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The Human Myt1 Kinase Preferentially Phosphorylates Cdc2 on Threonine 14 and Localizes to the Endoplasmic Reticulum and Golgi Complex

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Entry into mitosis requires the activity of the Cdc2 kinase. Cdc2 associates with the B-type cyclins, and the Cdc2-cyclin B heterodimer is in turn regulated by phosphorylation. Phosphorylation of threonine 161 is required for the Cdc2-cyclin B complex to be catalytically active, whereas phosphorylation of threonine 14 and tyrosine 15 is inhibitory. Human kinases that catalyze the phosphorylation of threonine 161 and tyrosine 15 have been identified. Here we report the isolation of a novel human cDNA encoding a dual-specificity protein kinase (designated Myt1Hu) that preferentially phosphorylates Cdc2 on threonine 14 in a cyclin-dependent manner. Myt1Hu is 46% identical to Myt1Xe, a kinase recently characterized from *Xenopus laevis*. Myt1Hu localizes to the endoplasmic reticulum and Golgi complex in HeLa cells. A stretch of hydrophobic and uncharged amino acids located outside the catalytic domain of Myt1Hu is the likely membrane-targeting domain, as its deletion results in the localization of Myt1Hu primarily to the nucleus.

The replication cycle of a typical eukaryotic somatic cell consists of four phases: G_1, S (DNA synthesis), G_2, and M (mitosis) (for a review, see reference 40). The result of this process is the generation of two daughter cells that are equivalent both in genetic makeup and in size to the original parental cell. Feedback controls operating at checkpoints ensure the faithful replication and segregation of the genetic material. In eukaryotic organisms, a general paradigm has emerged in which a family of cyclins and cyclin-dependent protein kinases (Cdks) regulate cell cycle progression (for a review, see reference 46). These mechanisms are at the level of reversible phosphorylation, binding to low-molecular-weight inhibitors, transcription, intracellular compartmentalization, and protein degradation (for a review, see reference 32, 52).

The transition from G_2 to M phase requires the activity of M-phase-promoting factor, which is composed of Cdc2, an evolutionarily conserved serine/threonine-specific protein kinase, and B-type cyclins (for reviews see references 38, 39, and 53). The activity of Cdc2 is regulated not only by its association with B-type cyclins but also by reversible phosphorylation (for a review, see reference 1). Throughout the early phases of the cell cycle, Cdc2 exists as an underphosphorylated monomer and is inactive as a protein kinase. During S phase, the B-type cyclins accumulate and bind to Cdc2 to form heterodimers. Cyclin B binding facilitates the phosphorylation of Cdc2 on three regulatory sites in higher eukaryotes: threonine 14, tyrosine 15, and threonine 161 (3, 10, 11, 25, 31, 33, 41, 54). Phosphorylation on Tyr 15 and Thr 14 maintains Cdc2 in an inactive state throughout the S and G_2 phases of the cell cycle, and Thr 161 phosphorylation is required for the kinase activity of the complex. Dephosphorylation of both Thr 14 and Tyr 15 by the Cdc25 phosphatase in late G_2 activates Cdc2 and is an obligate step for the onset of mitosis (12, 17, 27, 51, 55). Exit from mitosis requires the proteolytic degradation of the B-type cyclins, which is mediated by ubiquitination (18, 36).

In *Schizosaccharomyces pombe*, Cdc2 is not detectably phosphorylated on Thr 14 except under unusual circumstances (9). Tyr 15 phosphorylation is regulated by *wee1*$^+$ and *mik1*$^+$, which encode protein tyrosine kinases (13, 26, 41, 42). In higher eukaryotic organisms, Cdc2 is negatively regulated by phosphorylation of both Thr 14 and Tyr 15 (25, 37, 54). A human homolog of *wee1*$^+$ (*Wee1*Hu) has been isolated and shown to encode a tyrosine-specific protein kinase that phosphorylates Cdc2 exclusively on Tyr 15 (21, 22, 29, 30, 43, 44, 59). Although Wee1Hu is a nuclear protein (6, 20, 30), Cdc2-cyclin B complexes are phosphorylated on Thr 14 and Tyr 15 when they assemble in the cytoplasm throughout the S and G_2 phases of the cell cycle (47). These results suggest that higher eukaryotic organisms encode one or more protein kinases that are distinct from Wee1Hu and that regulate Tyr 15 and Thr 14 phosphorylation. This hypothesis is supported by studies that show at least two kinase activities are present in *Xenopus* interphase extracts that phosphorylate Cdc2 on Thr 14 and Tyr 15 (2, 24). These activities can be separated by ultracentrifugation into a soluble and insoluble fraction. The soluble fraction contains an activity that phosphorylates Cdc2 exclusively on Tyr 15, whereas the pelleted fraction contains activities that phosphorylate Cdc2 on both Thr 14 and Tyr 15. Interestingly both kinase activities present in the pelleted fraction are solubilized in detergent and copurify through several successive column fractionation steps, suggesting the existence of a dual-specificity protein kinase (2). Recently a *Xenopus* oocyte cDNA whose protein product (designated Myt1Xe) has the properties described above has been reported (35). Myt1Xe is a membrane-bound, dual-specificity protein kinase that phosphorylates Cdc2 on both Thr 14 and Tyr 15. Here we report the isolation of a human cDNA which encodes a protein kinase that localizes to both the endoplasmic reticulum (ER) and Golgi complex and preferentially phosphorylates Cdc2 on Thr 14.

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MATERIALS AND METHODS

Cell culture. All procedures relating to *Spodoptera frugiperda* Sf9 insect cell culture, generation of recombinant baculoviruses, and propagation of recombinant baculoviruses were performed as described elsewhere (48). HeLa cells were cultured in Dulbecco's modified Eagle medium (Gibco-BRL) supplemented with 10% calf serum and 2% L-glutamine (Gibco-BRL); Jurkat cells were cultured in RPMI 1640 (Gibco-BRL) supplemented with 5% fetal calf serum and 2% L-glutamine. Peripheral blood lymphocytes (PBLs) were stimulated by phytohemagglutinin-P (1 μg/ml; Sigma) and cultured in 5% CO_2 and 37°C at 2×10^6 to 5×10^6 cells/ml in RPMI 1640 supplemented with 10% fetal calf serum, 2% L-glutamine, 1% penicillin-streptomycin, and 25% Jurkat cell conditioned medium. Jurkat cell conditioned medium was prepared by stimulating cells with phorbol 12-myristate 13-acetate (10^{-8} M; Sigma) and ionomycin (1 μM; Calbiochem) for 48 h, collecting conditioned medium, and sterilizing it through a 0.22-μm-pore-size filter.

Recombinant baculoviruses. Recombinant viruses encoding Cdc2, Cdc2 (K33R), Cdc2(A14F15), glutathione *S*-transferase (GST)–cyclin B1 have been described previously (2, 3, 41, 44). Viruses encoding Myt1Hu and His-Myt1Hu were generated by using the BAC-TO-BAC baculoviral expression system (Gibco-BRL) and vector pFASTBAC1 (see below). Recombinant baculoviruses encoding Cdc2(A14Y15) and Cdc2(T14F15) were made by subcloning a 1.2-kb *Bam*HI-*Eco*RI fragment encoding each mutant from pVL1393Cdc2(Ala14) and pVL1393Cdc2(Phe15) (2, 3) into the *Bam*HI and *Eco*RI sites of pFASTBAC1 (Gibco-BRL). Viruses were generated by using the BAC-TO-BAC baculovirus expression system (Gibco-BRL) and protocols suggested by the manufacturer.

