Human Myt1 Is a Cell Cycle-regulated Kinase That Inhibits Cdc2 but Not Cdk2 Activity*

(Received for publication, April 3, 1997, and in revised form, June 3, 1997)

Robert N. Booher†, Patricia S. Holman, and Ali Fattaey

From Onyx Pharmaceuticals, Richmond, California 94806-5206

Activation of the Cdc2/cyclin B kinase is a pivotal step of mitotic initiation. This step is mediated principally by the dephosphorylation of residues threonine 14 (Thr14) and tyrosine 15 (Tyr15) on the Cdc2 catalytic subunit. In several organisms homologs of the Wee1 kinase have been shown to be the major activity responsible for phosphorylating the Tyr15 inhibitory site. A membrane-bound kinase capable of phosphorylating residue Thr14, the Myt1 kinase, has been identified in the frog *Xenopus laevis* and more recently in human. In this study, we have examined the substrate specificity and cell cycle regulation of the human Myt1 kinase. We find that human Myt1 phosphorylates and inactivates Cdc2-containing cyclin complexes but not complexes containing Cdk2 or Cdk4. Analysis of endogenous Myt1 demonstrates that it remains membrane-bound throughout the cell cycle, but its kinase activity decreased during M phase arrest, when Myt1 become hyperphosphorylated. Further, Cdc2/cyclin B1 was capable of phosphorylating Myt1 in *vitro*, but this phosphorylation did not affect Myt1 kinase activity. These findings suggest that human Myt1 is negatively regulated by an M phase-activated kinase and that Myt1 inhibits mitosis due to its specificity for Cdc2/cyclin complexes.

The cyclin-dependent kinases (Cdks)1 are a family of highly conserved serine/threonine kinases that mediate many of the cell cycle transitions that occur during duplication. Each of these Cdk catalytic subunits associates with a specific subset of regulatory subunits, termed cyclins, to produce a distinct Cdk/cyclin kinase complex that, in general, functions to execute a specific cell cycle event (1). The placement of a specific Cdk/cyclin complex function with a particular cell cycle transition has led to the identification of key downstream substrates as well as upstream regulatory mechanisms. Well characterized Cdk/cyclin-regulated cell cycle transitions that have been identified thus far include Cdk4/cyclin D and Cdk6/cyclin D complexes with the pRB-gated G1/S transition, Cdk2/cyclin E and Cdk2/cyclin A complexes with the initiation and progression of DNA replication and Cdc2/cyclin A and Cdc2/cyclin B complexes with the initiation of mitosis (reviewed in Refs. 2–4).

Activation of the Cdk2/cyclin kinases during these transitions is controlled by a variety of regulatory mechanisms. For the Cdc2/cyclin B complex, inhibition of kinase activity during S phase and G2 is accomplished by phosphorylation of Cdc2 residues Thr14 and Tyr15, which are positioned within the ATP-binding cleft (5–7). Phosphorylation of Thr14 and/or Tyr15 is believed to suppress catalytic activity by disrupting the orientation of the ATP molecule present within this cleft (8, 9). The abrupt dephosphorylation of these residues by the Cdc25 phosphatase results in the rapid activation of Cdc2/cyclin B kinase activity and downstream mitotic events (10). While phosphorylation/dephosphorylation of the conserved Tyr15 site in Cdk2 likely plays an important role in regulating the G1/S transition, the role that Cdk2 Thr14 phosphorylation plays is less clear (11, 12). Phosphorylation of the corresponding inhibitory tyrosine residue in Cdk4 has also been observed (13, 14). It has been proposed that Thr14/Tyr15 phosphorylation functions to permit a cell to attain a critical concentration of inactive Cdk/cyclin complexes, which, upon activation, induces a rapid and complete cell cycle transition (15). There is evidence in mammalian cells that Thr14/Tyr15 phosphorylation also functions, in part, to delay Cdk activation after DNA damage (7, 13, 16–18).

The *Schizosaccharomyces pombe* wee1 gene product was the first kinase identified that is capable of phosphorylating Tyr15 in Cdc2 (19). Homologs of the Wee1 kinase have been subsequently identified and biochemically characterized from a wide range of species including human, mouse, frog, *Saccharomyces cerevisiae*, and *Drosophila* (20–26). In vertebrate systems, where Thr14 in Cdc2 is also phosphorylated, the Wee1 kinase was capable of phosphorylating Cdc2 on Tyr15, but not Thr14, indicating that another kinase was responsible for Thr14 phosphorylation (21, 27). Direct evidence for the existence of a kinase activity that phosphorylated Cdc2 on Thr14 in the membrane fractions of *Xenopus* egg extracts has been reported (28). The *Xenopus* gene encoding this membrane-associated kinase, the Myt1 kinase, has been isolated, and its gene product was shown to be capable of phosphorylating Thr14 and, to a lesser extent, Tyr15 in Cdc2 (29). A human Myt1 homolog displaying similar properties has been recently identified (30) (see “Results”). An apparently unrelated, non-membrane-associated Thr14 kinase activity has also been identified in bovine thymus cytosol (31).

