Cdc25M2 activation of cyclin-dependent kinases by dephosphorylation of threonine-14 and tyrosine-15

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ABSTRACT Recent evidence has suggested that human cyclin-dependent kinase 2 (CDK2) is an essential regulator of cell cycle progression through S phase. CDK2 is known to complex with at least two distinct human cyclins, E and A. The kinase activity of these complexes peaks in G₁ and S phase, respectively. The vertebrate CDC2/cyclin B1 complex is an essential regulator of the onset of mitosis and is inhibited by phosphorylation of CDC2 on Thr-14 and Tyr-15. *In vitro*, CDC2/cyclin B1 is activated by treatment with the members of the Cdc25 family of phosphatases. We found that, like CDC2, CDK2 is also phosphorylated on Thr-14 and Tyr-15 and that treatment of cyclin A or cyclin E immunoprecipitates with bacterially expressed Cdc25M2 (the mouse homolog of human CDC25B) increased the histone H1 kinase activity of these immune complexes 5- to 10-fold. Tryptic peptide mapping demonstrated that Cdc25M2 treatment of cyclin A or cyclin B1 immune complexes resulted in the specific dephosphorylation of Thr-14 and Tyr-15 on CDK2 or CDC2, respectively. Thus, we have confirmed that Cdc25 family members comprise a class of dual-specificity phosphatases. Furthermore, our data suggest that the phosphorylation and dephosphorylation of CDKs on Thr-14 and Tyr-15 may regulate not only the G₂/M transition but also other transitions in the cell cycle and that individual cdc25 family members may regulate distinct cell cycle checkpoints.

The vertebrate cell cycle is regulated by a series of cyclin-dependent-kinase (CDK)/cyclin complexes. CDC2/cyclin B regulates entry into M phase (1, 2). Human cyclin B is periodically expressed in the cell cycle, accumulating in G₂ until it is abruptly degraded in anaphase (3). As cyclin B accumulates, it associates with CDC2, which then becomes phosphorylated at Thr-14, Tyr-15, and Thr-161 (4–8). Phosphorylations of CDC2 at Thr-14 and Tyr-15 negatively regulate the activity of the CDC2/cyclin B complex (4, 5, 7), whereas Thr-161 phosphorylation is essential for kinase activity (6, 8, 9). In fission yeast, the products of *wee1* and *mik1* phosphorylate cdc2 on Tyr-15 (10, 11). A human gene with homology to *wee1* has been identified (12) and can inhibit CDC2/cyclin B kinase activity by phosphorylation of Tyr-15 (13). The protein kinase that phosphorylates Thr-14 is unknown. Homologs of the fission yeast mitotic inducer cdc25 are protein phosphatases which can activate CDC2/cyclin B1 phosphorylated at Thr-14 and Tyr-15 (14–19). CDC2/cyclin B is activated at the G₂/M boundary following dephosphorylation of Thr-14 and Tyr-15. This event is presumably regulated by activation of Cdc25 (20) and possibly also by inactivation of Wee1 (21).

The role of CDK2/cyclin A and CDK2/cyclin E in regulating S-phase progression has been established by a number of recent reports. CDK2 is essential for S-phase progression in the *Xenopus* egg cell-free system (22). A role for cyclin E

in regulating entry into S phase is suggested by reports that cyclin E-associated kinase activity peaks in G₁ (23, 24) and that overexpression of cyclin E decreases the length of G₁ and diminishes the dependency of proliferating human cells on growth factors (23). Although both CDK2 and CDC2 associate with cyclin E, the predominant complex appears to be CDK2/cyclin E. A role for CDK2/cyclin A in regulating progression through S phase is suggested by observations that microinjection of either anti-cyclin A antibodies or plasmids encoding antisense cyclin A cDNA inhibits the completion of DNA synthesis (25, 26). Overexpression of either cyclin A or cyclin E overcomes the ability of transfected wild-type retinoblastoma protein (RB) to suppress the growth of SAOS-2 human osteosarcoma cells (which lack functional endogenous RB), presumably by phosphorylating RB (27).

