (Control in the middle panel of Fig. 1). It is unknown whether this comes from auto-phosphorylation by a residual activity of the mutant kinase, or from an unidentified insect kinase in the preparation of nickel resin. The recWT-p70 was highly phosphorylated by itself, which was likely due to autophosphorylation of the kinase, but Cdc2-cyclin B complex still increased the phosphorylation of recWT-p70 by about 3-fold (data not shown). It should also be noted that there was an unidentified phosphorylated band below recT229A-p70 (Fig. 1, middle panel) which had a similar mobility to recACT-p70.

In vitro phosphorylated recT229A-p70 bands in the gel shown in Fig. 1, were cut out and subjected to trypsin/chymotrypsin digestion. The phosphopeptides were analyzed by two-dimensional thin layer electrophoresis/chromatography (TLE/TLC). D, a synthetic peptide (EPKIRSPRRFIG, the Ser<sup>411</sup> peptide) representing residues 406–417 of human p70<sup>s6k</sup>, was phosphorylated in vitro by Cdc2-cyclin B, digested with trypsin/chymotrypsin, and separated by TLE/TLC as described above. E and F, cells expressing recWT-p70 (E) or recACT-p70 (F) were labeled with [53P]orthophosphate for 3 h. The rec-p70<sup>s6k</sup> proteins were recovered and subjected to the same TLE/TLC procedure as described above.

FIG. 1. Cdc2-cyclin B, but not Cdk2-cyclin E, phosphorylates a recombinant p70<sup>65k</sup>. Recombinant p70<sup>65k</sup> (recT229A-p70; Thr<sup>229</sup> is replaced by Ala, rec<sub>CT</sub>-p70; the carboxyl-terminal 109 amino acids are deleted) were prepared by a baculovirus protein expression system. In vitro kinase reaction by purified Xenopus Cdc2-cyclin B or Cdk2-cyclin E complex were performed using [γ-<sup>32</sup>P]ATP. The radiolabeled substrates were separated by 10% SDS-polyacrylamide gels and visualized by autoradiography. The exposure times were 2 h and 1 h for histone H1 phosphorylation and rec70 phosphorylation, respectively.

FIG. 2. Cdc2-cyclin B, but not Cdk2-cyclin E, phosphorylates the carboxyl terminus of p70<sup>65k</sup>, especially at Ser<sup>411</sup>. A–C, the recT229A-p70 bands in the gel shown in Fig. 1, were cut out and subjected to trypsin/chymotrypsin digestion. The phosphopeptides were analyzed by TLE/TLC. D, a synthetic peptide (EPKIRSPRRFIG, the Ser<sup>411</sup> peptide) representing residues 406–417 of human p70<sup>65k</sup>, was phosphorylated in vitro by Cdc2-cyclin B, digested with trypsin/chymotrypsin, and separated by TLE/TLC as described above. E and F, cells expressing recWT-p70 (E) or recACT-p70 (F) were labeled with [53P]orthophosphate for 3 h. The rec-p70<sup>s6k</sup> proteins were recovered and subjected to the same TLE/TLC procedure as described above.
Vitro with approximately 10⁻⁶ M to 10⁻⁸ M) were measured as described under “Experimental Procedures.” Briefly, 1 μl of the kinase complex (with a specific activity of about 1 pmol/μl/min to transfer phosphate to whole histone H1 peptide) was incubated with the Ser⁴¹¹ peptide (approximately 10⁻⁶ M to 10⁻⁸ M) and 15 min at 30 °C. The specific activities (cpm) were divided by the activities (cpm) to phosphorylate 10⁻⁸ M histone H1 peptide (49,019 cpm for Cdc2-cyclin B complex; 84,208 cpm for Cdc2-cyclin E complex).