While *Xenopus* Myt1 clearly plays an important role in regulating the rapid early embryonic cell cycles, its role in cells that have distinct G1 and G2 phases as well as growth and checkpoint controls is not known. To investigate the role of Cdk Thr14 phosphorylation in somatic cell cycle control, we have initiated a biochemical analysis of the human Myt1 kinase. The results presented reveal that Myt1, unlike Wee1, exhibits a restricted substrate specificity; it was capable of phosphorylating Thr14 only on Cdc2/cyclin and not Cdk2/cyclin complexes. We also found that endogenous Myt1 kinase activity was reduced during a drug-induced M-phase arrest, concomitant with hyperphosphorylation of the Myt1 protein, implying that Myt1 may be negatively regulated by phosphorylation.

---

1 The abbreviations used are: Cdk, cyclin-dependent kinase; PAGE, polyacrylamide gel electrophoresis; DTT, dithiothreitol.

† To whom correspondence should be addressed: Onyx Pharmaceuticals, 3031 Research Dr., Richmond, CA 94806-5206; Tel.: 510-222-9700; Fax: 510-222-9758.

‡ This work was supported by Parke-Davis Pharmaceutical Research, Ann Arbor, MI. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
EXPERIMENTAL PROCEDURES

Human Myt1 Cloning—A 325-bp pair DNA fragment, corresponding to an internal region of EST clones 332541 and 336264, was polymerase chain reaction-regenerated using oligos 5'-AGCAGCTCTCCGACAATGCT-3' and 5'-CAGGACAAGATCATGCGG3' (5' and 3' primers, respectively) and the first strand cDNA synthesis of fetal brain RNA (Clonetech) as a template. This DNA fragment was 32P-labeled by primer extension. The resulting radiolabeled Myt1-containing DNA fragments were cloned into a derivative of the mammalian expression vector pcDNA3 (Invitrogen) that contained a 5'-HA epitope-coding sequence such that the expressed Myt1 proteins encoded by these clones contained a C-terminal HA tag and have been used in this study contained a C-terminal HA tag and have been used in this study.

Small Scale Baculovirus/Sf9 Lysate Preparation and Histone H1 Kinase Assays—Polymerase chain reaction-generated using the full-length Wee1 clone 1E-12 as a template, the complete DNA sequence of this 1.98-kilobase pair insert was determined using an ABI sequencer. The 1.98-kilobase pair insert was excised by cleaving with HindIII and NdeI into two parts, one that encoded p49Wee1 (0.78 mg/ml) or Myt1 (2.2 mg/ml) in 4.5 ml of KAB containing 5 mM ATP, 10 mM Hepes, pH 7.5, 10 mM MgCl2, 1 mM DTT, and 1 mg/ml of protein A-Sepharose (1:1 slurry, Pierce). After 1 h at 4 °C, the beads were collected, washed three times with EB, and lysed by Dounce homogenization in EB containing 10 mM MgCl2, 1.5 mM MgCl2, protease inhibitors). This lysate was ultracentrifuged for 30 min at 40,000 rpm in a TLA45 rotor at 4 °C to obtain the S100 fraction. The S100 fraction was mixed with the P100 fraction to prepare the S100/P100 fraction, which was used in the kinase assays. The S100 fraction contained the longest cDNA insert. The complete DNA sequence of this insert was determined using an ABI sequencer.

Histone H1 Immunoprecipitation and In Vitro Kinase Assays—Histone H1 lysates were prepared from Myt1-expressing cell lines that had been lysed by Dounce homogenization in EB containing 5 mM KCl, 1.5 mM MgCl2, protease inhibitors and incubated by microcentrifugation at 8200 × g for 15 min. Myt1 was immunoprecipitated by incubating 2 μl of anti-Myt1 antibody serum in 0.2–0.4 ml of lysate containing 1 mg of sample buffer for 30 min at 4 °C, followed by addition of 25 μl of protein A-Sepharose (1:1 slurry, Pierce). After 1 h at 4 °C, the beads were collected, washed three times with EB, and lysed in EB. To assay kinase activity, 10 μl of Cdc2/cyclin B1 kinase reaction (1.5 μl of Cdc2/cyclin B1 Sf9 lysates mix, see above, in 8.5 μl of KAB containing 7.5 μM ATP) was added to the beads and incubated for 45 min at room temperature with periodic mixing. Additionally, 10 μl of the corresponding peptide was included during kinase assays of C-terminal antibody immunoprecipitates. This reaction was then assayed for histone H1 kinase activity by adding 5 μl of a histone H1 kinase mix (50 mM Tris, pH 7.5, 10 mM MgCl2, 1 mM DTT, 5 μg of histone H1, 1 μCi of [γ-32P]ATP (3000 Ci/mmol)) and incubating for 15 min at 30 °C with agitation. This reaction was stopped with SDS sample buffer, resolved by SDS-PAGE, and analyzed by autoradiography.

