The Effects of Changing the Site of Activating Phosphorylation in CDK2 from Threonine to Serine*

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Cyclin-dependent kinases (CDKs) that control cell cycle progression are regulated in many ways, including activating phosphorylation of a conserved threonine residue. This essential phosphorylation is carried out by the CDK-activating kinase (CAK). Here we examine the effects of replacing this threonine residue in human CDK2 by serine. We found that cyclin A bound equally well to wild-type CDK2 (CDK2Thr-160) or to the mutant CDK2 (CDK2Ser-160). In the absence of activating phosphorylation, CDK2Ser-160-cyclin A complexes were more active than wild-type CDK2Thr-160-cyclin A complexes. In contrast, following activating phosphorylation, CDK2Ser-160-cyclin A complexes were less active than phosphorylated CDK2Thr-160-cyclin A complexes, reflecting a much smaller effect of activating phosphorylation on CDK2Ser-160. The kinetic parameters for phosphorylating histone H1 were similar for mutant and wild-type CDK2, ruling out a general defect in catalytic activity. Interestingly, the CDK2Ser-160 mutant was selectively defective in phosphorylating a peptide derived from the C-terminal domain of RNA polymerase II. CDK2Ser-160 was efficiently phosphorylated by CAKs, both human p40MO15(CDK7)-cyclin H and budding yeast Cak1p. In fact, the kcat values for phosphorylation of CDK2Ser-160 were significantly higher than for phosphorylation of CDK2Thr-160, indicating that CDK2Ser-160 is actually phosphorylated more efficiently than wild-type CDK2. In contrast, dephosphorylation proceeded more slowly with CDK2Ser-160 than with wild-type CDK2, either in HeLa cell extract or by purified PP2Cβ. Combined with the more efficient phosphorylation of CDK2Ser-160 by CAK, we suggest that one reason for the conservation of threonine as the site of activating phosphorylation may be to favor unphosphorylated CDKs following the degradation of cyclins.

Cyclin-dependent kinases (CDKs), a subfamily of protein kinases, promote cell cycle progression. In mammals, nine different CDKs (CDK1 to CDK9; CDK1 is better known as CDC2) have been identified (for a review, see Ref. 1). The activity of CDKs is regulated on various levels including binding of proteins (cyclins, inhibitors, and assembly factors), protein degradation, transcriptional control, localization, and multiple phosphorylations (2–5). This leads to timely regulation of CDK activity, allowing progression from one cell cycle phase to the next.

CDKs are regulated by both inhibitory and activating phosphorylations. Threonine 14 and tyrosine 15 (in human CDK2) were identified as inhibitory phosphorylation sites that are phosphorylated by WEE1-like kinases and dephosphorylated by the CDC25 dual specificity phosphatases. For full activation, the cell cycle CDKs need to bind a cyclin and to be phosphorylated on a conserved threonine residue, located in the T-loop (Thr-160 in CDK2). Activating phosphorylation is essential for CDK activity in vitro (6, 7) and for growth of yeast cells (8, 9). Activating phosphorylation is carried out by the CDK-activating kinase (CAK; for a review, see Ref. 10), an enzyme that, except in budding yeast, is composed of a catalytic subunit, p40MO15 (see Refs. 11–14; also called CDK7); a regulatory subunit, cyclin H (15, 16); and an assembly factor, MAT1 (17–19). In vitro, MO15 phosphorylates CDC2 (11, 12), CDK2 (11–13, 15, 20, 21), CDK3 (22), CDK4 (23–25), and CDK6 (21, 26, 27).