Antibodies. Antibodies used in this study include Cdc2 (41), a monoclonal antibody to the Myc epitope (9E10; gifts of Larry Feig and Michael Lieber), and antiphosphotyrosine antibody (gift of Brian Druker), anti-Cdc2 (pY15; New England Biolabs), an anti-γ-adaptin monoclonal antibody (Sigma), an anti-PTP1B (tyrosine-specific protein phosphatase 1B) monoclonal antibody (gift of Ben Neel), an anti-14-3-3 antibody (K19; Santa Cruz), a fluorescein isothiocyanate (FITC)-conjugated donkey anti-mouse antibody (Jackson Immuno Research), and an indocarbocyanine (Cy3)-conjugated donkey anti-rabbit antibody or donkey anti-mouse antibody (Jackson Immuno Research). An antibody to Myt1Hu (R5084) was prepared by using a peptide antigen composed of the C-terminal 13 amino acids of Myt1Hu (NLLSLFEDTLDPT) coupled to a cysteine residue at the N terminus. The peptide was cross-linked to keyhole limpet hemocyanin and used for immunizing rabbits.

Affinity purification of a Myt1Hu C-terminal peptide antibody. Twelve milligrams of the synthetic Myt1Hu C-terminal peptide was coupled to 8 ml of SulfoLink coupling gel (Pierce) according to the protocol suggested by the manufacturer. Fifteen milliliters of polyclonal antiserum was diluted to 50 ml with phosphate-buffered saline (PBS) and circulated through the peptide affinity column overnight at 4°C. After antibody binding, the column was washed sequentially with 10 ml of PBS, 30 ml of radioimmunoprecipitation assay buffer (50 mM Tris [pH 8.0], 150 mM NaCl, 1% Nonidet P-40 [NP-40], 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate [SDS]), 30 ml of 10 mM Tris (pH 7.5) containing 500 mM NaCl, and finally 15 ml of 10 mM Tris (pH 7.5). Bound antibodies were first eluted with 100 mM glycine (pH 2.5), and the column was washed with 10 mM Tris (pH 8.8) prior to the second antibody elution with 100 mM triethylamine (pH 11.5). Eluted antibodies were neutralized with 1/10 volume of 1 M Tris (pH 8.0), adjusted to 0.1 mg of bovine serum albumin per ml, pooled, and precipitated by the addition of solid ammonium sulfate to 50% saturation. Precipitated antibodies were then resuspended in PBS–100 mM NaCl and dialyzed in two changes of the same buffer overnight at 4°C.

Northern analysis of Myt1Hu expression. Total RNA was isolated from HeLa S3 cells, Jurkat cells, and activated PBLs by using the TRIzol reagent (Gibco-BRL) as recommended by the manufacturer. Thirty micrograms of each RNA preparation was analyzed by electrophoresis on a 1% agarose–6% formaldehyde gel (50) and then transferred to a GeneScreen Plus hybridization transfer membrane (DuPont-NEN) as recommended by the manufacturer. The membrane was hybridized with a full-length Myt1Hu cDNA probe purified from pGEX2TNMyt1 (1.5-kb *Nde*I fragment). The cDNA probe was labeled with [α-^{32}P]dCTP by using the Megaprime DNA labeling system (Amersham). The blot was prehybridized at 42°C for 4 h in buffer containing 5× SSPE (1× SSPE is 0.15 M NaCl, 10 mM NaH_2PO_4, and 1 mM EDTA [pH 7.4]), 7× Denhardt's solution, 1% SDS, 50% formamide, and 100 μg of denatured salmon sperm DNA per ml (50). Labeled cDNA probe was then added to 3×10^6 cpm/ml, and the blot was hybridized for 16 h at 42°C. The blot was then washed twice in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate),–0.1% SDS at room temperature for 20 min and twice in 0.5× SSC–0.1% SDS at 60°C for 20 min. To probe for actin, the blot was stripped by incubation in 0.5% SDS at 95°C for 10 min and rehybridized with a labeled human β-actin probe as described above.

Cloning of an internal fragment of Myt1Hu. A fragment of the Myt1Hu cDNA was cloned by using a reverse transcriptase reaction followed by PCR in which degenerate oligonucleotides were used as primers. Primers 1 and 2 were designed based on regions of high amino acid conservation between members of the Wee1 family of protein kinases and were used to isolate *Xenopus* Wee1 (35). The final nested primer (primer 3) was again designed based on conserved residues within the Wee1 family of protein kinases including *Xenopus* Myt1. The nucleotide sequences of the primers are as follows: primer 1, 5′-CGCCATATG (C/T)(T/A)IGT(I/C)CA(C/T)(A/C/T)TIGA(C/T)(I/C)T(I/C)AA(A/G)CC-3′; primer 2, 5′-GGACATATGTGCCAI(T/G/C)II(A/T)(C/G)ICC(A/G)TT (I/C)(C/T)(G/T/C)(I/C)GG-3′; and primer 3, 5′-CCGCGGATCC(T/C)(T/G)IA A(A/G)(C/A/T)TIGGIGA(T/C)(T/C)T(I/C)GG-3′. The reverse transcriptase reaction was performed by using a first-strand cDNA synthesis kit (Clontech) and conditions recommended by the manufacturer; 1.5 μg of total RNA prepared from Jurkat cells by using an RNeasy total RNA kit (Qiagen) was used as the template. Primary PCR was then performed on an aliquot of the reverse transcriptase reaction, using 150 pmol of primers 1 and 2, 1× *Taq* DNA polymerase buffer (50 mM KCl, 10 mM Tris HCl [pH 9.0], 0.1% Triton X-100), and 0.2 mM deoxynucleoside triphosphates. The reaction mixture was heated to 85°C for 4 min before 1.25 U of *Taq* DNA polymerase (Promega) was added. The reaction was initiated with three cycles at low stringency (94°C, 30 s; 37°C, 30 s; ramp to 72°C for 2 min 30 s; 72°C, 90 s), followed by 30 cycles at higher stringency (94°C, 45 s; 50°C, 30 s; 72°C, 60 s) and a final extension phase of 7 min. A secondary PCR was then performed on an aliquot of the primary PCR product, using primers 2 and 3. The reaction conditions were identical to those used in the primary PCR except that the cycling conditions used were as follows: the reaction was heated to 94°C for 2.5 min and then cycled 35 times (94°C, 30 s; 52°C, 2 min; 72°C, 2 min) followed by an additional 7-min extension. By using a QIAquick gel extraction kit (Qiagen), a 210-bp DNA fragment was isolated, reamplified by using primers 2 and 3, and cloned into the pCR II cloning vector (Invitrogen).