Immunoblotting—Clariﬁed mammalian cell lysates, immunoprecipitates, and S9 cell lysates were fractionated by SDS-PAGE (Novex) and transferred to Immobilon polyvinylidene difluoride membranes (Millipore) using a Mini Trans-Blot cell (Bio-Rad). The blots were blocked with 5% nonfat dry milk in TBST (0.15 mM NaCl, 0.1% Tween 20, and 10 mM Tris, pH 8.0), washed with TBST, and incubated with primary antibodies (1:1000) and secondary antibodies, horseradish peroxidase-conjugated goat anti-mouse or horseradish peroxidase-conjugated goat anti-rabbit (1:10,000 dilution, Bio-Rad) in blocking solutions. Immunodetection was performed using an enhanced chemiluminescence system (Amersham Corp).

Human Myt1 Analysis in Xenopus Egg Extracts—Analysis of Myt1 proteins in Xenopus interphase and mitotic egg extracts was performed essentially as described by Stukenberg et al. (35) except that the Myt1 proteins were produced from pcDNA-containing Myt1 plasmids in a coupled transcription/translation system (TNT, Promega) in the absence of radiolabel.

RESULTS

Isolation of the Human Myt1 Kinase—To identify a human homolog of the Xenopus Myt1 kinase, we performed a TBLASTN search of the EST data base using the amino acid sequence encoded by Xenopus Myt1 as the query (29). As expected, the majority of high scoring matches were protein kinases, owing to the extensive conservation of residues within the catalytic domain of protein kinases (36). However, two nearly identical human ESTs (dbEST Id: 326988 and 326999) were found to match signiﬁcantly amino acid tracts within the C-terminal (noncatalytic domain) 130 residues of the Xenopus Myt1 kinase. A full-length cDNA clone was isolated and found to encode the recently described human Myt1 kinase (30). Myt1 inactivates Cdc2 but Not Cdk2 Kinase Activity—To analyze human Myt1 activity, we examined its ability to inactive Cdc2/cyclin complexes in a cell-free extract system. We used the baculovirus expression system to produce insect cell

2 R. N. Boorer, P. S. Holman, and A. Fattaey, unpublished data.
extracts that individually contained high levels of either human Cdk, cyclin, Wee1, or Myt1 proteins. It has been shown previously that Cdk-cyclin kinase activation can be reconstituted upon mixing individual Cdk and cyclin-containing lysates (33); thus, addition of an inhibitory activity would be expected to block this activation. We first assessed the ability of this assay system to detect Wee1 activity. Lysates containing wild-type Cdc2 were mixed with cyclin B1 lysates and with either mock-infected or Wee1-containing lysates. After a brief incubation, an aliquot of this lysate mixture was removed and Cdc2-cyclin B1 kinase activity was determined by performing a histone H1 kinase reaction. As shown in Fig. 1A, addition of Wee1 lysates effectively inhibited Cdc2 phosphorylation and decreased kinase activity (29). To determine whether human Myt1 can inactivate Cdk2 requires cyclin association, we performed a Myt1 kinase reaction using monomeric Cdc2 as substrate. As shown in Fig. 2A, Myt1 did not phosphorylate monomeric Cdc2. However, reconstitution of the Cdc2-cyclin B1 complex, by preincubating monomeric Cdc2 with monomeric cyclin B1, enabled Myt1 to phosphorylate Cdc2, indicating that Myt1 can only phosphorylate Cdc2 that is complexed with a cyclin subunit. The truncated Wee1 kinase was capable of $^{32}$P-labeling monomeric Cdc2, but not Cdc2 complexed with cyclin A or E, as well as Cdk4 complexed with cyclin D1 (Fig. 2B). In contrast, Wee1 readily phosphorylated Cdk2 complexed with cyclins A or E, but not Cdk4 complexed with cyclin D1, as has been previously demonstrated (22). Thus, using either a crude lysate system or an in vitro kinase reaction with purified components, we consistently observed that Myt1 only phosphorylated and inactivated Cdk2/cyclin complexes in which Cdc2 was the catalytic subunit.