SPRRFIG) was phosphorylated by Cdc2-cyclin B, digested with trypsin/chymotrypsin, and analyzed by phosphopeptide mapping. The digested peptide containing phospho-Ser⁴¹¹ localized to the identical spot as the most highly phosphorylated peptide from recT229A-p70 (Fig. 2D). Co-migration of the peptides were confirmed by applying mixture of the phosphopeptides used in Fig. 2 (C and D) (data not shown). These data indicate that Cdc2-cyclin B complex phosphorylates p70⁶⁸k in vitro at Ser⁴¹¹.

Cdc2-cyclin B phosphorylates the Ser⁴¹¹ peptide better than Cdk2-cyclin E—Consistent with our data that Cdc2 phosphorylates p70⁶⁸k at Ser⁴¹¹ at Ser⁴¹¹, the sequence surrounding Ser⁴¹¹ has the best match among the carboxyl-terminal phosphorylation sites in p70⁶⁸k with the consensus phosphorylation site sequence for Cdc2 (K/R-S-P-R/P-R/K/H) determined by a peptide sequence for Cdc2 (K/R-S-P-R/P-R/K/H) determined by a peptide sequence for Cdc2 (K/R-S-P-R/P-R/K/H).

The Phosphorylation of p70⁶⁸k on Ser⁴¹¹ in Vivo Increases at Late Phases of the Cell Cycle—The results above indicate that Cdc2 is the best kinase among Cdk's we investigated for phosphorylation of the carboxyl terminus of p70⁶⁸k in vitro. To further explore the relevance of the finding in vivo, we initially examined the phosphorylation status of p70⁶⁸k using mitogen-activated primary human T lymphocytes. Primary T lymphocytes provide a population of cells that are about 99% in the G₀ status of the cell cycle (33). Cells were stimulated with PDBu and ionomycin in the presence or absence of rapamycin (10 ng/ml) and harvested at the indicated times (up to 48 h). As reported previously, the activity of p70⁶⁸k kinase was induced within 3 h after mitogenic activation of cells (Fig. 5D), and it was paralleled by the mobility shift of the protein in the immnoblots (Fig. 5A, upper panel). This mobility shift of p70⁶⁸k is known to correspond to phosphorylation of the kinase at Thr³⁸⁹⁹ and Thr³⁹⁰² (15, 14). Activation and the mobility shift of the kinase were inhibited by addition of rapamycin. The cell lysates were also immunoblotted using an antibody raised against a synthetic phospho-Ser⁴¹¹ peptide corresponding to residues 406–417 (EKIRSPRRFIG) of p70⁶⁸k (anti-phospho-Ser⁴¹¹ antibody, New England Biolabs) (Fig. 5A, lower panel). p70⁶⁸k detected by anti-phospho-Ser⁴¹¹ antibody was increased only at late phases of the cell cycle (Fig. 5A), where Cdc2 activity was high (Fig. 5C) and cells were beginning to enter S and G₂/M phases (Fig. 5D). In contrast, total amount of p70⁶⁸k protein detected by anti-carboxyl terminus antibody, increased

### Table I

| Antibodies | Histone H1 peptide | Ser⁴¹¹ peptide |
|------------|--------------------|----------------|
| Anti-Cdc2  | 100                | 56.3 ± 14.2    |
| Anti-Cdk2  | 100                | 5.4 ± 4.0      |
| Anti-cyclin A | 100             | 4.4 ± 0.3      |
| Anti-cyclin B | 100             | 42.5 ± 12.0    |
| p13⁶⁸k     | 100                | 45.9 ± 10.9    |

Lyse from T cells stimulated with PDBu/ionomycin for 48 h were treated with specific antibodies against Cdc2, Cdk2, cyclin A, or cyclin B, and immune complexes were recovered using protein G-Sepharose beads. For P3⁴¹¹-associated kinases, lymphocytes were incubated with p3⁴¹¹-agarose beads. Kinase activity of the immune complexes to phosphorylate the histone H1 peptide or the Ser⁴¹¹ peptide was measured as described under “Experimental Procedures.” Data are shown by percent activity to the specific activity to phosphorylate the histone H1 peptide.