To expedite the identification of clones containing a fragment of the Myt1Hu cDNA, 25 ng of each recombinant plasmid (25 selected) was blotted onto a charged nylon membrane (Du Pont-NEN), which was then probed with radiolabeled cDNA fragments derived from either Wee1Hu (*Xba*I-*Acc*I fragment) or Myt1Xe (*Bam*HI-*Dde*I fragment). The cDNA fragments were labeled with [α-^{32}P]dCTP by using the Megaprime DNA labeling system (Amersham). For the Wee1Hu hybridization, the membrane was prehybridized at 42°C for 2 h in 6× SSPE–0.5% SDS–5× Denhardt's solution–50% formamide–100 μg of denatured salmon sperm DNA per ml (50). Labeled probe was added to 10^6 cpm/ml, and the hybridization was performed under the conditions described above for 14 to 16 h. Following hybridization, the membrane was washed twice in 6× SSC–0.1% SDS for 15 min at room temperature and then twice in 0.1× SSC–0.1% SDS for 15 min at 65°C. Following the Wee1Hu hybridization, the membrane was stripped as described above for the Northern blot. For the Myt1Xe hybridization, the membrane was prehybridized at 55°C for 4 h in 6× SSPE–0.1% SDS–5× Denhardt's solution–100 μg of denatured salmon sperm DNA per ml (50). Again, the labeled probe was added to 10^6 cpm/ml and hybridized under the conditions described above for 14 to 16 h. The membranes were then washed twice for 10 min each in 6× SSC–0.1% SDS at room temperature and then twice for 10 min each in 2× SSC–0.1% SDS at room temperature. Recombinant plasmids that hybridized preferentially to the Myt1Xe probe were sequenced by using a Perkin-Elmer DNA sequencing kit and an Applied Biosystems automated DNA sequencer.

Isolation of a full-length Myt1Hu cDNA. The 210-bp PCR product described above was labeled using the Megaprime DNA labeling system (Amersham) and was used to screen a lambda ZAP II HeLa cDNA library (Stratagene) according to the manufacturer's instructions. A total of 8.6×10^5 phage were plated. After growing for 10 h at 37°C, phage were lifted onto Colony/Plaque Screen nylon membranes (Du Pont-NEN), incubated twice with 0.5 N NaOH, once with 1 M Tris (pH 7.5), and once with 0.5 M Tris (pH 7.5)–1.25 M NaCl, and UV irradiated to cross-link the DNA to the membrane. Prehybridization was performed at 42°C for 4 h in 2× piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (PIPES) buffer (20 mM PIPES [pH 6.5]), 0.8 M NaCl) containing 0.5% SDS, 50% formamide, and 100 μg of denatured salmon sperm DNA per ml (50). Labeled probe was added to 10^6 cpm/ml, and hybridization was performed under the conditions described above for 14 to 16 h. The hybridized membranes were then washed twice in 2× SSC–0.1% SDS for 10 min at room temperature and once in 0.1× SSC–0.1% SDS for 15 min at 65°C. Sixteen positive plaques were picked and subjected to two additional rounds of hybridization. pBluescript phagemids containing the cDNA inserts were excised *in vivo* from the parental lambda ZAP II vector. The sequence of a representative cDNA clone, clone 6-1, was determined by direct automated DNA sequencing as described above.

Generation of recombinant Myt1Hu constructs. Myt1Hu cDNA (6-1) was amplified by PCR using primers N (5′-CCGGATCCATATGCTAGAACGG CCTCC-3′) and C (5′-CCAGTATCATATGTTAACTCAGGTTGGGTCTAG GGTGTC-3′). To facilitate cloning, primer N was designed to contain *Bam*HI and *Nde*I sites and primer C was designed to contain *Hpa*I and *Nde*I sites. PCR was carried out with 400 ng of clone 6-1 DNA, 100 pmol of each primer, 0.2 mM deoxynucleoside triphosphates, and 1 U of Vent DNA polymerase (New England Biolabs) in Vent DNA polymerase buffer [10 mM KCl, 20 mM Tris HCl (pH 8.8), 10 mM $(NH_4)_2SO_4$, 2 mM $MgSO_4$, 0.1% Triton X-100] supplemented with 10% dimethyl sulfoxide. PCR was initiated with three cycles of 94°C for 1 min, 48°C for 1 min, and 72°C for 2 min, followed by 20 identical cycles with an annealing temperature of 60°C. The 1.5-kb PCR product was gel isolated, digested with *Nde*I, and cloned into the *Nde*I site of either pET15b (Novagen) to generate pETHisMyt1Hu or pGEX2TN to generate pGEX2TNMyt1Hu. pFASTBACHisMyt1Hu was constructed by subcloning an *Xba*I-*Xho*I fragment of pETHisMyt1Hu into the *Xba*I and *Xho*I sites of plasmid pFASTBAC1 (Gibco-BRL). pFASTBACMyt1Hu was created by subcloning a *Bst*EII-*Eco*RI fragment

of the human Myt1 cDNA (where the *Bst*EII end was blunt ended with Klenow polymerase) into *Bam*HI and *Eco*RI sites of pFASTBAC1 (where the *Bam*HI site was also blunt ended with Klenow polymerase). pGEX2TNMytΔC137 was constructed by subcloning a single *Nde*I fragment from pcDNAmycMytΔC137 (see below) into the *Nde*I site of pGEX2TN.

Purification of hexahistidine-tagged p49^{wee1}Hu from bacteria. The polylinker of pET15b was modified by inserting a 10-mer oligonucleotide (5′-TCGAGGT ACC) into the *Xho*I site, creating a *Kpn*I site [pET15b(ΔXho I to Kpn I)]. This changes the reading frame of the *Bam*HI site in the polylinker, making it compatible with the *Bam*HI site in the polylinker of pGEX2T. A 1.4-kb *Bam*HI fragment encoding p49^{wee1Hu} was excised from pGEX2Tp49Wee1Hu(ΔEco RI to Bam HI) (43) and inserted into the *Bam*HI site of pET15b(ΔXho I to Kpn I) to generate pET15b(ΔXho I to Kpn I)p49Wee1Hu. Four-tenths liter of *Escherichia coli* BL21(DE3) transformed with pET15b(ΔXho I to Kpn I)p49Wee1 was grown to an optical density at 600 nm of 0.6. Isopropyl-1-thio-β-D-galactopyranoside (IPTG) was added to a final concentration of 0.1 mM, and protein expression was induced for 11 h at 25°C. Bacteria were pelleted and resuspended in buffer I (20 mM Tris [pH 7.9], 500 mM NaCl) containing 5 mM imidazole. Cell lysis was achieved by one passage through a French press at 1,000 lb/in^2. Lysates were clarified by centrifugation at 25,000 × *g* for 15 min at 4°C and incubated with 0.6 ml of Ni^{2+}-nitrilotriacetic acid (NTA) agarose beads (Qiagen) for 2 h at 4°C. The beads were washed with buffer I containing 50 mM imidazole. Bound proteins were eluted with buffer I containing 250 mM imidazole and dialyzed in 1 liter of dialysis buffer (25 mM Tris [pH 8.0], 1 mM EDTA, 1 mM dithiothreitol [DTT], 10% glycerol) at 4°C for 8 h.