To assess whether Myt1 phosphorylation of Cdc2 requires cyclin association, we performed a Myt1 kinase reaction using monomeric Cdc2 as substrate. As shown in Fig. 2C, Myt1 did not phosphorylate monomeric Cdc2. However, reconstitution of the Cdc2-cyclin B1 complex, by preincubating monomeric Cdc2 with monomeric cyclin B1, enabled Myt1 to phosphorylate Cdc2, indicating that Myt1 can only phosphorylate Cdc2 that is complexed with a cyclin subunit. The truncated Wee1 kinase was capable of $^{32}$P-labeling monomeric Cdc2, but this phosphorylation was enhanced when the cyclin-bound form was reconstituted, which is consistent with previous studies (37).

**Human Myt1 Is Hyperphosphorylated during Mitosis**—To examine the regulation of endogenous Myt1, rabbit polyclonal antibodies were raised against three different peptides corresponding to Myt1 residues 10–23, 472–487, and 486–499. These antibodies are designated N2-1, C1-1, and C2-1, respectively. When used for immunoblotting of total cell lysates, cytosolic fractions, and detergent-solubilized membrane fractions prepared from asynchronously growing C-33A human cells, each of the anti-Myt1 antibodies recognized a 66-kDa membrane-associated protein (Fig. 3). This 66-kDa protein comigrated with baculovirus-expressed human Myt1 as well as Myt1 that was produced in an *in vitro* transcription/translation-coupled reaction (data not shown).

In *Xenopus* mitotic extracts, the electrophoretic mobility of Xe-Myt1 is drastically decreased, concomitant with Myt1 hyperphosphorylation and decreased kinase activity (29). To determine whether human Myt1 exhibits similar cell cycle regulation, we examined Myt1 in lysates prepared from unsynchronized human CEM cells as well as cells that had
been arrested at G1, S, and M phases of the cell cycle by treatment with mimosine, hydroxyurea, and nocodazole, respectively. Immunoblot analysis using anti-Myt1 C-terminal peptide antibodies (C1-1 and C2-1) showed that Myt1 shifted to a slower migrating form in mitotic lysates (Fig. 4A). The main contribution to this modification is phosphorylation, since phosphatase treatment of the mitotic lysate increased the migration of Myt1 to nearly that observed in G1- and S-phase extracts (Fig. 4B). At least five ladder Myt1 forms were detectable in the phosphatase-treated mitotic lysate, indicating that multiple residues are phosphorylated during mitosis. Further, immunoblot analysis of cytosolic and detergent-extracted membrane fractions prepared from nocodazole-arrested CEM cells revealed that human Myt1 remained membrane-associated during M-phase arrest (Fig. 4C).

Interestingly, anti-Myt1 antibody N2-1 failed to recognize the slower migrating Myt1 protein in mitotic lysates but was capable of detecting Myt1 in lysates prepared from unsynchronized as well as mimosine, or hydroxyurea-arrested CEM cells (Fig. 4A). Phosphatase treatment of the mitotic lysate alleviated this Myt1 masking effect, suggesting that this antibody recognized only unphosphorylated Myt1 (data not shown). Consistent with the immunoblot results, this N-terminal antibody was incapable of immunoprecipitating mitotic Myt1 protein (Fig. 5A).

Since Myt1 likely spans the endoplasmic reticulum membrane (30), it is possible that membrane association is required for Myt1 hyperphosphorylation observed during mitosis. To investigate this question, we established a U2-OS cell line that stably expressed a non-membrane-associated Myt1 mutant, Myt1ΔT.M., which lacks the 19-residue transmembrane domain (see “Experimental Procedures”). This Myt1ΔT.M.-expressing cell line was arrested in mitosis with nocodazole, and the soluble (S100) and detergent-solubilized particulate (P100) fractions were prepared. Immunoblot analysis of these fractions revealed that the S100-containing Myt1ΔT.M. protein exhibited a reduced mobility shift similar to that seen for the endogenous Myt1 protein (Fig. 4D) present in mitotic extracts. This result indicates that a kinase exists in the cytosol that is capable of phosphorylating Myt1 during mitosis, independent of Myt1 association with endoplasmic reticulum membranes. We cannot, however, rule out the possibility that this unidentified kinase is also present in the endoplasmic reticulum lumen.