Budding yeast CAK, Cak1p, is very different from MO15-type CAKs. Cak1p is active as a monomer and is only distantly related to CDKs (28–30). Cak1p is an essential gene product that phosphorylates and activates Cdc28p (the major CDK in budding yeast, a homolog of CDC2) in vivo (28, 29). In vitro, Cak1p can phosphorylate Cdc28p (28–31), CDC2 (32), CDK2 (21, 28–30), CDK6 (21), and Kin28p (33). Activating phosphorylation is reversed by type 2C protein phosphatases (34). Specifically, budding yeast Ptc2p and Ptc3p dephosphorylate Cdc28p in vivo. Deletion of the corresponding genes partially rescues a cak1 mutant at restrictive temperature, and overexpression renders a cak1 mutant inviable at semirestrictive temperature (34), indicating that Ptc2p and Ptc3p are the physiological phosphatases that dephosphorylate the activating threonine in Cdc28p. PP2Cs are also responsible for dephosphorylating human CDK2 in HeLa cell extracts (34).

Human CDK2 was identified by complementation of cdc2 mutants in Schizosaccharomyces pombe (35) and cdc28 mutants in Saccharomyces cerevisiae (36, 37), indicating that CDK2 can function in all phases of the yeast cell cycle and that it is a substrate for Cak1p. In human cells, CDK2 binds to cyclins A and E and promotes entry into and progression through S phase. The substrate specificity of CDKs is defined by at least two factors: (i) the intrinsic kinase specificity and (ii) a docking site in the cyclin subunit. Most CDKs phosphorylate serines and threonines within the general consensus sequence (S/T)PXR(K/R), although there are strong differences among the various CDKs (38–41), and, in addition, many CDKs have not

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The abbreviations used are: CDK, cyclin-dependent kinase; GST, glutathione S-transferase; DTT, dithiothreitol; CAK, CDK-activating kinase; PAGE, polyacrylamide gel electrophoresis; CTD, C-terminal domain.
been tested in such assays. Some CDK substrates such as Rb, p27, and CDC25 require docking to the cyclin subunit for efficient phosphorylation (42–46), whereas other substrates are independent of docking. On a structural level, the understanding of substrate specificity has been helped by a recent co-crystal structure of a substrate peptide with CDK2-cyclin A3 (47). The basic residue at the P-3-position (with respect to the phosphorylation site) interacts with the phosphate on threonine 160 and the preference for a basic residue at the P+3-position of the substrate (47).

It is curious that all cell cycle CDKs contain a threonine, and never a serine, at the site of activating phosphorylation. Replacement of Thr-160 by serine leads to serine-phosphorylated CDK2 in cell lines and indicates that this molecule is generally active (48). In this study, we investigated the detailed biochemical effects of replacing the activating threonine in human CDK2 by a serine residue. We found that this mutant bound cyclin A like wild-type CDK2 and displayed elevated activity in the absence of CAK phosphorylation. Following phosphorylation, CDK2Ser-160 had a normal affinity for ATP and histone H1 but was compromised in phosphorylation of Rb, the CTD peptide, and a synthetic peptide substrate GST-KSPRK. Furthermore, CDK2Ser-160 was more efficiently phosphorylated by CAKs and less efficiently dephosphorylated by PP2C than wild-type CDK2.

**EXPERIMENTAL PROCEDURES**

**Mutagenesis and Constructs—**QuickChange mutagenesis (Stratagene, La Jolla, CA) was performed to introduce the T160S mutation of substrate specificity has been helped by a recent co-crystal structure of a substrate peptide with CDK2-cyclin A3 (47). The basic residue at the P-3-position (with respect to the phosphorylation site) interacts with the phosphate on threonine 160 and the preference for a basic residue at the P+3-position of the substrate (47).

**Preparation of CAK-phosphorylated CDK2—**8.9 µM (0.15 pmol) of GST-cyclin A173–432 was incubated at room temperature with 2.9 µg (41.5 pmol) of GST-cyclin A173–432. After incubation for 30 min at room temperature, reactions were stopped by adding 7 µl of 5× SDS-PAGE sample buffer. Some CDK substrates such as Rb, p27, and CDC25 require docking to the cyclin subunit for efficient phosphorylation (42–46), whereas other substrates are independent of docking. On a structural level, the understanding of substrate specificity has been helped by a recent co-crystal structure of a substrate peptide with CDK2-cyclin A3 (47). The basic residue at the P-3-position (with respect to the phosphorylation site) interacts with the phosphate on threonine 160 and the preference for a basic residue at the P+3-position of the substrate (47).