Plasmids for HeLa cell transfection. To make pcDNAmycMyt1, human Myt1 cDNA 6-1 was amplified by PCR using primer C (described above) and primer N-myc (5′-CCGGATCCATATGGAGCAGAAGCTCATCTCAGAAGAAGA CCTCCTAGAACGGCCTCCTGC-3′), using the same conditions as described in the construction of pETHisMyt1. The N-myc primer fuses sequences encoding EQKLISEEDL (Myc epitope tag; underlined above) in frame with N-terminal human Myt1 sequences and also contains *Bam*HI and *Nde*I sites. The PCR product was digested with *Bam*HI and cloned into the *Bam*HI and *Eco*RV sites of the pcDNA3 vector (Invitrogen). For the determination of the Myt1 membrane-targeting domain, pcDNAmycMytΔC137 was constructed by digesting pcDNAmycMyt with *Hpa*I and *Sac*II, blunt ended with Klenow polymerase, and recircularized by self-ligation. To make pcDNAmycMytΔC98, a *Bam*HI-*Xba*I fragment from pcDNAmycMyt was partially digested with *Pvu*II. A fraction of the partial digestion reaction was ligated to the *Bam*HI and *Eco*RV sites of pcDNA3, and a positive clone containing a 1.2-kb insert was selected. pcDNAmycMytΔ378-399 was constructed by PCR-mediated deletion. Myt1 cDNA clone 6-1 was amplified by PCR (94°C for 1 min, 55°C for 1 min, and 72°C for 1 min; 20 cycles), using a combination of primer N-myc and primer ΔTM5′ (5′-GGCC CTGAGCCCTGCCAGCTGGCTACAGCC-3′) or primer ΔTM3′ (5′-AGCTG GCAGGGCTCAGGGCCTCCGCTGCCA-3′) and primer C. The PCR products were gel purified, combined, and reamplified with primers N-myc and C by PCR (94°C for 1 min, 55°C for 1 min; and 72°C for 1 min; 25 cycles). The resulting 1.5-kb product was gel isolated, digested with *Bam*HI, and cloned into the *Bam*HI and *Eco*RV sites of pcDNA3. All constructs were sequenced to confirm the orientation and junctions between the insert and the vector.

Coproduction of wild-type and mutant forms of Cdc2 with cyclin B1 and Myt1Hu in Sf9 insect cells. Sf9 cells were infected with baculoviruses encoding GST-cyclin B1, wild-type (T14Y15) or mutant forms of Cdc2 where Thr 14 was replaced with alanine (A14Y15), Tyr 15 was replaced with phenylalanine (T14F15), or codons encoding Thr 14 and Tyr 15 were both mutated (A14F15), and either 150 or 300 μl of Myt1 recombinant virus. At 42 h after infection, cells were washed with PBS and lysed in Cdc2 lysis buffer (50 mM Tris [pH 7.4], 250 mM NaCl, 5 mM NaF, 10 mM sodium pyrophosphate, 0.1% NP-40) supplemented with 1 mM DTT, 1 mM sodium orthovanadate, and the following protease inhibitors: 2 mM phenylmethyl sulfonyl fluoride, 10 μg of aprotinin per ml, 20 μM leupeptin, and 5 μg of pepstatin per ml. GST-cyclin B1–Cdc2 complexes were precipitated by using glutathione agarose beads and washed three times with LiCl wash buffer (50 mM Tris [pH 7.5], 0.5 M LiCl). Proteins were resolved on an SDS–12% polyacrylamide gel, transferred to nitrocellulose membranes, and analyzed for the phosphorylation status of Cdc2 by immunoblotting with an anti-Cdc2 antibody or antiphosphotyrosine antibody.

Labelings in vivo. Sf9 cells (3 × 10^6) were infected with recombinant baculoviruses. At 36 h postinfection, cells were rinsed twice with phosphate-free minimum essential medium (pH 6.2) and then incubated for 4 h in the same medium supplemented with 1.5% dialyzed calf serum, 2 mM glutamine, and 1.5 mCi of ^{32}P-labeled inorganic phosphate per ml at 27°C.

Dephosphorylation of Myt1 by PP1 in vitro. Lysates were prepared from HeLa cells and from Sf9 insect cells overproducing Myt1Hu. HeLa (750 μg) and Sf9 (3 μg) cell proteins were incubated with affinity-purified Myt1Hu antibody. Immunoprecipitates were washed twice with NETN buffer and three times with phosphatase buffer (50 mM Tris [pH 7.4], 0.2 mM $MnCl_2$, 1 mM DTT). Immunoprecipitates were divided into three aliquots and incubated in 250 μl of phosphatase buffer alone or containing either protein phosphatase 1 (PP1; 0.25 μg) or PP1 and microcystin (5 μM). Reaction mixtures were supplemented with 0.5% Brij 35 and were incubated at room temperature for 45 min.

Purification of bacterially produced GST-MytΔC137 and MytΔC137. *E. coli* JM109 cells transformed with pGEX2TNMytΔC137 were induced by the addition of IPTG to a final concentration of 0.5 mM for 4.5 h at 30°C. Induced cultures were centrifuged, and bacterial pellets were resuspended in lysis buffer (25 mM Tris [pH 7.5], 250 mM NaCl, 2 mM EDTA, 1 mM DTT) supplemented with protease inhibitors and lysed by one pass through a French press at 1,000 lb/in^2. Lysates were clarified by centrifugation at 25,000 × *g* for 15 min at 4°C, and the equivalent of 15 ml of the original culture was incubated with 1.5 ml of packed glutathione-agarose beads (Sigma) at 4°C for 40 min. The beads were then washed with lysis buffer, with LiCl wash buffer, and finally with 50 mM Tris (pH 8.0). GST-MytΔC137 was eluted from the beads with 50 mM Tris (pH 8.0) containing 20 mM reduced glutathione. The eluate was then dialyzed at 4°C against two changes of 400 ml of dialysis buffer (20 mM Tris (pH 7.5), 25 mM NaCl, 0.05% Brij 35, 1 mM DTT, 10% glycerol), aliquoted, and stored at −80°C. To cleave the GST moiety, 7 ml of glutathione-agarose beads bound with GST-MytΔC137 (from 250 ml of culture lysate) was incubated in thrombin cleavage buffer (25 mM Tris [pH 7.5], 150 mM NaCl, 2.5 mM $CaCl_2$, 0.01% Brij 35) containing 10 mM reduced glutathione and 50 U of thrombin (Sigma). The cleavage reaction mixture was incubated for 45 min at room temperature. The eluate was then separated from the beads by centrifugation, diluted with an equal volume of buffer A (25 mM Tris [pH 7.5], 1 mM EDTA, 0.01% Brij 35), and loaded onto a 1-ml Resource Q column (Pharmacia) equilibated with buffer A containing 75 mM NaCl. Bound MytΔC137 was eluted with a 15 ml of gradient of 75 to 400 mM NaCl in buffer A, dialyzed in two changes of dialysis buffer, and stored in aliquots at −80°C.