We chose to investigate the possibility that CDK2 complexes were regulated by Thr-14 and Tyr-15 phosphorylation in a manner similar to CDC2/cyclin B. There are three human homologs of cdc25, CDC25A, -B, and -C (28–30). Microinjection of CDC25C antibodies into HeLa cells causes cell cycle arrest in G₂ (31), and a *Xenopus* homolog of CDC25C is activated at the G₂/M transition point (20). Thus, CDC25C has been implicated as a positive regulator of entry into mitosis in vertebrate cells. CDC25A, on the other hand, appears to be required for progression through M phase (30). Since the function of CDC25B in regulating the cell cycle is unknown, we examined the ability of Cdc25M2 [the murine homolog of CDC25B (32)] to activate and dephosphorylate cyclin A, cyclin B1, and cyclin E immune complexes. For this purpose, we used a purified, bacterially expressed glutathione S-transferase (GST)–Cdc25M2 fusion protein that contains the predicted catalytic domain of this protein (described in ref. 32).

MATERIALS AND METHODS

Immunoprecipitations and GST–Cdc25M2 Treatment. Anti-cyclin B1 serum (3) was used at a 1:500 dilution, C160 anti-cyclin A monoclonal antibody was used at a 1:100 dilution of hybridoma supernatant (33), and anti-cyclin E serum (kindly provided by A. Koff and J. Roberts) was used at a 1:200 dilution (23). Immunoprecipitates were prepared from growing unlabeled HeLa cells, which were lysed and clarified as described (34). Washes with radioimmunoprecipitation assay (RIPA) buffer (34) were followed by a final wash in glutathione elution buffer (32), and the washed immunoprecipitates were treated with GST–Cdc25M2 or GST at pH 8.2 at 30°C (≈0.3 μg for 15 min for histone H1 kinase assays, ≈3 μg for 1 hr for ³²P-labeled samples). Purified GST–Cdc25M2 was prepared as described (32).

Histone H1 Kinase Assays. After GST–Cdc25M2 treatment, the immunoprecipitates were washed in kinase buffer, and

histone H1 kinase assays were performed as described (3). The cyclin E experiment in Fig. 1 was performed separately.

Metabolic Labeling of HeLa Cells. Asynchronously growing HeLa cells were incubated for 4 hr with [32 P]orthophosphate (ICN) at 1 mCi (37 MBq)/ml, as described (3).

Tryptic Phosphopeptide Mapping. Immune complexes were resolved by SDS/17.5% PAGE, transferred to Immobilon-P (Millipore), and analyzed by autoradiography. Peptide mapping was performed as described (35). 32 P-labeled tryptic digests were applied to 100- μ m thin-layer cellulose plates, and subjected to electrophoresis at pH 1.9 for 25 min at 1 kV and subsequently to ascending chromatography in "phospho-chromo" buffer (35). Recovery of spot X_{TY} and subsequent phospho-amino acid analysis by acid hydrolysis were performed as described (35).

RESULTS AND DISCUSSION

Treatment of human cyclin A immunoprecipitates with GST-Cdc25M2 resulted in an increase in histone H1 kinase activity similar to that of GST-Cdc25M2-treated cyclin B1 immunoprecipitates (Fig. 1). In both cases this activation was inhibited by the inclusion of sodium orthovanadate (data not shown). We routinely observed a 5- to 10-fold increase in CDK2/cyclin A kinase activity upon treatment with GST-Cdc25M2. Cdc25M2 treatment also led to a similar level of activation of cyclin E immunoprecipitates (Fig. 1).

Our initial observation that Cdc25 treatment activated the CDK2 kinase suggested that, like CDC2, CDK2 is negatively regulated by phosphorylation on Thr-14 and Tyr-15, and that these residues are dephosphorylated by Cdc25M2. To prove this, we performed tryptic peptide mapping on 32 P-labeled CDK2 isolated from cyclin A immunoprecipitates. Cyclin A immunoprecipitates from asynchronously growing HeLa cell lysates contained CDK2 and no detectable levels of CDC2 (Fig. 2A and ref. 36). A tryptic digest of 32 P-labeled human CDK2 (Fig. 2B) contained a species which comigrated with the CDC2 peptide containing phosphorylated Thr-14 and Tyr-15 [Fig. 2C, spot X_{TY} (6–8); for comparison with a tryptic digest of CDC2 alone, refer to Fig. 3B]. The sequences of the predicted tryptic peptides derived from p33^{CDK2} and p34^{CDC2} containing Thr-14 and Tyr-15 are identical. The identity of X_{TY} was confirmed by acid hydrolysis, which revealed that X_{TY} contained phosphothreonine, phosphotyrosine, and a species with a mobility similar to that predicted for a dipeptide phosphorylated on both tyrosine and threonine (Fig. 2D). Because others have observed association between cyclin A and CDC2 (33), we confirmed that spot X_{TY} was indeed derived from CDK2 and not CDC2 by verifying its presence in a tryptic peptide map of CDK2 isolated from a lysate precleared with a monoclonal antibody (provided by T. Hunt,