**Human Myt1 Kinase Activity Is Reduced during Mitosis**—To characterize the kinase activity of endogenous human Myt1, anti-Myt1 antibody immunoprecipitates from CEM cell extracts were tested for their ability to inactivate various Cdk-cyclin complexes. Fig. 5A shows that the C-terminal anti-Myt1 antibodies were capable of immunoprecipitating Myt1 from lysates prepared from nonsynchronized and mitotic cells, whereas, as described above, the N-terminal antibody failed to recognize mitotic Myt1. Consistent with our analysis of recombinant Myt1, anti-Myt1 immunoprecipitates dramatically reduced the histone H1 kinase activity of Cdc2-cyclin B1 and

**Cell Cycle Regulation of the Human Myt1 Kinase**

**FIG. 2. In vitro phosphorylation of Cdk-cyclin complexes by Myt1.** A, purified Myt1 or Wee1 (p49Wee1) kinases were incubated either alone (lanes 10 and 11) or in combination with purified Cdc2-cyclin B1 (lanes 1–3), Cdc2-ACF-cyclin B1 (lanes 4–6), or Cdc2-K-cyclin B1 (lanes 7–9) complexes in an in vitro kinase reaction containing γ-32P-ATP. The reaction was subjected to SDS-PAGE and analyzed by autoradiography. The basis of the low level of 32P-labeled Cdc2 in the untreated wild-type Cdc2-cyclin B1 kinase reaction (lane 1) is unknown, but a similar observation has been reported by Watanabe et al. (22). B, purified Cdk2-cyclin A (lanes 1–3), Cdk2-cyclin E (lanes 4–6), and Cdk4-cyclin D1 (lanes 7 and 8) complexes were incubated either alone or in combination with purified Myt1 or Wee1Δ (p49Wee1) in an in vitro kinase reaction and analyzed as in panel A. C, cyclin-dependent phosphorylation of Cdc2 by Myt1. Affinity-purified Cdc2-cyclin B1 complexes (lanes 1–3), monomeric Cdc2 (lanes 4–6), monomeric Cdc2 plus monomeric cyclin B1 (lanes 7–9), or monomeric cyclin B1 (lanes 10 and 11) proteins were incubated either alone or in combination with purified Myt1 or Wee1Δ (p49Wee1) in an in vitro kinase reaction and analyzed as in panel A. The faint band in lane 11 may represent an endogenous insect Cdk that co-purified with the human cyclin B1 protein.

**FIG. 3. Immunoblot detection of Myt1 in human cell lysates.** Asynchronously growing C-33A cells were collected, and the whole cell lysate (WCE, 10 μg), cytosolic fraction (S100, 30 μg), and detergent-extracted membrane fraction (P100, 20 μg) were prepared, resolved on a 4–20% SDS-PAGE, and immunoblotted with the indicated anti-Myt1 antibody. The 66-kDa Myt1 protein is indicated.
antibodies C1-1, N2-1, and C2-1, as indicated. Resolved on a 4–20% SDS-PAGE and immunoblotted with anti-Myt1 zole- and mimosine-treated cells from panel A were incubated either with or without lambda phosphatase. Equal amounts (20 μg) of nocodazole- and mimosine-treated cells from panel A were incubated either with or without lambda phosphatase. Equal amounts (20 μg) of nocodazole (lanes 1), mimosine (lanes 4), and lambda phosphatase-treated nocodazole (lanes 2 and 3) lysates were subjected to gel electrophoresis and immunoblotted with anti-Myt1 C1-1 antibody. C, the cytosolic S100 (lanes 1 and 2) and detergent-solubilized P100 fractions (lanes 3 and 4) from asynchronously growing (lanes 1 and 3) and nocodazole-arrested (lanes 2 and 4) CEM cells were prepared, subjected to SDS-PAGE, and immunoblotted with anti-Myt1 antibody, C1-1, D, hyperphosphorylation of the cytosolic Myt1-ATM protein during mitosis. The S100 and detergent-extracted P100 fractions were prepared from asynchronously or nocodazole-arrested C2-OS cell lines that stably expressed HA-tagged Myt1-1b or Myt1-2TM proteins. These fractions were subjected to SDS-PAGE and blotted with anti-HA monoclonal antibody (top) and anti-Myt1 C2-1 antibody (bottom).

Cdc2-F15-cyclin B1 complexes, partially inhibited Cdc2-A14-cyclin B1 complexes, and had no effect on the Cdc2-AF-cyclin B1 complex (Fig. 5B). Likewise, anti-Myt1 immunoprecipitates failed to immunoprecipitate Cdk2/cyclin A and Cdk2/cyclin E complexes (Fig. 5C). These results demonstrate that the endogenous Myt1 kinase phosphorylates preferentially Cdc2 on residue Thr160 and does not phosphorylate Cdk2.

To determine if the kinase activity of human Myt1 was cell cycle-dependent, we compared the kinase activity of Myt1 immunoprecipitates isolated from nonsynchronized and nocodazole-arrested CEM cells. Using two different C-terminal Myt1 peptide antibodies, Myt1 immunoprecipitated from mitotic extracts had approximately 40–50% reduced kinase activity (Fig. 6A). Analysis of Myt1 immunoprecipitates using an N-terminal Myt1 peptide antibody showed that Myt1 was active in asynchronous, as well as mimosine- and hydroxyurea-arrested cells (Fig. 6C). As discussed above, this N-terminal peptide antibody failed to immunoprecipitate Myt1 from nocodazole-arrested cells. These results strongly suggest that Myt1 is active during G1, S, and G2 but becomes inactive during M phase, concomitant with Myt1 hyperphosphorylation.