Purification of full-length GST-Myt1Hu from bacteria. Two hundred fifty milliliters of *E. coli* JM109 cells transformed with pGEX2TNMyt1 was induced with 0.5 mM IPTG for 4.5 h at 30°C. Bacteria were pelleted and then suspended in 15 ml of STE buffer (25 mM Tris [pH 8.0], 150 mM NaCl, 1 mM EDTA) supplemented with protease inhibitors, 1 mM DTT, and 1 mg of lysozyme per ml. Then 2.6 ml of 10% sodium *N*-lauroylsarcosine in STE buffer was added, and the suspension was rocked at 4°C for 20 min. Bacteria were lysed by sonication, 4.5 ml of 10% Triton X-100 in STE buffer was added, and the lysate was centrifuged to remove insoluble material. Clarified lysates were incubated with 3 ml of packed glutathione-agarose beads for 45 min at 4°C. Beads were pelleted and then washed with NETN buffer containing 1 M NaCl, followed by 50 mM Tris (pH 7.5)–0.1% NP-40. Bound proteins were eluted with 50 mM Tris (pH 7.5)–0.1% NP-40 containing 20 mM glutathione and dialyzed in buffer containing 20 mM Tris (pH 8.0), 25 mM NaCl, 0.1% NP-40, and 10% glycerol.

Purification of GST-cyclin B1–Cdc2 from Sf9 cells. Sf9 cells (1.4 × 10^8) were infected with baculoviruses encoding GST-cyclin B1 and Cdc2(K33R) for 40 h and then lysed in Cdc2 lysis buffer. The cleared lysate was incubated with 7 ml of glutathione-agarose beads for 1 h at 4°C with rocking. Beads were then washed sequentially with 10 ml of Cdc2 lysis buffer, 10 ml of 0.5 M LiCl in 50 mM Tris (pH 8.0), and 10 ml of Tris buffer (20 mM Tris [pH 8.0], 2 mM EDTA, 2 mM DTT, 150 mM NaCl, 0.1% Brij 35). GST-cyclin B–Cdc2(K33R) complexes were eluted from the column with Tris buffer containing 20 mM reduced glutathione and dialyzed at 4°C overnight in 20 mM Tris (pH 8.2)–10% glycerol.

Phosphorylation of Cdc2 by recombinant GST-Myt1Hu *in vitro*. Kinase reactions were carried out with 2 μg of GST-Myt1Hu purified from bacteria and 0.7 μg of GST-cyclin B–Cdc2(K33R) in 50 μl of incomplete kinase buffer (50 mM Tris [pH 7.5], 10 mM $MgCl_2$) supplemented with 1 mM DTT and various amounts of ATP. The reaction mixtures were incubated for 25 min at 30°C, and the reactions were terminated by the addition of SDS sample buffer. Proteins were resolved on an SDS–12% polyacrylamide gel, and Cdc2 was detected by immunoblotting with a polyclonal antibody specific for Cdc2 and an enhanced chemiluminescence detection system (Amersham).

Purification of monomeric Cdc2. Insect cells were infected with recombinant virus encoding Cdc2(K33R). Lysates were prepared and incubated with p13^{suc1}-conjugated beads as described previously (41). Beads were washed three times with Cdc2 lysis buffer and twice with incomplete kinase buffer (50 mM Tris [pH 7.5], 10 mM $MgCl_2$, 0.05% Brij 35).

Substrate specificity of Myt1Hu. Two-tenths microgram of bacterially produced GST-Myt1Hu or 5 ng of bacterially produced His-p49^{wee1} was used to phosphorylate 0.7 μg of GST-cyclin B1–Cdc2(K33R) or 1 μg of monomeric Cdc2(K33R) bound to 20 μl of p13^{suc1} beads. Kinase reactions were carried out in 50 μl of complete kinase buffer (50 mM Tris [pH 7.4], 10 mM $MgCl_2$, 50 μM ATP, 1 mM DTT, 0.05% Brij 35, 15 μCi of [γ-^{32}P]ATP) at 30°C for 15 min. Reactions were stopped by the addition of SDS-polyacrylamide gel electrophoresis (PAGE) loading buffer, resolved on an SDS–10% polyacrylamide gel, and analyzed by autoradiography.

Phosphoamino acid analysis. One-half microgram of soluble GST-Myt1Hu purifed from *E. coli* was incubated either alone or in the presence of 0.5 μg of purified Cdc2(K33R)–cyclin B1 in kinase buffer (50 mM Tris [pH 7.4], 10 mM $MgCl_2$, 50 μM ATP, 1 mM DTT) containing 10 μCi of [γ-^{32}P]ATP. Kinase reactions were carried out at 30°C for 20 min, terminated by the addition of SDS sample buffer, and resolved on an 10% SDS–polyacrylamide gel. Proteins were transfered to an Immobilon-P membrane (Millipore), and samples were prepared for two-dimensional phosphoamino acid analysis as described previously (41). Phosphoamino acids were separated in the first dimension by thin-layer electrophoresis in a pH 1.9 buffer (58) and by chromatography in the second dimension in a buffer consisting of isopropanol-hydrochloric acid-water in a ratio of 70/15/15. Phosphoamino acid standards were visualized by staining with ninhydrin.

Inhibition of Cdc2-cyclin B1 histone H1 kinase activity by phosphorylation of Thr 14 and Tyr 15. Insect cells were coinfected with baculoviruses encoding GST-cyclin B1 and Cdc2. Cells were lysed in Cdc2 lysis buffer supplemented with protease inhibitors. GST-cyclin B1–Cdc2 complexes were precipitated from clarified lysates by using glutathione-agarose beads and then washed with lysis buffer, LiCl wash buffer, and incomplete kinase buffer. The beads were then incubated in the presence of both an ATP-regenerating system (50 mM Tris [pH 7.4], 10 mM $MgCl_2$, 2 mM ATP, 1 mM DTT, 0.05% Brij 35, 10 mM creatine phosphate, 50 μg of creatine kinase per ml) and 2 μg of soluble His-p49^{wee1} and/or 2 μg of soluble MytΔC137. After a 15-min incubation at 30°C, an additional 2 μg of each kinase was added to the reactions and a further 15-min incubation was performed. Following the kinase reaction, supernatants were removed and the beads were washed three times with incomplete kinase buffer. Three-fourths of each sample was analyzed by Western blotting, and the remainder was assayed for histone H1 kinase activity as previously described (43).

Preparations of membrane and cytosol fractions. All fractionation steps were carried out at 4°C. Approximately 3.5×10^7 HeLa cells were rinsed three times with PBS and scraped from tissue culture dishes in PBS by using a rubber policeman. Cells were collected by centrifugation at 500 × *g* for 10 min. The cell pellet (~0.5 ml) was resuspended in HB (0.2 M sucrose, 20 mM HEPES [pH 7.3], 0.2 mM $MgCl_2$, 5 mM KCl, 1 mM EDTA) supplemented with protease inhibitors (2 mM phenylmethylsulfonyl fluoride, 10 μg of aprotinin per ml, 20 μM leupeptin, and 5 μg of pepstatin per ml) and Dounce homogenized (~30 strokes). Homogenization was monitored by trypan blue staining and generally resulted in >90% cell lysis. The homogenate was centrifuged at 1,000 × *g* for 10 min to yield a postnuclear supernatant and a pellet consisting mainly of nuclei and unbroken cells. The postnuclear supernatant was then centrifuged at 100,000 × *g* for 1 h in a Beckman TLA 100.3 ultracentrifuge rotor, yielding a cytosolic supernatant and a pellet consisting of cellular membranes. The membranes were resuspended in HB, divided into two fractions, and centrifuged again at 100,000 × *g* in the same rotor. One of the membrane pellets was resuspended in PBS, while the other membrane pellet was resuspended in PBS containing 0.5 M NaCl. Both suspensions were incubated on ice for 30 min. The membranes were pelleted as described above, and then both pellets were resuspended in PBS. Fractions were resolved on an SDS–10% polyacrylamide gel, transferred to a nitrocellulose membrane, and analyzed by Western blotting. The primary antibodies were affinity-purified Myt1Hu peptide antibody (R5084; 1:10,000 dilution) or anti-14-3-3 antibody (K19; 1:40 dilution). Secondary antibodies were horseradish peroxidase-conjugated donkey anti-rabbit antibodies (1:2,000 dilution; Jackson Immuno Research). Secondary antibodies were detected with enhanced chemiluminescence (Amersham) and visualized by autoradiography.