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Effects of T160S Mutation in CDK2

CDK2Ser-160 has been shown previously to function normally, at least to a first approximation, following transfection into cell lines (48). However, in preliminary experiments we observed clear differences between CDK2Ser-160 and wild-type CDK2. We explored the causes of these observations. Since cyclin binding is an essential step in the activation of CDKs, we first compared the ability of cyclin A to bind to CDK2Ser-160 and to wild-type CDK2 (CDK2Thr-160). In vitro translated, radiolabeled cyclin A was incubated for 1 h with identical amounts of CDK2Ser-160 or CDK2Thr-160 that were unphosphorylated or prephosphorylated by Cak1p (see below). The CDK2 was preprecipitated, and the amount of bound cyclin A was determined by SDS-PAGE. Both unphosphorylated and prephosphorylated CDK2Ser-160 bound similar amounts of cyclin A as the corresponding forms of CDK2Thr-160 (Fig. 1A, lanes 2–5). Phosphorylation of either CDK2 protein stimulated the binding to cyclin A as has been reported previously for wild-type CDK2 (53, 54). We next examined cyclin binding after short incubation times to determine whether there was a kinetic difference in the ability of CDK2Thr-160 and CDK2Ser-160 to bind cyclin (Fig. 1B). Since activating phosphorylation strengthens the cyclin-CDK2 interaction (Fig. 1A), we performed this experiment with unphosphorylated CDK2, reasoning that a kinetic effect would be more apparent. Cyclin binding occurred very quickly with half-maximal binding at 18 s for CDK2Thr-160 and at 13 s for CDK2Ser-160. CDK2Thr-160 bound approximately 15% more cyclin A than CDK2Ser-160 (Fig. 1B), even when the time course was extended to 4 h (data not shown). We do not know if these differences are significant. Nevertheless, they were small and should not affect the outcome of the following experiments, which involve longer incubation times in the presence of excess cyclin.

We tested the effects of threonine 160 mutations on the low but detectable kinase activity of unphosphorylated CDK2-cyclin A complexes (55, 56). Detection of this low activity required the use of a higher specific activity of radiolabeled ATP than was used in other experiments. In addition to wild-type CDK2 and CDK2Ser-160, we also tested a nonphosphorylatable mutant (threonine 160 replaced by alanine; Ala160) and a mutant designed to mimic constitutive phosphorylation (threonine 160 replaced by glutamic acid; Glu160). CDK2Ala-160 displayed lower activity than CDK2Thr-160 toward all substrates tested (histone H1 (Fig. 2A), CTD peptide (Fig. 2B), and Rb (Fig. 2C)), whereas CDK2Glu-160 displayed higher activity (Fig. 2, compare lanes 13–16 with lanes 1–4). Interestingly, CDK2Glu-160 was more active than CDK2Thr-160, similar to CDK2Thr-160 (compare lanes 5–8 with lanes 1–4 and lanes 13–16).

We next compared the abilities of two CAs, budding yeast Cak1p (Fig. 3A) and human p40MO15-cyclin H (Fig. 3B), to phosphorylate CDK2Thr-160 and CDK2Ser-160. The assays were performed over a range of CDK2 concentrations so that we could derive the kinetic parameters $K_{\text{on}}$ (substrate concentration that yields half-maximal velocity) and $k_{\text{cat}}$ (maximal velocity at saturating substrate concentrations divided by the enzyme concentration). The $K_{\text{on}}$(CDK2) for Cak1p was approximately 4-fold higher for CDK2Thr-160 than for CDK2Ser-160 (Table 1). However, the $K_{\text{on}}$(CDK2) for MO15 was very similar for both substrates. Interestingly, the $k_{\text{cat}}$ of each CA using CDK2Ser-160 was approximately 2.5 times as high as when using CDK2Thr-160.

