Phosphorylation of Casein Kinase II by p34\textsuperscript{cdc2}

IDENTIFICATION OF PHOSPHORYLATION SITES USING PHOSPHORYLATION SITE MUTANTS IN VITRO*

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The \( \alpha \) and \( \beta \) subunits of casein kinase II are dramatically phosphorylated in cells that are arrested in mitosis (Litchfield, D. W., Lüscher, B., Lozeman, F. J., Eisenman, R. N., and Krebs, E. G. (1992) J. Biol. Chem. 267, 13943-13951). Comparative phosphopeptide mapping experiments indicated that the mitotic phosphorylation sites on the \( \alpha \) subunit of casein kinase II can be phosphorylated in vitro by p34\textsuperscript{cdc2}. In the present study, we have demonstrated that a glutathione S-transferase fusion protein encoding the C-terminal 126 amino acids of the \( \alpha \) subunit is phosphorylated by p34\textsuperscript{cdc2} at the same sites as intact casein kinase II, indicating that the mitotic phosphorylation sites are localized within the C-terminal domain of \( \alpha \). Four residues within this domain, Thr-344, Thr-360, Ser-362, and Ser-370, conform to the minimal consensus sequence for p34\textsuperscript{cdc2} phosphorylation. Synthetic peptides corresponding to regions of \( \alpha \) that contain each of these residues are phosphorylated by p34\textsuperscript{cdc2} at these sites. Furthermore, alterations in the phosphorylation of the glutathione S-transferase proteins encoding the C-terminal domain of \( \alpha \) are observed when any of the four residues are mutated to alanine. When all four residues are mutated to alanine, the fusion protein is no longer phosphorylated by p34\textsuperscript{cdc2} at any of the sites that are phosphorylated in mitotic cells. These results indicate that Thr-344, Thr-360, Ser-362, and Ser-370 are the sites on the \( \alpha \) subunit of casein kinase II that are phosphorylated in mitotic cells.

Biological and genetic studies have demonstrated that the p34\textsuperscript{cdc2} protein kinase is an indispensable regulator of events leading to the division of eukaryotic cells (for reviews see Refs. 1-4). To ensure that the division of cells is very precisely regulated, the activity of this protein serine/threonine kinase is exquisitely controlled through its interactions with regulatory cyclins and through phosphorylation of p34\textsuperscript{cdc2} itself. p34\textsuperscript{cdc2} is defined as a cyclin-dependent kinase since it is inactive unless it is associated with a regulatory cyclin subunit. Furthermore, p34\textsuperscript{cdc2} is inhibited by phosphorylation of Tyr-15 and/or Thr-14 (5, 6) but requires phosphorylation of Thr-161 to be activated (7, 8). CAK (the p34\textsuperscript{cdc2} activating kinase) is responsible for phosphorylation of Thr-161 (9–11), while the phosphorylation state of Thr-14 and/or Tyr-15 is at least in part controlled by the relative activities of the cdc25 protein kinase (12, 13) and cdc25 protein phosphatase (14, 15).

Concomitant with the activation of p34\textsuperscript{cdc2} at the G2-M transition of eukaryotic cells is a massive burst of protein phosphorylation. Many of the events that are associated with entry into mitosis including nuclear envelope breakdown, transcriptional termination, nuclear disassembly, cytoskeletal reorganization, and chromosome condensation appear to be associated with protein phosphorylation. While it is evident that p34\textsuperscript{cdc2} directly phosphorylates a number of proteins at the G2-M transition, there are also indications that p34\textsuperscript{cdc2} could indirectly regulate phosphorylation events through its phosphorylation of other protein kinases (16–23).