Indirect immunofluorescence. To visualize endogenous Myt1Hu and γ-adaptin (Golgi marker), HeLa cells grown on 12-mm-diameter coverslips were fixed with 2% paraformaldehyde in PBS for 10 min at room temperature. Coverslips were then incubated with 50 mM NH_4Cl in PBS for 5 min, washed twice with PBS, and permeabilized with 0.1% NP-40 in PBS for 10 min. All subsequent incubations and washes were carried out at room temperature in 0.1% NP-40 in PBS. After blocking for 30 min with 5% normal donkey serum (Jackson Immuno Research), coverslips were incubated with affinity-purified Myt1Hu peptide antibody (R5084; 1:1,000 dilution) and with a monoclonal antibody against γ-adaptin (1:250 dilution; Sigma) for 60 min, washed four times for 10 min each, then incubated with Cy3-conjugated donkey anti-rabbit antibody (1:1,000 dilution) and fluorescein isothiocyanate (FITC)-conjugated donkey anti-mouse antibody (1:200 dilution) for 45 min, and finally washed four times for 10 min each. To localize Myt1Hu with an ER marker, HeLa cells were transfected with a plasmid encoding PTP1B (pJ3MPTP1B) by using LipofectAMINE reagent (Bethesda Research Laboratories) in Opti-MEM reduced serum medium (Bethesda Research Laboratories) for 4.5 h. Transfected cells were then incubated in Dulbecco's modified Eagle medium supplemented with 10% calf serum at 37°C for 18 h. Immunofluorescent staining was performed as described above, using affinity-purified Myt1Hu antibody (1:1,000 dilution) and a monoclonal antibody against PTP1B (1:500 dilution). To localize the membrane-targeting domain of Myt1Hu, HeLa cells were transfected with plasmids expressing Myc epitope-tagged deletion mutants of Myt1Hu. Cells were fixed in 3.7% formaldehyde and stained by using a monoclonal antibody against the Myc epitope (9E10; 1:250 dilution) and Cy3-conjugated donkey anti-mouse secondary antibody (1:500 dilution). Coverslips were then mounted on glass slides for analysis using either a conventional fluorescence microscopy (model BX60; Olympus) or the confocal laser scanning imaging system (model MRC-1024; Bio-Rad).

Nucleotide sequence accession number. The sequence shown in Fig. 1A has been assigned GenBank accession number U56816.

RESULTS

Isolation of a Myt1Hu cDNA. A PCR-based approach was used to obtain a cDNA encoding the human homolog of the Myt1Xe protein kinase (designated Myt1 for membrane-associated, tyrosine- and threonine-specific, Cdc2-inhibitory kinase) (35). The primary PCR reaction was carried out by using degenerate oligonucleotides designed to hybridize to conserved regions within the catalytic domain of Wee1 family members (Fig. 1A, primers 1 and 2). The primary PCR product was further amplified by using primers containing sequences conserved between Wee1 family members and the recently cloned Myt1Xe kinase. PCR products of the expected size were subcloned and hybridized with a DNA probe derived from Wee1Hu followed by hybridization at lower stringency to a probe derived from Myt1Xe cDNA sequences. Several clones that hybridized to the Myt1Xe probe but not to the Wee1Hu probe were identified.

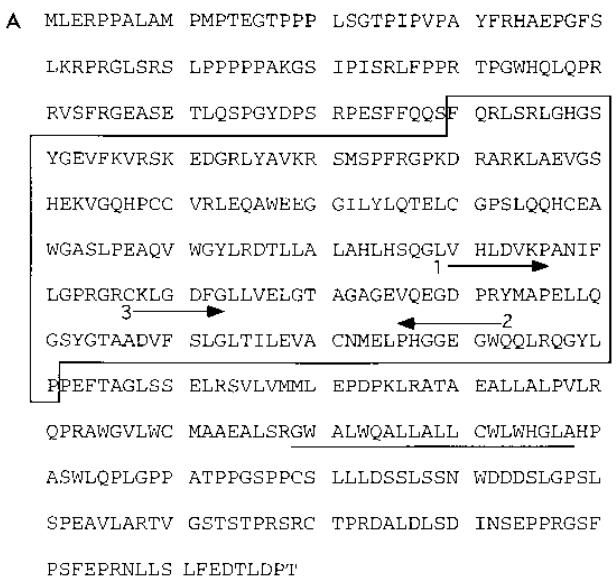

FIG. 1. (A) Predicted amino acid sequence of Myt1Hu. The nucleotide sequence of the Myt1Hu cDNA is predicted to encode a protein of 499 amino acids. The putative catalytic domain corresponds to amino acid residues 110 to 321 and is boxed. Regions used to design the degenerate PCR primers are underlined, with numbers and leftward and rightward arrows representing the coding and noncoding DNA sequences, respectively. A putative membrane-targeting domain is underlined. (B) Alignment of Myt1Xe and Myt1Hu. Residues conserved by both kinases are boxed. The alignment was performed by using the Clustal method in the MegAlign program of DNA STAR. (C) Alignment of the catalytic domains of Myt1Hu (amino acids 110 to 321), Myt1Xe (amino acids 103 to 315), SpWee1 (amino acids 566 to 781), *Xenopus* Wee1 (amino acids 210 to 443), and Wee1Hu (amino acids 299 to 532). The first amino acid in subdomain I was chosen as the amino-terminal boundary, and a proline conserved among Myt1 and Wee1 family members in subdomain X was chosen as the carboxy-terminal boundary. Residues conserved by at least three family members are boxed. The alignment was performed by using the Clustal method in the MegAlign program of DNA STAR.

Clones whose predicted open reading frames showed significant sequence homology to Myt1Xe were used to screen a HeLa cell cDNA library. The longest cDNA obtained was ~1.9 kb. Evidence that this was a full-length cDNA was suggested by the presence of a termination codon upstream of and in frame with the assigned open reading frame. The predicted open reading frame of the human cDNA encodes a protein of 499 amino acids with an expected molecular mass of 55 kDa (Fig. 1A). Based on its structural (Fig. 1B) and biochemical (see below) similarities to Myt1Xe, the kinase encoded by this cDNA was named Myt1Hu.