We compared the activities of phosphorylated CDK2-cyclin A complexes toward histone H1 (Fig. 4A, upper panel), the CTD peptide (Fig. 4A, middle panel), and Rb (Fig. 4A, lower panel). The activities toward histone H1 were similar for CDK2Thr-160 and CDK2Ser-160 (Fig. 4A, upper panel). However, the activities of CDK2Ser-160 toward the CTD peptide and Rb were substantially lower than those of CDK2Thr-160 (Fig. 4A, middle and lower panels), suggesting that phosphorylation of the activating threonine selectively affects phosphorylation of different substrates. Since phosphorylation of the CTD peptide was car-

**Fig. 1.** Cyclin A binding to wild-type and mutant CDK2. Cyclin A was translated in vitro and labeled with [35S]methionine. A, after mixing with buffer (column 1), unphosphorylated wild-type CDK2Thr-160 (column 2), CAK-phosphorylated CDK2Thr-160 (column 3), unphosphorylated mutant CDK2Ser-160 (column 4), or CAK-phosphorylated CDK2Ser-160 (column 5), CDK2 was pulled down via its GST tag, and bound cyclin A was measured by phosphorimaging following SDS-PAGE. Each column represents the average of three different CDK2 concentrations. Immunoblotting confirmed that identical amounts of CDK2 were precipitated (data not shown). B, time course of cyclin A binding to unphosphorylated wild-type CDK2Thr-160 (C) and unphosphorylated mutant CDK2Ser-160 (D). Each time point corresponds to the average of three independent measurements.

**Fig. 2.** Activity of unphosphorylated CDK2-cyclin A. 0.1 μg of wild-type CDK2Thr-160 (Thr160, lanes 1–4), mutant CDK2Ser-160 (Ser160, lanes 5–8), mutant CDK2Ala-160 (Ala160, lanes 9–12), and mutant CDK2Glu-160 (Glu160, lanes 13–16) was bound to cyclin A, and the activity toward histone H1 (A), CTD peptide (B), and GST-Rb605–928 (C) was determined. The following GST-cyclin A complexes toward histone H1 (A), CTD peptide (B), and GST-Rb605–928 (C) was determined. The following GST-cyclin A complexes toward histone H1 (A), CTD peptide (B), and GST-Rb605–928 (C) was determined. The following GST-cyclin A complexes toward histone H1 (A), CTD peptide (B), and GST-Rb605–928 (C) was determined. The following GST-cyclin A complexes toward histone H1 (A), CTD peptide (B), and GST-Rb605–928 (C) was determined. The following GST-cyclin A complexes toward histone H1 (A), CTD peptide (B), and GST-Rb605–928 (C) was determined. The following GST-cyclin A complexes toward histone H1 (A), CTD peptide (B), and GST-Rb605–928 (C) was determined. The following GST-cyclin A complexes toward histone H1 (A), CTD peptide (B), and GST-Rb605–928 (C) was determined.
TABLE I

Kinetic parameters for phosphorylation of CDK2 by Cak1p and p40MO15(CDK7)

| Enzyme          | $K_{cat}(Thr-160)$ | $k_{cat}(Thr-160)$ | $K_{cat}(Ser-160)$ | $k_{cat}(Ser-160)$ |
|-----------------|-------------------|-------------------|-------------------|-------------------|
| Cak1p           | 246 nm            | 0.055 min$^{-1}$  | 1270 nM           | 0.222 min$^{-1}$  |
| p40MO15(CDK7)   | 297 nm            | 0.022 min$^{-1}$  | 365 nM            | 0.100 min$^{-1}$  |

Because of the striking effects of the T160S mutation on the phosphorylation of the CTD peptide and Rb by phosphorylated CDK2 (Fig. 4), we performed a kinetic analysis to determine whether this effect was due to an increase in $K_{cat}$, a decrease in $k_{cat}$, or a combination of the two. We could not use Rb or the CTD peptide for this analysis. At high concentrations, Rb binds to CDK2-cyclin A and inhibits its activity (data not shown). The CTD peptide was phosphorylated too weakly at high ATP concentrations and at lower CTD peptide concentrations (data not shown). Therefore, we turned to a systematic panel of CDK substrates in which a substrate peptide (KSPRK) was fused to the C terminus of glutathione S-transferase (GST-KSPRK; Ref. 40). 19 such substrates, containing all possible amino acids except for isoleucine at the P+3-position with respect to the serine, were analyzed. The initial assays were carried out at low (below the $K_{cat}$) concentrations of substrates, yielding phosphorylation efficiencies relative to the phosphorylation of the KSPRK substrate by CDK2Ser160, which was defined as 100%.