FIG. 1. Cdc25M2 activation of histone H1 kinase associated with cyclin A and cyclin E immunoprecipitates from asynchronous HeLa cells. Lane 1, cyclin A immunoprecipitate treated with GST; lane 2, cyclin A immunoprecipitate treated with GST-Cdc25M2; lane 3, cyclin B1 immunoprecipitate treated with GST; lane 4, cyclin B1 immunoprecipitate treated with GST-Cdc25M2; lane 5, cyclin E immunoprecipitate treated with GST; lane 6, cyclin E immunoprecipitate treated with GST-Cdc25M2. The dried gels were exposed to Kodak XAR film for 12 hr at room temperature without presensitization (cyclin A and B1) or for 12 hr at -70°C with presensitization and an intensifying screen (cyclin E).

ICRF) specific for CDC2 (data not shown). In addition to X_{TY} , we observed a second major peptide in the p33^{CDK2} tryptic peptide map (Fig. 2B, spot X_Y). This peptide comigrated with the tryptic peptide derived from a synthetic peptide corresponding to residues 8–22 of CDC2 and CDK2, which had been phosphorylated on Tyr-15 alone (data not shown).

Having established that CDK2, like CDC2, is phosphorylated on Thr-14 and Tyr-15, we confirmed that these sites were specifically dephosphorylated by Cdc25M2. Treatment of 32 P-labeled cyclin A immunoprecipitates with GST-Cdc25M2 did not produce a mobility shift in CDK2 but did result in a decrease in 32 P content (Fig. 2A). A tryptic digest of CDK2 isolated from a cyclin A immunoprecipitate treated with GST-Cdc25M2 did not contain spot X_{TY} , but spot X_Y was still present (Fig. 2E).

The inability of Cdc25M2 to dephosphorylate Tyr-15 completely raises a number of possibilities. One is that Cdc25M2 is primarily a threonine phosphatase and does not efficiently dephosphorylate Tyr-15. We ruled out this possibility by treating 32 P-labeled cyclin B1 immunoprecipitates with GST-Cdc25M2 (Fig. 3A) and performing tryptic peptide mapping on treated and untreated CDC2. While a tryptic peptide map of untreated CDC2 revealed two spots, X_{TY} and Y [the peptide corresponding to phosphorylated Thr-161 (6, 8)], a tryptic peptide map of CDC2 immunoprecipitated with anti-cyclin B1 serum and treated with GST-Cdc25M2 contained only peptide Y (Fig. 3C). We did not observe accumulation of peptide X, which is phosphorylated on Thr-14 or Tyr-15 alone, indicating that Cdc25M2 had quantitatively dephosphorylated both Thr-14 and Tyr-15 but not Thr-161. Cdc25 would not be expected to dephosphorylate Thr-161, since phosphorylation of Thr-161 has been shown to stimulate CDC2/cyclin B kinase activity (6, 8, 9). These data confirm previous reports which have suggested that cdc25 family members can act as "dual specificity" phosphatases (14–19). The remaining explanations for the inability of Cdc25M2 to dephosphorylate CDK2 on Tyr-15 completely are either that Cdc25M2 dephosphorylates CDC2/cyclin B1 more efficiently than it does CDK2/cyclin A, or that Cdc25M2 does not efficiently recognize CDK2/cyclin A phosphorylated on Tyr-15 alone.

While cyclin A-associated kinase activity increases in S phase in approximate correlation with the increase in cyclin A mRNA (36), our results suggest that the activity of CDK2 complexes is also regulated by phosphorylation/dephosphorylation of Thr-14 and Tyr-15. Phosphorylation of CDC2 on either Thr-14 or Tyr-15 results in inactivation of the kinase. One of the kinases which has been proposed to phosphorylate these residues, WEE1Hu, has now been shown to phosphorylate Tyr-15 and not Thr-14 (13). This raises the possibility of a signal transduction mechanism in which distinct kinases phosphorylate Thr-14 and Tyr-15, while a single phosphatase (Cdc25) dephosphorylates both residues. Interestingly, cyclin A-associated CDK2 appears to be highly phosphorylated on Tyr-15 alone (Fig. 2B), whereas CDC2 isolated from cyclin B1 immunoprecipitates is not (Fig. 3B). This suggests that there may be important differences in the ways in which CDK2 and CDC2 Thr-14/Tyr-15 phosphorylation is regulated.