One protein serine/threonine kinase that could be regulated by p34\textsuperscript{cdc2} is casein kinase II (CKII), which has been shown to be dramatically phosphorylated in mitotic cells (19–21). CKII is a tetrameric enzyme composed of two catalytic (\( \alpha \) and/or \( \alpha' \)-subunits) and two additional subunits (\( \beta \) subunits) (for reviews, see Refs. 24–26). Our previous studies demonstrated that p34\textsuperscript{cdc2} phosphorylates the \( \beta \) subunit of CKII at Ser-209, a site that is maximally phosphorylated in mitotic cells (20). Interestingly, the \( \alpha \) subunit (but not the \( \alpha' \)-subunit) of CKII is also dramatically phosphorylated in mitotic avian and mammalian cells (21). This result suggests that there may be differences in the functional or regulatory properties of the isozymic forms of the catalytic subunit of CKII. Our analyses demonstrated that the mitotic phosphorylation sites on \( \alpha \) can be phosphorylated in vitro by p34\textsuperscript{cdc2}. To facilitate efforts to examine the functions of CKII during mitosis and how phosphorylation may affect these functions, the objective of the present study was directed toward identification of the sites on the \( \alpha \) subunit of CKII that are phosphorylated by p34\textsuperscript{cdc2} so that non-phosphorylatable forms of CKII could be prepared by mutagenesis. Utilizing synthetic peptides and glutathione S-transferase (GST) fusion proteins containing the C-terminal domain of the human CKII \( \alpha \) subunit, p34\textsuperscript{cdc2} phosphorylation sites were identified as Thr-344, Thr-360, Ser-362, and Ser-370. Furthermore, following mutagenesis of each of these residues to alanine residues, fusion proteins containing the C-terminal domain of CKII \( \alpha \) were no longer phosphorylated by p34\textsuperscript{cdc2} at any of the sites that are phosphorylated in mitotic cells.

**EXPERIMENTAL PROCEDURES**

**Materials**

Synthetic peptides (peptide 1, Pro-Gly-Gly-Ser-Thr-Pro-Val-Ser-Ser-Ala; peptide 2, Ile-Ser-Ser-Val-Pro-Thr-Pro-Pro-Leu-Gly-Pro-Leu-Ala-Gly; peptide 3, Ile-Ser-Ser-Val-Pro-Thr-Pro-Leu-Gly-Pro-Leu-Ala-Gly; peptide 4, Leu-Gly-Pro-Leu-Ala-Gly-Ser-Pro-Val-Ile-Ala-Ala) were synthesized with an Applied Biosystems model 431A peptide synthesizer (Applied Biosystems, Foster City, CA). Peptides were purified by reversed-phase HPLC and analyzed by amino acid analysis and MALDI-TOF mass spectrometry. Peptide purity was determined to be greater than 95% by HPLC analysis.

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\(^{1}\) The abbreviations used are: CKII, casein kinase II; GST, glutathione S-transferase.
tively, fusion proteins were recovered from phosphorylation reactions by glutathione-agarose bead purification (29). Bovine CKII was purified (37) and phosphorylated using purified p34<sup>cdc2</sup> as described previously (20, 21). Synthetic peptides (1 mM, final concentration) were similarly phosphorylated with purified p34<sup>cdc2</sup> in kinase buffer containing γ-[<sup>32</sup>P]ATP (30–60 μCi/nmol). After terminating the reaction by the addition of EDTA, phosphopeptides were separated from the free γ-[<sup>32</sup>P]ATP using thin layer cellulose chromatography as described below using Scheidtmann buffer (33) and visualized with autoradiography.

To determine the stoichiometry of phosphorylation of fusion proteins, the following procedure was followed. Known amounts (2, 4, 6 μg) of each fusion protein were subjected to SDS-polyacrylamide gel electrophoresis and visualized by staining with Coomassie Blue. Typically, each fusion protein displayed one major band corresponding to undergraded GST-fusion protein since it reacted on immunoblots with antibodies directed against the C terminus of CKII α (anti-α<sup>376–391</sup>) as well as bands representing degradation products that were not reactive with anti-α<sup>376–391</sup> antibodies. Densitometry of the Coomassie Blue-stained gel was performed to determine the proportion of intact GST fusion protein. The phosphate incorporation into the undergraded GST fusion proteins were subsequently determined by analysis on a PhosphorImager (Molecular Dynamics). Stoichiometry of phosphorylation was calculated by the following formula: pmol of phosphate incorporated into intact fusion protein/pmol of intact fusion protein.

Two-dimensional Phosphopeptide Mapping and Phosphoamino Acid Analysis

Phosphorylated proteins were separated by two-dimensional gel electrophoresis (20, 21). Synthetic peptides (1 mM, final concentration) were similarly phosphorylated with purified p34<sup>cdc2</sup> in kinase buffer containing γ-[<sup>32</sup>P]ATP (30–60 μCi/nmol). After terminating the reaction by the addition of EDTA, phosphopeptides were separated from the free γ-[<sup>32</sup>P]ATP using thin layer cellulose chromatography as described below using Scheidtmann buffer (33) and visualized with autoradiography.