We further analyzed the mutant CDK2Ser160 by performing a kinetic analysis. The $K_{cat}(Thr-160)$ for CDK2Ser160 was 34% higher than for wild-type CDK2Thr160 (Fig. 5A and Table II), and the $K_{cat}(histone H1)$ was increased by 92% (Fig. 5B and Table II). These are modest differences considering the high concentrations of ATP and histone H1 used in Fig. 4; these differences cannot explain the lower activity of CDK2Ser160 toward the CTD peptide and Rb (Fig. 3A). Interestingly, the $k_{cat}$ of CDK2Ser160 toward histone H1 was only 33% that of wild-type CDK2Thr160 (Fig. 5B, Table II). This low $k_{cat}$ does not reflect lower levels of phosphorylation by CAK, since CDK2Ser160 is actually phosphorylated more efficiently than CDK2Thr160 (see Fig. 3).
was analyzed using dilution series of CDK2Thr-160 and CDK2Ser-160 to phosphorylate histone H1 (Fig. 7, A and B). As observed in Fig. 2, CDK2Thr-160 was less active than CDK2Ser-160 when unphosphorylated (Fig. 7A) but more active than CDK2Ser-160 following phosphorylation by CAK (Fig. 7B). Specific activities calculated from the slopes in Fig. 7, A and B, indicated that wild-type CDK2Thr-160 was activated approximately 46.1-fold by CAK phosphorylation (Fig. 7C, compare columns 1 and 2), whereas mutant CDK2Ser-160 was stimulated only 9.8-fold (Fig. 7C, compare columns 3 and 4). Thus, the dynamic range of activation of mutant CDK2Ser-160 upon CAK phosphorylation was only 21% as much as for wild-type CDK2Thr-160, providing less room for regulation by phosphorylation.

Dephosphorylation of the activating threonine in CDKs is carried out by type 2C phosphatases (34). Since PP2C has been reported to display a 20-fold preference for a phosphothreonine peptide substrate compared with an equivalent phosphoserine substrate (57), we tested if CDK2Ser-160 could serve as a substrate for PP2C. Mutant and wild-type CDK2 proteins were phosphorylated in vitro and added to a HeLa cell extract. Although both CDK2Thr-160 and CDK2Ser-160 were dephosphorylated in a Mg2+-dependent (Fig. 8A) and cyclin-inhibitable fashion (data not shown), mutant CDK2Ser-160 was dephosphorylated more slowly than wild-type CDK2Thr-160. To obtain quantitative data, the linear phase of this experiment was repeated three times (Fig. 8B). Mutant CDK2Ser-160 was dephosphorylated approximately 25% as rapidly as wild-type CDK2Thr-160 by a HeLa cell extract. A similar value (~17%) was obtained using purified recombinant human PP2C (Fig. 8C), indicating that the slower dephosphorylation of CDK2Ser-160 was a direct effect and not due to other factors in the cell extract.

**DISCUSSION**

Superficially, CDK2 seems to function well with a serine residue in place of threonine 160 (our results; Ref. 48). However, closer examination revealed effects on its activity, its phosphorylation by CAK, and on its dephosphorylation by PP2C. These results have implications for the functions of the activating threonine.

Site-directed mutagenesis is widely used to elucidate the functions of proteins. Replacement of a threonine residue by serine is considered to be a "conservative" mutation that should...
mimic the functions of the replaced threonine. The serine residue can be phosphorylated and dephosphorylated like the threonine residue. Nevertheless, every mutation has effects, even if they are subtle. Our results demonstrate that the serine replacement of threonine 160 in human CDK2 displays a variety of defects that can only be detected by detailed quantitative analysis.