**Phosphorylation of Human Myt1 in Xenopus Extracts and by Cdc2/cyclin B1**—The similarity of cell cycle-dependent kinase activity and phosphorylation state for both human and Xenopus Myt1 suggested that Myt1 kinases may be regulated by a conserved mechanism. As an initial test of this possibility we compared the electrophoretic mobility of human Myt1 that had been incubated in Xenopus interphase and mitotic egg extracts. As shown in Fig. 7A, Xenopus mitotic extracts contained an activity that drastically reduced the electrophoretic mobility of each of the four human Myt1 proteins tested. The reduced mobility observed for Myt1 truncation mutants lacking the N-terminal 57 residues (-1A, lane 4) and C-terminal 206 residues2 indicates that phosphorylated residues responsible for the Myt1 mobility shift are not contained exclusively within either of these regions.

Since the Xenopus mitotic extracts were derived from interphase egg extracts that had been induced into an M-phase state...
noprecipitated Myt1 and purified Cdc2 showed that the Myt1-K human Myt1 kinase. We found that human Myt1 was capable of phosphorylating and inactivating Cdc2 associated with cyclin A or cyclin B1. In contrast, however, Myt1 was unable to phosphorylate or inactivate Cdc4-cyclin complexes that function earlier in the cell cycle. In particular, Myt1 did not phosphorylate Cdk4-cyclin D1, Cdk2-cyclin E, or Cdk2-cyclin A complexes. The inability of Myt1 to phosphorylate Cdk4 was not unexpected since Cdk4 contains an alanine residue at the corresponding Thr^14 position. Since Myt1 readily phosphorylated Cdc2-cyclin A complexes, Myt1 specificity appears to be determined principally by the Cdk subunit. In vitro, Cdk2 Tyr^15 is phosphorylated to much greater extent than Thr^14 (11, 12), consistent with our in vitro labeling of Myt1: Cdc2-AF cyclin B1. The indicated Myt1 proteins were immunoprecipitated from Sf9 lysates and incubated with purified Cdc2-AF cyclin B1 in an in vitro kinase reaction containing [γ-^32P]ATP.

FIG. 6. Human Myt1 kinase activity during the cell cycle. A, lysates from nonsynchronized and nocodazole-arrested CEM cells were prepared and subjected to immunoprecipitation with anti-Myt1 antibodies C1-1 and C2-1. The washed anti-Myt1 immunoprecipitates were assayed for Myt1 kinase activity by incubating with Cdc2-cyclin B1 complexes and then measuring histone H1 kinase activity. These results represent the mean of duplicate kinase assays. B, a portion of the anti-Myt1 immunoprecipitates from panel A was subjected to immunoblotting using a mouse anti-Myt1 serum as probe. C, kinase assays of Myt1 immunoprecipitated from lysates prepared from nonsynchronized and mimosine-, hydroxyurea-, and nocodazole-arrested CEM cells. Myt1 was immunoprecipitated using the N-terminal anti-Myt1 antibody, N2-1, and assayed for kinase activity as described Fig. 5B. These results represent the mean of duplicate kinase assays. D, a portion of the anti-Myt1 immunoprecipitates from panel C was subjected to immunoblotting using a mouse anti-Myt1 polyclonal serum as probe.

by addition of exogenous cyclin B1, it is possible that Cdc2-cyclin B1 may phosphorylate Myt1 directly, perhaps acting as an autocatalytic feedback mechanism such as has been proposed for Xenopus Cdc25 and Wee1 (24, 38). To address this possibility, we first determined whether Cdc2-cyclin B1 could affect the electrophoretic mobility of a kinase-defective Myt1 (Myt1-K^-). Sf9 lysates containing Cdc2-cyclin B1 were incubated together with a Myt1-K^-lysat in the presence of an ATP regeneration system. Immunoblot analysis of this reaction showed that the Myt1-K^-protein migrated with a reduced mobility after Cdc2-cyclin B1 treatment (Fig. 7B), albeit to a lesser degree than observed for Myt1 in mitotic extracts derived from human CEM cells (data not shown). Interestingly, Fig. 7B also shows that the Myt1 N2-1 antibody exhibited a reduced ability to recognize Myt1 that had been phosphorylated by the Cdc2-cyclin B1 kinase. The direct phosphorylation of Myt1 by Cdc2-cyclin B1 was confirmed by including [γ-^32P]ATP in an in vitro kinase reaction that contained immunoprecipitated Myt1 and purified Cdc2-cyclin B1 (Fig. 7C). Phosphoamino acid analysis showed that Cdc2-cyclin B1 phosphorylated Myt1 on both threonine and serine residues (data not shown).