A comparison of the complete Myt1Hu protein sequence with sequences of other members of the Wee1 family indicated the greatest degree of sequence identity with full-length Myt1Xe (46% [Fig. 1B]). Within the predicted kinase domains of Myt1Hu and Myt1Xe, the sequence identity was even greater (~64% [Fig. 1C]). Lower levels of sequence identity

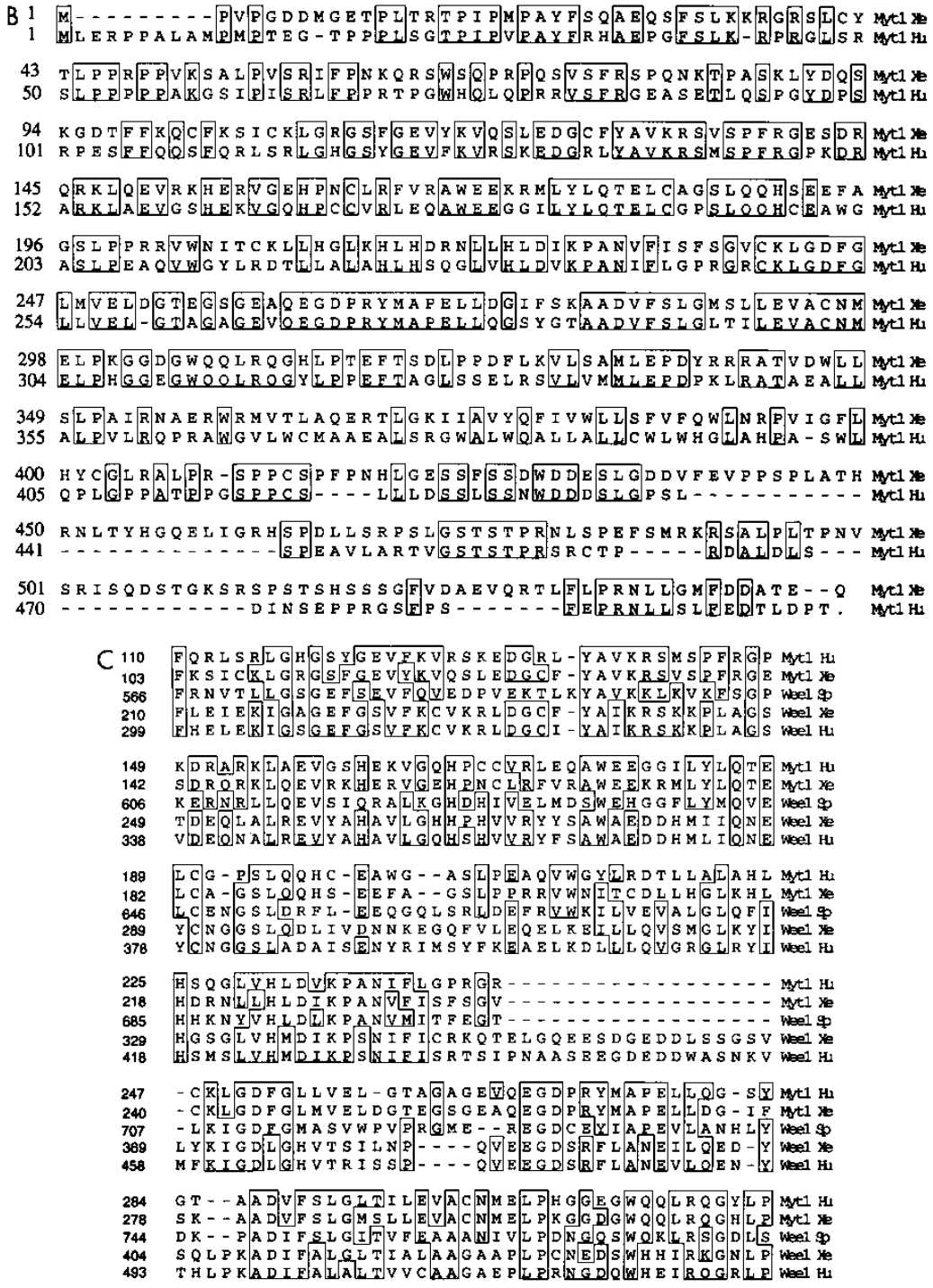

FIG. 1—*Continued.*

were noted between the kinase domains of Myt1Hu and *S. pombe* Wee1 (SpWee1) (35% [49]), *Xenopus* Wee1 (32% [34]), and Wee1Hu (32% [22]). Interestingly, the human and *Xenopus* Wee1 protein kinases contain a 19-amino-acid insertion in their catalytic domains that is absent in SpWee1 and human and *Xenopus* Myt1. C terminal to the catalytic domain of Myt1Hu is a sequence of 20 amino acids, most of which are either hydrophobic or uncharged and which are bordered by an arginine and histidine (Fig. 1A, underlined). This sequence is predicted to form an alpha-helical structure and likely accounts for the membrane localization of Myt1Hu, as C-terminal truncations removing this region result in the nuclear localization of Myt1Hu in HeLa cells (see below).

Northern analysis was performed on total RNA isolated from primary activated PBLs and transformed cells (HeLa and Jurkat cells) to assess the size and relative abundance of

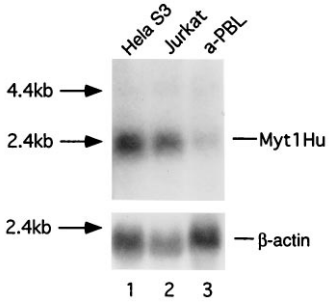

FIG. 2. Northern analysis of Myt1Hu expression in primary and transformed cells. Thirty-microgram aliquots of total RNA isolated from HeLa cells (lane 1), Jurkat cells (lane 2), and activated PBLs (a-PBL; lane 3) were probed with either radiolabeled Myt1Hu cDNA (top panel) or radiolabeled human β-actin probe (bottom panel).

Myt1Hu RNA. As seen in Fig. 2, a transcript of approximately 2.0 to 2.4 kb was detected in each case. Lowest Myt1Hu expression levels were detected in the activated PBLs (lane 3).

Phosphorylation of human Myt1 *in vivo* and *in vitro*. A recombinant baculovirus encoding human Myt1 was generated to study the phosphorylation of Myt1Hu *in vivo* and *in vitro* (Fig. 3). Insect cells were infected with viruses encoding Myt1Hu, and infected cells were incubated with ^{32}P-labeled inorganic phosphate. Lysates were prepared, and Myt1Hu was immunoprecipitated with a Myt1Hu-specific antibody. Immunoprecipitates were resolved by SDS-PAGE and analyzed by autoradiography. As seen in Fig. 3A (lane 1), Myt1Hu was phosphorylated *in vivo* and phosphoamino acid analysis revealed primarily phosphoserine, although low levels of phosphothreonine were also detected (Fig. 3B). The kinase activity of human Myt1 was assessed by performing kinase assays *in vitro* (Fig. 3A, lane 2). Myt1Hu was produced in insect cells as a hexahistidine fusion protein and isolated on Ni^{2+}-NTA agarose. Kinase assays were performed *in vitro*, and the phosphorylation status of Myt1Hu was assessed by autoradiography. As seen in Fig. 3A (lane 2), Myt1Hu was phosphorylated under these conditions. In contrast, Myt1Xe reportedly does not autophosphorylate (35). Phosphoamino acid analysis of Myt1Hu revealed approximately equal levels of phosphoserine and