Our results suggest that CDK2_{Ser-160} displays no general defects in catalytic activity, since the kinetic parameters for ATP and histone H1 were similar to those of wild-type CDK2 (Table II). Surprisingly, phosphorylated CDK2_{Ser-160} was compromised specifically in phosphorylating the CTD peptide, Rb (Fig. 4A), and the GST-KSPRK model substrate (Fig. 6). However, unphosphorylated CDK2_{Ser-160}-cyclin A complexes phosphorylated the CTD peptide and Rb much better than did CDK2_{Thr-160}-cyclin A (Fig. 2). Rb is one of the substrates that requires a docking site located on the cyclin subunit (RXL; Refs. 44–47 and 58), whereas phosphorylation of histone H1, presumably of the CTD peptide, and of GST-KSPRK are independent of the docking site. These results suggest that the T160S mutation selectively affects the phosphorylation of different substrates.

A recent report of the crystal structure of CDK2-cyclin A3 with a substrate peptide (47) indicates that the phosphate group on threonine 160 makes direct contact with the P_1 residue of the substrate peptide. Although the absence of a methyl side group on serine appears unlikely to have a significant effect on the distance between the substrate phosphorylation site and the phosphate on CDK2, it could have subtle but important effects on the orientation of this side chain. For instance, the added methyl group of threonine might constrain the movement of the side chain. This constraint might place the phosphate in an optimal orientation for efficient substrate binding. With a serine, however, the side chain might be less constrained and might spend less time in the optimal position. In contrast, the weak substrate phosphorylation by unphosphorylated CDK2_{Thr-160} might reflect a low time-averaged occupancy of the most favorable orientation in the absence of phosphorylation. By allowing greater motion, serine 160 might spend a greater fraction of time in a favorable orientation for substrate phosphorylation. Thus, greater motion of serine 160 might account for the paradoxical observation that CDK2_{Ser-160}...
Effects of T160S Mutation in CDK2

has higher activity than CDK2Thr-160 when unphosphorylated but lower activity when phosphorylated. Such speculative ideas are, of course, difficult to test in the absence of crystal structures of phosphorylated and unphosphorylated CDK2Ser-160. Interestingly, threonine is also found as the catalytic residue in all protease subunits of the proteasome. Although replacement with serine compromises activity, no structural explanation of this surprising effect is apparent (59).

A previous in vivo study confirms some of the predictions of our analysis. Mutation of threonine 160 to serine in CDK2 led to a >20-fold increase of [32P] labeling in vivo (48), supporting our finding that CDK2Ser-160 is phosphorylated more efficiently by CAK (Fig. 3) and dephosphorylated less efficiently by PP2C (Fig. 8). In addition, Gu et al. (48) found that the specific activity of CDK2Ser-160 was only 50% that of CDK2Thr-160, consistent with the reduced activity we see in vitro (Fig. 7C).

Therefore, our findings reflect differences that can be observed in an in vivo situation. No phenotype was observed when CDK2Ser-160 was overexpressed up to 50-fold (48), excluding the possibility of a dramatic effect of CDK2Ser-160 function in vivo.

Examination of more subtle effects will require replacing both copies of CDK2Thr-160 with CDK2Ser-160.

Threonine is used as the site of activating phosphorylation in all known cell cycle CDKs from all species. Our results suggest the following possible explanations for this conservation: (i) CDK2Thr-160 has a broader substrate utilization than CDK2Ser-160; (ii) CDK2Thr-160 displays a greater dynamic range of activity upon phosphorylation than CDK2Ser-160; and (iii) the slower phosphorylation of CDK2Thr-160 by CAK (24-fold) combined with its faster dephosphorylation by PP2Cs (~4-fold) shifts the equilibrium toward unphosphorylated monomeric CDK2, which would prevent an immediate activation of CDK2 after cyclin binding. All of these changes could hinder the precise control of CDK2Ser-160 activity during the cell cycle.

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