To determine whether GST-α-C126 and intact CKII α are phosphorylated at the same sites, we performed comparative analysis of the two-dimensional electrophoresis as described (36).

RESULTS AND DISCUSSION

Previous work had shown that sites within the C-terminal domain of the α subunit of CKII were phosphorylated in cells arrested in mitosis with nocodazole and that immunopurified p34<sup>cdc2</sup> was capable of phosphorylating the same sites in vitro (21). To provide additional evidence that the p34<sup>cdc2</sup> phosphorylation sites are localized to the C-terminal domain of CKII α, we prepared GST fusion protein encoding the C-terminal 126 amino acids of CKII α (GST-α-C126) to express GST fusion proteins harboring mutations at the putative phosphorylation sites.

Fusion proteins were expressed in Escherichia coli JM109 and purified using glutathione-agarose as described previously (29). Fusion proteins were eluted from glutathione-agarose using buffer (20 mM Tris-Cl, pH 8.0, 100 mM NaCl, 1 mM EDTA) containing 5 mM reduced glutathione. Protein determinations were by the method of Bradford (38) using y-globulin as standard.

Phosphorylation Reactions—Purified fusion proteins (typically 4 μg/assay) were incubated with purified p34<sup>cdc2</sup> in kinase buffer (50 mM Tris-Cl, pH 7.4, 10 mM MgCl<sub>2</sub>, 1 mM dithiothreitol, 100 μM ATP) containing 10 μM γ-[<sup>32</sup>P]ATP (30–60 μCi/nmol) in the total volume of 3 μl. Kinase reactions were initiated by the addition of purified p34<sup>cdc2</sup> (typically 50 μM units of enzyme), and the assay performed at 30 °C with constant agitation for the indicated length of time. Reactions were terminated by the addition of EDTA to a final concentration of 20 mM. Phosphorylated fusion proteins were immunoprecipitated with the addition of anti-α<sup>376–391</sup>-GST (10 μg fusion protein) described previously (20, 21). Protein A-Sepharose beads in antibody buffer (20 mM Tris-Cl, pH 7.5, 50 mM NaCl, 0.5% Nonidet P-40, 0.5% deoxycholate, 0.5% SDS) supplemented with phosphatase inhibitors (20 mM NaF, 20 mM β-glycerophosphate). After incubation for 60 min on ice, the beads were collected by centrifugation and washed twice with L buffer (phosphate-buffered saline, pH 7.5, 1% Nonidet P-40, 0.5% deoxycholate) containing 1% aprotinin and phenylmethylsulfonyl fluoride. The washed beads were then resuspended in Laemmli sample buffer, and the samples were boiled for 3–5 min prior to analysis by SDS-polyacrylamide gel electrophoresis (32). Alternately, fusion proteins were recovered from phosphorylation reactions by glutathione-agarose bead purification (29). Bovine CKII was purified (37) and phosphorylated using purified p34<sup>cdc2</sup> as described previously (20, 21). Synthetic peptides (1 mM, final concentration) were similarly phosphorylated with purified p34<sup>cdc2</sup> in kinase buffer containing γ-[<sup>32</sup>P]ATP (30–60 μCi/nmol). After terminating the reaction by the addition of EDTA, phosphopeptides were separated from the free γ-[<sup>32</sup>P]ATP using thin layer cellulose chromatography as described below using Scheidtmann buffer (33) and visualized with autoradiography.

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Fig. 1. Phosphorylation of GST Fusion Proteins. A, schematic representation of GST and the GST-αC126 and GST-αC51 proteins. GST-αC126 is composed of GST fused to the 126 C-terminal amino acids of the α subunit of CKII (indicated by solid black rectangle), GST-αC51 encodes GST and the 51 C-terminal residues of CKII α (indicated by striped rectangle). B, equal amounts (4 μg) of purified GST, GST-αC126, and GST-αC51 proteins were incubated with purified p34<sup>cdc2</sup> (lanes 1, 3, and 6) and without purified p34<sup>cdc2</sup> (lanes 2, 4, and 5, respectively) for 90 min using the in vitro phosphorylation conditions described under “Experimental Procedures.” The proteins were recovered from the phosphorylation reactions using glutathione-agarose before analysis on a 12% SDS-polyacrylamide gel and were visualized by autoradiography.