![Fig. 3. Cdc2-cyclin B phosphorylates the Ser⁴¹¹ peptide better than Cdk2-cyclin E](image-url)
earlier (within 24 h) (Fig. 5A, upper panel). Anti-phospho-Ser\textsuperscript{411} antibody-reacted species were not detectable within 3 h even when 3-fold amounts of samples were applied (data not shown). In a previous study, we have shown that rapamycin prolonged G\textsubscript{1} phase and delayed resting T cells from entering the S phase by about 9 h when T cells were stimulated with PDBu and ionomycin (10). Of interest, Ser\textsuperscript{411} phosphorylation was seen in late cell cycle phases in rapamycin-treated samples as well. These results indicate that the in vivo phosphorylation of p70\textsuperscript{68k} on Ser\textsuperscript{411} is increased at late phases of the cell cycle, regardless of the activity of p70\textsuperscript{68k}. To confirm the efficacy of the anti-phospho-Ser\textsuperscript{411} antibody, COS7 cells were transfected with either wild type or S411A mutant p70\textsuperscript{68k} expression vector and the transfected p70\textsuperscript{68k} proteins were subjected to the same immunoblotting analysis. The antibody reactivity was markedly reduced with the S411A mutant as compared with wild type (data not shown).

**Nocodazole Treatment Enhances the Phosphorylation of p70\textsuperscript{68k} on Ser\textsuperscript{411}**—In order to further investigate where in the cell cycle Ser\textsuperscript{411} phosphorylation is enhanced, proliferating cells (COS7 cells) were treated for 16 h with aphidicolin (to arrest the cycle at the early S phase) or nocodazole (to arrest the cycle at the mitotic phase). Cell lysates were immunoblotted with either the anti-carboxy terminus antibody or the anti-phospho-Ser\textsuperscript{411} antibody as described above. As shown in Fig. 6A, the phospho-Ser\textsuperscript{411}-containing species of p70\textsuperscript{68k} was increased by addition of nocodazole but not by aphidicolin. Neither of the drugs alters the expression level of p70\textsuperscript{68k}, confirmed by immunoblotting with the anti-carboxy terminus antibody. In addition, effects of release of cells from nocodazole-block were examined by washing out nocodazole and leaving cells in the regular medium for 3 h. As shown in Fig. 6A (right panels), release from a nocodazole-block decreased Ser\textsuperscript{411} phosphorylation. Nocodazole treatment also enhanced Cdc2 activity, due to arrest of the cell cycle at metaphase, when Cdc2 activity was high (Fig. 6B). Release from nocodazole-block decreased Cdc2 activity (Fig. 6B). These data indicate that the phosphorylation of p70\textsuperscript{68k} on Ser\textsuperscript{411} is enhanced during the metaphase block induced by nocodazole where Cdc2 activity is high. In addition, the p70\textsuperscript{68k} activity was measured in the system. Of interest, the p70\textsuperscript{68k} activity was low in nocodazole-treated cells and increased by release from the drug (Fig. 6B).

**The p70\textsuperscript{68k} with Phosphorylated Ser\textsuperscript{411} Is Detected Only in Mitotic Cells**—In order to further investigate the phosphorylation status of p70\textsuperscript{68k} on Ser\textsuperscript{411}, NIH3T3 cells in normal growing culture were examined by indirect immunofluorescence microscopy using the anti-phospho-Ser\textsuperscript{411} antibody as a primary antibody and a Cy3-conjugated secondary antibody. The cells were simultaneously stained with Hoechst 33258 dye to visualize DNA and nuclei. As shown in Fig. 7, cells in the mitotic phase were stained with anti-phospho-Ser\textsuperscript{411} antibody. Especially, cells in prophase and metaphase of mitosis were highly stained, and cells in telophase were weakly stained. In contrast, cells not in mitotic phase were not stained at all with the antibody. Cells treated with nocodazole were also highly stained with the antibody (data not shown). These data suggest that p70\textsuperscript{68k} is phosphorylated on Ser\textsuperscript{411} exclusively in mitosis.