Using an Sf9 lysate system, we examined whether the activity of Myt1 was affected by Cdc2-cyclin B1 phosphorylation. A Myt1-containing lysate was first mixed with a Cdc2-2AF-cyclin B1 lysate, followed by subsequent addition of wild-type Cdc2-cyclin B1. The latter Cdc2-cyclin B1 complex was isolated by immunoprecipitation and its kinase activity was determined by performing a histone H1 kinase assay. We observed that phosphorylation of Myt1 by Cdc2-2AF-cyclin B1 had no effect on its ability to inactivate Cdc2 kinase activity (data not shown). Hence, phosphorylation by Cdc2 does not appear to directly block Myt1 catalytic activity.

DISCUSSION

In this study we report several biochemical properties of the human Myt1 kinase. We found that human Myt1 was capable of phosphorylating and inactivating Cdc2 associated with cyclin A or cyclin B1. In contrast, however, Myt1 was unable to phosphorylate or inactivate Cdc4-cyclin complexes that function earlier in the cell cycle. In particular, Myt1 did not phosphorylate Cdk4-cyclin D1, Cdk2-cyclin E, or Cdk2-cyclin A complexes. The inability of Myt1 to phosphorylate Cdk4 was not unexpected since Cdk4 contains an alanine residue at the corresponding Thr^14 position. Since Myt1 readily phosphorylated Cdc2-cyclin A complexes, Myt1 specificity appears to be determined principally by the Cdk subunit. In vivo, Cdk2 Tyr^15 is phosphorylated to much greater extent than Thr^14 (11, 12), consistent with our in vitro results that Myt1 does not phosphorylate Cdk2 on Thr^14. It is possible, however, that Myt1 can phosphorylate Cdk2 that has been initially phosphorylated on Tyr^15, as has been previously suggested (11). Alternatively, additional kinases may be responsible for phosphorylating Thr^14 in Cdk2, such as a non-membrane-associated Thr^14 kinase activity that is present in bovine thymus cytosol (31). In either case, the significantly greater extent of Tyr^15 phosphorylation on endogenous Cdk2 suggests that Wee1, or another Wee1-like kinase, is the major kinase activity that suppresses Cdk2-cyclin kinase activity during the G_2/M transition.

The kinase activity of Xenopus Myt1 was decreased during M-phase arrest. This decrease activity correlated with hyperphosphorylated and electrophoretically slower forms of Myt1. Indeed, phosphorylation of the high number of TP and SP amino acid doublets in both human and Xenopus Myt1, 10 and 12, respectively, could account for the dramatic Myt1 hyperphosphorylation observed during M phase. Many of these TP/SP doublets conform to the Cdc2-cyclin B1 phosphorylation
consensus site (39), consistent with our observation that Cdc2-cyclin B1 readily phosphorylates Myt1 in vitro. Our finding that an anti-Myt1 N-terminal peptide antibody recognized interphase but not M-phase Myt1, or Myt1 phosphorylated by Cdc2-cyclin B1 in vitro, strongly suggests that a residue near the N terminus is phosphorylated during mitosis.

Which kinase(s) phosphorylates Myt1 during mitosis? Our in vitro data demonstrate that Cdc2-cyclin B1 can phosphorylate Myt1 on sites that reduce its mobility on SDS-polyacrylamide gels. While this phosphorylation did not affect Myt1 kinase activity, we cannot rule out the possibility that, in vivo, Cdc2 phosphorylation of Myt1 may be a requisite for further modifications that directly block Myt1 catalytic activity. Additionally, the partial reduction in Myt1 gel mobility induced by Cdc2-cyclin B1 phosphorylation suggests that an additional kinase(s) may phosphorylate Myt1. Further experiments will be required to discern the mechanism of Myt1 regulation during the cell cycle.

Acknowledgments—We thank Brian Karlak and Eric Vermaas for assistance in identifying the initial human Myt1 clones. We are also grateful to the following: Dr. Janice Williams for generously providing purified Cdk2-cyclin A and Cdk2-cyclin E complexes; Dr. David Morgan (University of California, San Francisco) for providing numerous Cdc2 and Cdk2 clones and recombinant baculoviruses; Dr. Emma Lees (DNAx) for providing cyclin clones; Dr. Todd Stonkenberg (HIAM) for providing Xenopus extracts and cyclins; Drs. Michelle Garrett, Nancy Pryer, and Abdallah Fanidi for helpful discussions; David Lowe and Drs. Jim Litts and Barbara Belisle for expressing and purifying the recombinant baculovirus proteins used throughout this study.