Fig. 2. Comparative phosphopeptide maps of GST-αC126 and of the α subunit of CKII phosphorylated in vitro by purified p34<sup>cdc2</sup>. GST-αC126 and purified bovine CKII were phosphorylated in vitro using purified p34<sup>cdc2</sup> and were then immunoprecipitated from kinase reactions using anti-α<sup>376,392</sup> antisera. Immunoprecipitates were analyzed by autoradiography after separation of proteins on a 12% SDS-polyacrylamide gel. The phosphorylated fusion protein and the α subunit of CKII were recovered from homogenized gel slices eluted from the SDS-polyacrylamide gel. The samples were exhaustively digested with thermolysin and then separated by electrophoresis at pH 1.9 (horizontal dimension with anode to the left), followed by ascending chromatography as described under “Experimental Procedures.” The MIX phosphopeptide map was obtained by mixing aliquots (equal cpm) of each phosphorylated sample (GST-αC126 and the α subunit of CKII) prior to two-dimensional separation. The positions of the origins are marked by arrows and the letter O. Individual phosphopeptides are identified with letters as in Ref. 21.

Phosphorylation of purified CKII comigrates with phosphopeptides obtained following 32P labeling of mitotic J urkat cells (21), these results indicate that GST-αC126 is phosphorylated at sites that are phosphorylated in mitotic cells. We previously noted that purified CKII is phosphorylated at a site, represented by phosphopeptide d (Fig. 2), that was not detected from samples of CKII obtained from intact cells (21). It is apparent that this phosphopeptide, which is also present following phosphorylation of GST-αC126, does not represent an in vivo phosphorylation site.

Inspection of the amino acid sequence of the C-terminal domain of CKII α revealed the presence of four residues, Thr-344, Thr-360, Ser-362, and Ser-370, that conform to the minimal consensus sequence for p34<sup>cdc2</sup> phosphorylation (39). Synthetic peptides corresponding to portions of CKII α containing each of the putative phosphorylation sites were synthesized and phosphorylated in vitro with purified p34<sup>cdc2</sup> (see Table I). It is interesting to note that replacement of Ser-362 with alanine (compare peptide 3 with peptide 2) abolishes serine phosphorylation, suggesting that p34<sup>cdc2</sup> does not likely phosphorylate Ser-356 or Ser-357 within this peptide. In a similar vein, peptide 1 is exclusively threonine phosphorylated, demonstrating that p34<sup>cdc2</sup> does not likely phosphorylate Ser-343, Ser-348, or Ser-349 within the peptide.

To identify the p34<sup>cdc2</sup> phosphorylation sites on CKII α, a mutagenesis strategy was employed. We prepared fusion proteins in which one or more of the putative p34<sup>cdc2</sup> phosphorylation sites had been mutated to non-phosphorylatable alanine residues (see Table II for summary). Each of these fusion proteins was tested as an in vitro substrate for p34<sup>cdc2</sup> and analyzed by SDS-polyacrylamide gel electrophoresis followed by autoradiography (see Fig. 3). Each of the fusion proteins displays at least one phosphorylated band with noticeably reduced electrophoretic mobility, except for the fusion protein in which all four putative phosphorylation sites had been mutated (Fig. 3K). This fusion protein yields a single phosphorylated band of unaltered electrophoretic mobility. In addition to differences in the locations or shift in electrophoretic mobility of each protein, differences in the extent of phosphorylation were observed for each fusion protein. Interestingly, fusion proteins containing both residues Thr-344 and Ser-370 (lanes A, B, C and I; TTSS, TTAS, TASS, and TAAS, respectively) exhibited the most significant shifts in electrophoretic mobility and also produced some of the most intense bands. Taken together, these results suggest that Thr-344 and Ser-370 are important for the optimal phosphorylation of the C-terminal domain of CKII α by p34<sup>cdc2</sup>. Phosphorylation of the wild-type GST-αC126 fusion protein achieved an approximate stoichiometry of 3 mol of phosphate/mol of protein (Fig. 4), while the AAAA mutant was phosphorylated to a stoichiometry of approximately 0.3 mol of phosphate/mol of protein.