**A Dominant Negative Mutant of Cdc2 Suppresses the Phosphorylation of p70\textsuperscript{68k} on Ser\textsuperscript{411}**—In order to investigate further whether Cdc2 is essential for the phosphorylation of p70\textsuperscript{68k} on Ser\textsuperscript{411} in vivo, a dominant negative form of Cdc2 (D146N) was utilized. This form of Cdc2 has been demonstrated to inhibit endogenous Cdc2 activity in vivo (30). COS7 cells were co-transfected with HA-tagged wild type p70\textsuperscript{68k} expression vector and either wild type or D146N mutant Cdc2 expression vector. Cells were pulse labeled with [\textsuperscript{32}P]orthophosphate at 21–24 h after transfection, and transfected p70\textsuperscript{68k} was recovered by anti-HA antibody. The ratio of plasmids was 20:1 (Cdc2 vector: p70\textsuperscript{68k} vector), and it was confirmed that most (>80%) of the p70\textsuperscript{68k} overexpressing cells were also overexpressing Cdc2 by immunofluorescence staining (data not shown). Fig. 8A illustrates that D146N Cdc2 decreases total phosphorylation of p70\textsuperscript{68k} compared with the control (wild type Cdc2). Immunoblotting using the whole lysates demonstrates that D146N Cdc2 decreases Ser\textsuperscript{411} phosphorylation but not total p70\textsuperscript{68k} protein amount (Fig. 8, B and C). These data indicate that Cdc2 phosphorylates p70\textsuperscript{68k} on Ser\textsuperscript{411} in vivo.

**DISCUSSION**

Here, we have demonstrated that Cdc2, but not Cdk2, Cdk4, or Cdk6, phosphorylates p70\textsuperscript{68k} at Ser\textsuperscript{411} in vitro. Additionally, p70\textsuperscript{68k} is phosphorylated at Ser\textsuperscript{411} in vivo at mitosis, when Cdc2 is highly activated. Finally, a dominant negative mutant of Cdc2 suppressed the carboxy-terminal phosphorylation of p70\textsuperscript{68k}. Based on these findings, we conclude that Cdc2 is an important physiological kinase for the phosphorylation of p70\textsuperscript{68k} at Ser\textsuperscript{411}, and p70\textsuperscript{68k} is a relevant mitotic substrate for Cdc2. However, the study does not totally exclude the involvement of other kinases for phosphorylation of p70\textsuperscript{68k} at Ser\textsuperscript{411}. For example, some Ser\textsuperscript{411} phosphorylation was detected in cells treated with PDBu/ionomycin and rapamycin for 39 h, where there was little substantial increase in Cdc2 activity (Fig. 5, A and C). This may suggest that another kinase in late cell cycle phases also takes part in this event.

Although previous reports have claimed that the carboxy-terminal phosphorylation of p70\textsuperscript{68k} including Ser\textsuperscript{411} residues...
occurs soon after mitogenic stimulation (13, 15, 23), those studies were done using serum-starved cell lines that are not completely in resting (G0) status. In fact, Mukhopadhyay et al. demonstrated insulin-induced Cdc2 activity in serum-starved H4 hepatoma cells within 30 min, in parallel to an increase in the activity to phosphorylate the carboxyl terminus of p70s6k in vitro by the cell lysates (23). Taken together with the fact that Cdc2 is active exclusively in late cell cycle phases (25), the increase in Ser411 phosphorylation soon after mitogenic stimulation in these cell lines may not represent an event occurring in early G1 phase. It may come from a cell fraction already in late phases of the cell cycle. In contrast, primary T lymphocytes provide a population of cells that are about 99% in the G0 phase of the cell cycle (33). In mitogen activated primary T cells, neither Cdc2 activity nor cells containing greater than 2N DNA is detectable within 30 h after mitogenic stimulation (33, 34).