REFERENCES
1. Nigg, E. A. (1995) Bioessays 17, 471–480
2. Nasmyth, K. (1996) Science 274, 1643–1645
3. Stillman, B. (1996) Science 274, 1659–1664
4. Sherr, C. J. (1996) Science 274, 1672–1677
5. Gould, K. L., and Nurse, P. (1989) Nature 342, 39–45
6. Krek, W., and Nigg, E. A. (1991) EMBO J. 10, 3331–3341
7. Jin, P., Gu, Y., and Morgan, D. O. (1996) J. Cell Biol. 134, 963–970
8. Atherton-Fessler, S., Parker, L. L., Geahlen, R. L., and Piwnica-Worms, H. (1993) Mol. Cell. Biol. 13, 1675–1685
9. De Bondt, H. L., Rosenblatt, J., Jancarik, J., Jones, H. D., Morgan, D. O., and Kim, S.-H. (1993) Nature 363, 595–902
10. Dunphy, W. G. (1994) Trends Cell Biol. 4, 202–207
11. Gu, Y., Turek, C. W., and Morgan, D. O. (1995) Nature 366, 707–710
12. Sebastian, B., Kakizuka, A., and Hunter, T. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 3521–3524
13. Terada, Y., Tateoka, M., Jinno, S., and Okayama, H. (1995) Nature 376, 358–362
14. Iavarone, A., and Massague, J. (1997) Nature 387, 418–421
15. Solomon, M. J., Gleiter, M., Lee, T. H., Philippe, M., and Kirschner, M. W. (1990) Cell 63, 1013–1024
16. Lock, R. B., and Ross, W. E. (1990) Cancer Res. 50, 3761–3766
17. Poon, R. Y. C., Jiang, W., Toyoshima, H., and Hunter, T. (1996) J. Biol. Chem. 271, 13263–13269
18. Wang, Q., Fan, S. F., Eastman, A., Wolarand, P. J., Sausville, E. A., and O’Connor, P. M. (1996) J. Natl. Cancer Inst. 88, 956–965
19. Parker, L. L., Atherton-Fessler, S., Lee, M. S., Oggi, S., Fulk, J. L., Swenson, K. I., and Piwnica-Worms, H. (1991) EMBO J. 10, 1255–1263
20. Igashiri, M., Nagata, A., Jinno, S., Suto, K., and Okayama, H. (1991) Nature 353, 80–83
21. Parker, L. L., and Piwnica-Worms, H. (1992) Science 257, 1955–1957
22. Watanabe, N., Broomo, M., and Hunter, T. (1995) EMBO J. 14, 1878–1891
23. Honda, R., Tanaka, H., Ohba, Y., and Yasuda, H. (1995) Chromosome Res. 3, 300–308
24. Mueller, P. R., Coleman, T. R., and Dunphy, W. G. (1995) Mol. Biol. Cell 6, 119–134
25. Booser, R. N., Deshaies, R. J., and Kirschner, M. W. (1993) EMBO J. 12, 3417–3426
26. Campbell, S. D., Sprenger, F., Edgar, B. A., and O’Farrell, P. H. (1995) Mol. Biol. Cell 6, 1333–1347
27. McGowan, C. H., and Russell, P. (1993) EMBO J. 12, 75–85
28. Kornbluth, S., Sebastian, B., Hunter, T., and Newport, J. (1994) Mol. Biol. Cell 5, 273–282
29. Mueller, P. R., Coleman, T. R., Kumagai, A., and Dunphy, W. G. (1995) Science 270, 86–90
30. Liu, F., Stanton, J. J., Wu, Z., and Piwnica-Worms, H. (1997) Mol. Cell. Biol. 17, 571–580
31. Matsuura, I., and Wang, J. H. (1996) J. Biol. Chem. 271, 5443–5450
32. Rubinfeld, B., Souza, B., Albert, I., Munemitsu, S., and Polakis, P. (1995) J. Biol. Chem. 270, 5549–5555
33. Dein, D., Gu, Y., and Morgan, D. O. (1992) Mol. Biol. Cell 3, 571–582
34. Fisher, R. P., and Morgan, D. O. (1994) Cell 78, 713–24
35. Stensson, T. P., Lustig, K. D., McGarry, T. J., King, R. W., Kuang, J., and Kirschner, M. W. (1997) Cell 8, 338–348
36. Hanski, S. K., Quinn, A. M., and Hunter, T. (1988) Science 241, 42–52
37. Parker, L. L., Sylvestre, P. J., Byrnes, M. J., Liu, F., and Piwnica-Worms, H. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 9638–9642
38. Iavarone, A., and Massague, J. (1997) Nature 387, 418–421
39. Songyang, Z., Blechner, S., Haagland, N., Hockstra, M. F., Piwnica-Worms, H., and Cantley, L. C. (1994) Curr. Biol. 4, 973–982