Altering the intensity of phosphorylation and in the extent of the electrophoretic mobility shift of individual GST fusion proteins (GST-αC126 and corresponding mutants) suggested that some of the p34<sup>cdc2</sup> sites had been eliminated by mutation. To directly examine the phosphorylation pattern of individual mutants, two-dimensional phosphopeptide mapping procedures were utilized (Fig. 5). In all cases, the most highly phosphorylated form (i.e. the uppermost phosphorylated band observed in Fig. 3) was subjected to analysis. A number of observations are apparent from examination of these phosphopeptide maps. As evidenced by examination of maps D, F, G, J, K, and L (ATSS, ATSA, AASS, AASA, AAAA, and AAAAA, respectively), mutation of Thr-344 to alanine results in loss of phosphopeptide a, indicating that this phosphopeptide contains Thr-344. Phosphoamino acid analysis demonstrated that phosphopeptide a contains exclusively phosphothreonine (data not shown) supporting this interpretation. Phosphopeptide b is absent on all phosphopeptide maps obtained from fusion proteins that are mutated at both Ser-362 and Ser-370 (panels H and K; TTAA and AAAA, respectively), suggesting that phosphopeptide b is derived from similar, if not identical, peptides that are phosphorylated at either Ser-362 or Ser-370. Phosphoamino acid analysis of phosphopeptide b shows that this peptide is composed exclusively of phosphoserine (data not shown).

The region of CKII α that contains the putative p34<sup>cdc2</sup> phosphorylation sites does not contain any charged amino acids...
Phosphoamino acid analysis of phosphorylated synthetic peptides

| Peptide | Residues | Synthetic peptide sequence | Residue phosphorylated | Phosphorylation rate |
|---------|----------|---------------------------|-----------------------|---------------------|
| 1       | 340-349  | P-G-G-S-T-P-V-S-S-A        | T                     | 2.9                 |
| 2       | 355-369  | I-S-V-P-T-P-S-P-L-G-P-L-A-G| S,T                   | 4.8                 |
| 3      | 355-369  | I-S-V-P-T-P-A-P-L-G-P-L-A-G| T                     | 5.6                 |
| 4       | 364-375  | L-G-P-L-A-G-S-P-V-I-A-A    | S                     | 4.3                 |

* Amino acid numbering of human CKII α (as per Ref. 28).
* Residues in bold are the residues that conform to the minimal consensus for p34cdc2 phosphorylation.
* Synthetic peptides were assayed at a concentration of 1 μM using purified p34cdc2 with an activity of 52 pmol/min/μl with 1 μM Ser-209 peptide as substrate.
* Residue underlined in peptide 3 represents a serine to alanine change.

**TABLE II**

Phosphorylation site mutants of GST-αC126

| Designation | Sequence (CKII α residues 344–371) | Mutations |
|-------------|------------------------------------|-----------|
| TTSS        | TPVSSANMSGISSVPTPSPLAGSP            | None (wild type) |
| TTAS        | **************************A********* | T360A     |
| ATSS        | A***************A*A*********        | T344A     |
| TSTA        | A***************A*A*********        | S370A     |
| ATSA        | A***************A*A*********        | T344A, S370A |
| AASS        | A***************A*A*********        | T344A, S362A |
| TAAH        | A***************A*A*********        | S362A, S370A |
| TAAS        | A***************A*A*********        | T360A, S362A |
| AASA        | A***************A*A*********        | T344A, T360A, S370A |
| AASS        | A***************A*A*********        | T344A, T360A, S362A |
| AAAA        | A***************A*A*********        | T344A, T360A, S362A, S370A |

* Asterisks signify residues identical to the wild-type sequence of human CKII α (Ref. 28).

**Fig. 3.** Phosphorylation of the GST-αC126 fusion proteins by p34cdc2. Wild-type and mutant GST-αC126 fusion proteins were phosphorylated in vitro for 90 min using purified p34cdc2 as described under “Experimental Procedures.” Equivalent amounts of each fusion protein were subsequently subjected to SDS-polyacrylamide gel electrophoresis on a 12% gel, and phosphoproteins were visualized by autoradiography. Lanes A–L contain the following GST fusion proteins, respectively: TTSS (i.e. wild-type GST-αC126), TTAS, TASS, ATSS, TSTA, ATSA, AASS, TAAH, TAAS, AASA, AASS, AAAA. Fusion protein designation is according to Table II and represents the identity of the amino acid residues present at positions 344, 360, 362, and 370, respectively (numbering according to the deduced sequence of human CKII α; see Ref. 28).