Cdc2 but not Cdk2, Cdk4, or Cdk6, phosphorylates p70s6k at the physiological phosphorylation sites on the carboxyl terminus, and the Ser411 peptide as well. The site that Cdc2 phosphorylates especially well (Ser411 in p70s6k) has a consensus motif for Cdc2/Cdk2 substrates (S/T-P-X-K/R) (31). The differential substrate specificity between Cdc2/Cdk2 and Cdk4/Cdk6 is well known; Cdc2/Cdk2 phosphorylates histone H1 and Rb well in vitro, but Cdk4/Cdk6 prefers Rb as an in vitro substrate. Among the numerous phosphorylation sites in Rb protein,
Cdc2 Phosphorylates p70⁶S6K at Mitosis

The carboxyl-terminal phosphorylation site in p70⁶S6K was originally proposed to be an autoinhibitory site for the kinase (22). The sequence at the phosphorylation site has homology with that seen in its physiological substrate S6. Moreover, the peptide derived from the carboxyl-terminal phosphorylation site can block the kinase activity of p70⁶S6K to phosphorylate S6 in vitro (36). Therefore, it was proposed that the phosphorylation of the carboxyl terminus will release this autoinhibitory effect, thus regulating the activity of p70⁶S6K. However, this model has not been fully supported by later experimental results. First, the carboxyl-terminal phosphorylation is in fact not essential for the major regulation of activity of p70⁶S6K. A mutant in which all four serine/threonine residue at the carboxyl-terminal site are replaced by alanine maintains the ability to phosphorylate ribosomal S6 protein. Moreover, the mutant was activated by growth factor stimulation and inactivated by rapamycin as well as wild type p70⁶S6K (20). In addition, another mutant of p70⁶S6K with a deleted carboxyl terminus has weaker activity in phosphorylating S6, and is also positively and negatively regulated by growth factors and rapamycin, respectively (21). Here, we demonstrated that the carboxyl-terminal phosphorylation occurs at late phases of the cell cycle, whereas activation of the kinase was induced rapidly after mitogenic stimulation. Taken together, these results suggest that the carboxyl-terminal phosphorylation may not be a factor involved in activation of the kinase.

The Cdc2-cyclin B complex plays a central role in mitosis. It phosphorylates, for instance, nuclear lamins thus leading to their disassembly, an important event in the initiation of nuclear envelope breakdown (37–39). The complex also phosphorylates histone H1, which is thought to promote chromosome condensation, an event that occurs at the onset of mitosis as well (40). In addition, nucleolin, RNA polymerase II, p53, elongation factors 1β and 1γ, Srf, and Cdc25-C serve as substrates for the complex (in vivo and/or in vitro) (41). Here, p70⁶S6K was demonstrated to be a new candidate for a physiological mitotic substrate of Cdc2-cyclin B. A recent study (42) has demonstrated, and we confirmed here that the activity of p70⁶S6K is low at mitotic phase, and release of cells from mitosis reactivates p70⁶S6K. Taken together with the proposed role of p70⁶S6K in protein synthesis, it is reasonable that p70⁶S6K is inactivated during mitosis where protein synthesis is generally suppressed (43, 44). Cdc2-cyclin B may phosphorylate and inactivate p70⁶S6K at mitosis. However, treatment of recombinant p70⁶S6K (wild type) with purified Xenopus Cdc2-cyclin B did not suppress (nor increase) the activity of p70⁶S6K (data not shown), suggesting that the phosphorylation of Ser⁴¹¹ by itself does not inactivate the kinase. A phosphoserine-binding protein may bind p70⁶S6K at phospho-Ser⁴¹¹, and inactivate the kinase at mitosis. An example of this kind of regulation has been shown for the cell cycle control protein Cdc25 C by Peng et al. (45). This phosphatase is phosphorylated on Ser⁴¹⁶ throughout interphase and has been shown to bind 14-3-3 proteins at this site, thereby sequestering and negatively regulating its activity until entry into mitosis (45). Proteins such as 14-3-3, pin 1, etc., may also play a role negatively regulating p70⁶S6K by a similar mechanism via Ser⁴¹¹ phosphorylation. Identification of such p70⁶S6K-binding factors are now in progress.

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