In summary, we have shown that CDK2, an important regulator of S-phase progression, is negatively regulated by phosphorylation on Thr-14 and Tyr-15. That dephosphorylation of these residues by Cdc25M2 results in a 5- to 10-fold increase in the kinase activity of cyclin complexes suggests that the phosphorylation and dephosphorylation of these residues are important means by which S-phase progression is regulated. Since CDK2 and CDC2 complexes are activated at different points in the cell cycle and appear to perform quite different functions, we propose that individual Cdc25 family members may regulate different cyclin complexes.

FIG. 2. Phosphorylation of CDK2 on Thr-14 and Tyr-15 and dephosphorylation of these residues by GST-Cdc25M2. (A) ^{32}P -labeled cyclin A immunoprecipitates from asynchronously growing HeLa cells were prepared and analyzed by SDS/17.5% PAGE electrophoresis. Lane 1, cyclin A immunoprecipitate incubated with GST elution buffer; lane 2, cyclin A immunoprecipitate incubated with GST-Cdc25M2. Position of the 33-kDa CDK2 protein ($p33^{\text{CDK2}}$) is indicated. (B) Tryptic phosphopeptide map of $p33^{\text{CDK2}}$ isolated from a ^{32}P -labeled cyclin A immunoprecipitate. (C) Tryptic phosphopeptide map of a mix of $p33^{\text{CDK2}}$ and $p34^{\text{CDC2}}$ isolated as described above. (D) Phospho-amino acid (PAA) analysis of spot X_{TY} in B (the spot with a mobility similar to that of a dipeptide phosphorylated on both tyrosine and threonine is to the left of the T and Y spots). (E) Tryptic peptide map of CDK2 isolated from a ^{32}P -labeled cyclin A immunoprecipitate and treated with GST-Cdc25M2. Amounts applied were 500 cpm in B, 1100 cpm of each protein in C, and ≈ 300 cpm in E. Exposures (at -10°C with presensitization and an intensifying screen) were 10 hr, 3 days, 3 days, 10 days, and 3 days for A-E, respectively.

Microinjection experiments, which suggest that CDC25C regulates the G_2/M transition (31) and that CDC25A is required for exit from mitosis (30), support the notion that cdc25 family members regulate multiple cell-cycle checkpoints.

While this work was in review, a recent report described the activation of a *Xenopus* CDK2 immunoprecipitate by

CDC25C (37). A similar study demonstrated that human CDK2 is inhibited by phosphorylation on Thr-14 and Tyr-15, and that Thr-160 (corresponding to Thr-161 in CDC2) phosphorylation is essential for CDK2 kinase activity (38). The authors also showed that treatment of human CDK2 immunoprecipitates with the string protein, a *Drosophila* homolog of Cdc25, dephosphorylated CDK2 on Thr-14/Tyr-15 and

FIG. 3. Dephosphorylation of $p34^{\text{CDC2}}$ by GST-Cdc25M2. (A) Cyclin B1 immunoprecipitates from $[^{32}\text{P}]$ orthophosphate-labeled HeLa cells were treated with GST (lane 1) or GST-Cdc25M2 (lane 2). (B and C) Tryptic peptide map of $p34^{\text{CDC2}}$ from lanes 1 and 2, respectively. Equal cpm were loaded for these two maps, and therefore spot Y is relatively darker in C than in B. Exposures were for 12 hr, 4 days, and 4 days for A-C, respectively, using an intensifying screen and presensitized Kodak XAR film at -70°C .

doubled the kinase activity of these immunoprecipitates. However, since both string and CDC25C appear to act as regulators of the G₂/M transition (31, 39, 40), we believe that they are unlikely to also regulate S-phase progression. Since the role of CDC25B/Cdc25M2 has not been established, it appears to be the better candidate for the CDK2 Thr-14/Tyr-15 phosphatase. In addition, we observed 5- to 10-fold activation of cyclin E and cyclin A immunoprecipitates, whereas Gu *et al.* (38) observed 2-fold activation of CDK2 immunoprecipitates. This suggests either that Cdc25M2 is a more efficient activator of CDK2 immune complexes or that cyclin E and cyclin A complexes are more tightly regulated by Thr-14 and Tyr-15 phosphorylation than the CDK2 population as a whole.

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