As a result, monophosphorylated peptides would be nearly neutral at pH 1.9 (36). Phosphopeptides containing more than one phosphate would have sufficient negative charge at pH 1.9 to migrate toward the positive electrode (to the left in Fig. 5). The minimal migration exhibited by phosphopeptides a and b in the electrophoretic dimension is consistent with the presence of only a single phosphate on each of these peptides. Phosphopeptide c, observed only in A (TTSS), exhibits the electrophoretic mobility of a peptide with a significant negative charge. Furthermore, this peptide contains both phosphothreonine and phosphoserine, suggesting that it is indeed a multiply phosphorylated peptide (data not shown). When Ser-362 (map B, TTAS) or Thr-360 (map C, TASS) were mutated to alanine residues, phosphopeptide c was not observed. Instead, phosphopeptide e was observed on map B (TTAS), and phosphopeptide f was observed on map C (TASS). Mixing experiments indicated that both phosphopeptides e and f were less negatively charged than phosphopeptide c (data not shown). These results indicate that phosphopeptides e and f are phosphorylated at more than one site but that they are phosphorylated to a lesser extent than phosphopeptide c. Furthermore, phosphoamino acid analysis indicated that spot f was phosphorylated exclusively on serine, whereas spot e contained a mixture of phosphoserine and phosphothreonine. These results suggest that the negatively charged phosphopeptide c is a triply phosphorylated peptide that had been phosphorylated at Thr-360, Ser-362, and Ser-370. Mutation of Thr-360 to alanine (map C, TASS) results in the disappearance of phosphopeptide c and a concomitant appearance of phosphopeptide f. Phosphopeptide f, which is exclusively serine phosphorylated, has a lesser negative charge than phosphopeptide c but still behaves as a multiply phosphorylated peptide, which is presumably phosphorylated at Ser-362 and Ser-370. Similarly, mutation of Ser-362 to alanine (map B, TTAS) results in the loss of phosphopeptide c with the gain of the less negatively charged phosphopeptide e,
which is most likely phosphorylated at both Thr-360 and Ser-370. The presence of phosphoserine and phosphothreonine in phosphopeptide e supports this conclusion. If phosphopeptides e and f are indeed diphosphorylated peptides, it would naturally follow that phosphopeptide c is a triphosphorylated peptide that is phosphorylated at Thr-360, Ser-362, and Ser-370. The greater negative charge and diminished chromatographic migration of phosphopeptide c in comparison to phosphopeptides e or f is consistent with the presence of an additional phosphate on the former peptide (36).

Phosphopeptide d, which is only observed on maps derived from CKII or GST fusion proteins that are phosphorylated in vitro, is present on all phosphopeptide maps, including the fusion protein in which all putative p34\(^{cdc2}\) phosphorylation sites, Thr-344, Thr-360, Ser-362, and Ser-370, have been mutated to non-phosphorylatable alanine residues. In fusion proteins with alanine instead of Thr-360 (maps C, G, I, J, K, L), it was noticed that spot d (denoted as d' on maps) only contained phosphoserine (data not shown). By comparison, when threonine was present at residue 360, phosphoserine and phosphothreonine were detected (data not shown) at spot d (designated as spot d on the maps). This result suggests that spot d, which is not observed following the phosphorylation of CKII in cells (21), was a mixture of comigrating or identical mono-phosphorylated peptides arising either from phosphorylation of Thr-360 or phosphorylation of an unidentified serine. The result that the four-site mutant (i.e. AAAA) was phosphorylated by p34\(^{cdc2}\) was somewhat unexpected since the most likely p34\(^{cdc2}\) phosphorylation sites had been eliminated through mutagenesis. It would therefore appear that the additional phosphorylation site does not conform to the minimal consensus for p34\(^{cdc2}\) phosphorylation since the C-terminal region of CKII \(\alpha\) does not contain any additional serine residues that are followed by proline with the exception of Ser-295. Elimination of this site by expressing a GST fusion protein encoding residues 300–391 of CKII \(\alpha\) did not abolish the ability of p34\(^{cdc2}\) to phosphorylate the fusion protein (data not shown), suggesting that Ser-295 is not the unknown phosphorylation site. The phosphorylation of non-consensus residues by p34\(^{cdc2}\) has been previously noted (39, 40). It is important to emphasize that phosphopeptide d is not observed on phosphopeptide maps derived from CKII \(\alpha\) that had been isolated from \(^{32}\)P-labeled human or chicken cells that had been arrested in mitosis.

Overall, the results obtained by phosphorylation of mutant fusion proteins and resultant phosphopeptide maps support the conclusion that the preferred sites of phosphorylation by p34\(^{cdc2}\) in cells are Thr-344, Thr-360, Ser-362, and Ser-370 on the CKII \(\alpha\) subunit (summarized in Table III). In fact, mutation of each of these residues to alanine results in elimination of all phosphopeptides that are detected following the phosphoryla-
tion of CKII α in mitotic cells. Since p34\textsuperscript{cdc2} also phosphorylates Ser-209 on CKII β in mitotic cells, the present results indicate that p34\textsuperscript{cdc2} could phosphorylate the CKII holoenzyme to a stoichiometry of up to 10 mol of phosphate/mol of αβ\textsubscript{2} tetramer in mitotic cells. The high stoichiometry of phosphorylation suggests that phosphorylation could regulate functional properties of CKII and that it could in some way participate in the burst of phosphorylation that accompanies the activation of p34\textsuperscript{cdc2} at the G\textsubscript{2}-M transition (1–4, 41–43).

At present, the role of phosphorylation in regulating the functions of CKII in mitotic cells remains speculative. There have been a number of studies in amphibian (44–46) or starfish oocytes (47) and in mammalian cells (48) that have suggested that the activity of CKII is regulated at different stages in the cell cycle. However, a direct link between these changes in the catalytic activity of CKII and the phosphorylation state of CKII has not been demonstrated. In vitro studies by Mulner-Lorillon et al. (19) demonstrated that CKII isolated from Xenopus laevis can be phosphorylated and activated by p34\textsuperscript{cdc2}. By comparison, when we examined the activity of CKII that had been isolated in its fully phosphorylated state by immunoprecipitation from mitotic cells, the catalytic properties of the enzyme were not significantly different from the unphosphorylated enzyme (21). The latter results suggest that phosphorylation of CKII in mammalian or avian cells may not have direct effects on the enzymatic properties of CKII. Despite the lack of evidence that directly links the phosphorylation of CKII to changes in its catalytic activity during mitosis, there are suggestions that the functions of CKII could be altered during mitosis. For example, independent studies using immunofluorescence have demonstrated that CKII is associated with the mitotic spindle in dividing cells (49, 50). The factors that control the interaction of CKII with the mitotic spindle remain uncharacterized. However, it should be noted that alterations in the ability of CKII to phosphorylate substrate proteins could be mediated by effects on its intracellular distribution or on its interaction with specific substrates without obvious effects on its enzymatic activity. In this regard, there are indications from the studies of Cardenas et al. (51) that the phosphorylation of topoisomerase II is increased in mitosis and that the major mitotic phosphorylation sites are CKII sites. At the repressive temperature in yeast harboring a temperature-sensing form of CKII, topoisomerase II is hypophosphorylated, and the cells fail to divide. In mammalian cells, the phosphorylation of topoisomerase II is also elevated in mitosis (52). With the exception of topoisomerase II, very little is known regarding the stage in the cell cycle when CKII phosphorylates its substrates. It will certainly be of interest to examine the phosphorylation of other CKII substrates to determine if any of these proteins are phosphorylated at the G\textsubscript{2}-M transition. It is also noteworthy that the mitotic phosphorylation sites that have been identified on the α subunit of human CKII are highly conserved between mammalian species and are even present in the α subunit of chicken CKII (28, 53). Interestingly, none of these sites are present of the α'-subunit of CKII from either species. The latter observation suggests that the regulation, and perhaps other functional properties, of the α and α'-isozymic forms of CKII could be distinct. Identification of the mitotic phosphorylation sites on the α subunit of CKII will undoubtedly facilitate efforts to define the role of CKII and its phosphorylation during cell division.

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| Phosphopeptide | Phosphoamino acid | Probable residue | Observed in vivo* |
|----------------|-------------------|------------------|------------------|
| a              | Thr               | Thr-344          | Yes              |
| b              | Ser               | Ser-362 or Ser-370 | Yes        |
| c              | Ser, Thr          | Thr-360 and Ser-362 and Ser-370 | Yes              |
| d              | Thr               | Thr-360, unknown Ser | No             |
| d'             | Ser               | Unknown Ser      | No               |
| e              | Ser, Thr          | Thr-360 and Ser-370 | No               |
| f              | Ser               | Ser-362 and Ser-370 | No               |

* Phosphopeptides obtained following isolation of CKII α from 32P-labeled cells arrested in mitosis (Ref. 21).

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