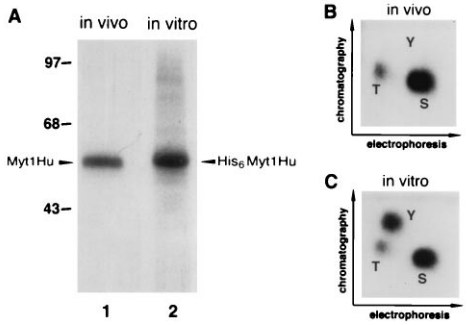

FIG. 3. Phosphorylation of Myt1Hu *in vivo* and *in vitro*. (A) Insect cells were infected with viruses encoding Myt1Hu and at 36 h after infection were incubated with ^{32}P-labeled inorganic phosphate. Lysates were prepared, and Myt1Hu was immunoprecipitated with a Myt1Hu-specific antibody (lane 1). Alternatively, Myt1Hu produced in insect cells as a hexahistidine fusion protein was isolated on Ni^{2+}-NTA agarose, and kinase assays were performed *in vitro* (lane 2). Precipitates were resolved by SDS-PAGE, and Myt1Hu was visualized by autoradiography. (B) Phosphoamino acid analysis of phosphorylated Myt1 from panel A, lane 1. (C) Phosphoamino acid analysis of phosphorylated Myt1 from panel A, lane 2. Positions of phosphoamino acid were determined by using unlabeled standards. S, phosphoserine; T, phosphothreonine; Y, phosphotyrosine.

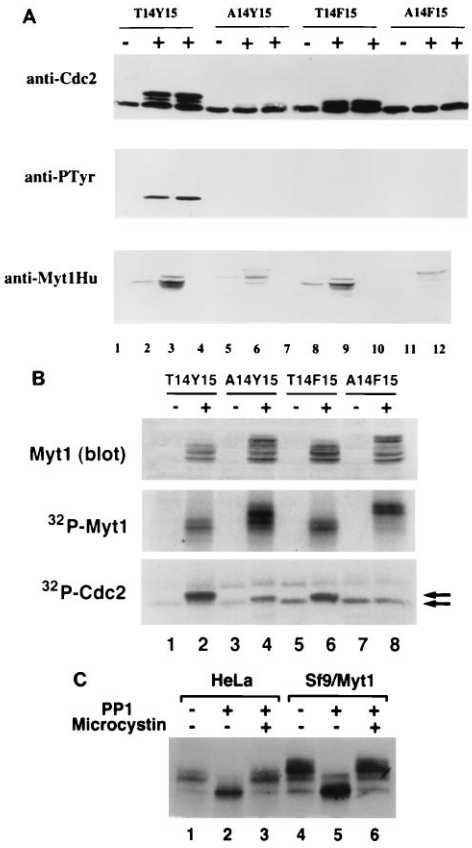

FIG. 4. Myt1Hu phosphorylates Cdc2 on Thr 14 and Tyr 15. (A) Sf9 insect cells were infected with baculoviruses expressing GST-cyclin B1 (lanes 1 to 12), Myt1Hu (+; lanes 2, 3, 5, 6, 8, 9, 11, and 12), and various forms of human Cdc2 [wild-type Cdc2 (lanes 1 to 3), Cdc2(A14Y15) (lanes 4 to 6), Cdc2(T14F15) (lanes 7 to 9), and Cdc2(A14F15) (lanes 10 to 12)]. Twice the amount of Myt1 virus was used in infections shown in lanes 3, 6, 9, and 12 compared with those shown in lanes 2, 5, 8, and 11. GST-cyclin B1–Cdc2 complexes were purified by using glutathione-agarose beads, then separated on an SDS–12% polyacrylamide gel, and immunoblotted for Cdc2 (anti-Cdc2), phosphotyrosine (anti-PTyr), or Myt1 (anti-Myt1Hu). (B) Sf9 insect cells were infected with baculoviruses expressing GST-cyclin B1 (lanes 1 to 8), Myt1Hu (+; lanes 2, 4, 6, and 8), and various forms of human Cdc2 [wild-type Cdc2 (lanes 1 and 2), Cdc2(A14Y15) (lanes 3 and 4), Cdc2(T14F15) (lanes 5 and 6), and Cdc2(A14F15) (lanes 7 and 8)]. Cells were incubated with ^{32}P-labeled inorganic phosphate for 4 h beginning at 36 h postinfection. GST-cyclin B1–Cdc2 complexes were purified by using glutathione-agarose, precipitates were resolved on an SDS–12% polyacrylamide gel, and Cdc2 was visualized by autoradiography (bottom panel). A portion of the remaining lysate was either resolved by SDS-PAGE and analyzed for Myt1Hu levels by immunoblotting (top panel) or immunoprecipitated with antibody specific for Myt1Hu and analyzed for Myt1Hu by autoradiography (middle panel). (C) Myt1Hu immunoprecipitated from HeLa cells (lanes 1 to 3) and overproducing insect cells (lanes 4 to 6) was treated with phosphatase buffer alone (lanes 1 and 4) or phosphatase buffer containing PP1 in either the absence (lanes 2 and 5) or presence (lanes 3 and 6) of 5 μM microcystin. Proteins were resolved on an SDS–10% gel, and Myt1 was visualized by immunoblotting.

phosphotyrosine and lower levels of phosphothreonine (Fig. 3C). Identical results were obtained for recombinant Myt1Hu overproduced and purified from bacteria (data not shown). Interestingly, both Myt1Hu and SpWee1 are capable of autophosphorylation on tyrosine yet *in vivo* are not phosphorylated on tyrosine to any significant level (41).

Myt1Hu preferentially phosphorylates Cdc2 on Thr 14. Several types of experiments were performed to determine whether Cdc2 serves as a substrate of Myt1Hu (Fig. 4 and 5). Insect cells were coinfected with recombinant baculoviruses encoding cyclin B1 and either wild-type or mutant forms of

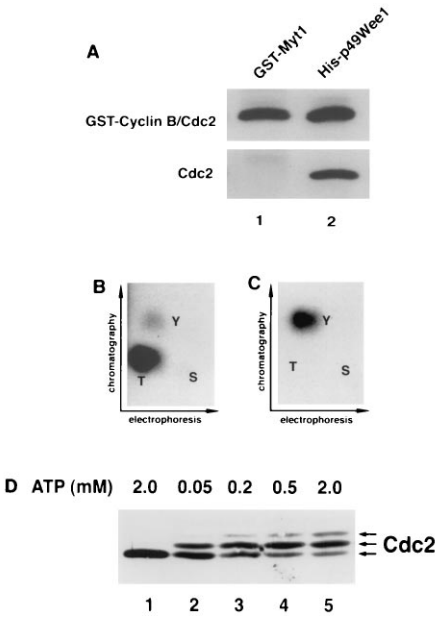

FIG. 5. Myt1Hu phosphorylates Cdc2 in a cyclin-dependent manner. (A) Full-length Myt1Hu was purified from bacteria as a GST fusion protein. The catalytic domain of Wee1Hu was purified from bacteria as a hexahistidine-tagged fusion protein (His-p49^{wee1}). Kinase assays were performed *in vitro* in the presence of [γ-^{32}P]ATP and either GST-cyclin B1–Cdc2(K33R) complex (top panel) or monomeric Cdc2(K33R) (bottom panel). Reactions were resolved by SDS-PAGE and subjected to autoradiography. (B and C) Phosphoamino acid analysis of Cdc2 phosphorylated by Myt1Hu (B) and p49^{wee1} (C). Positions of phosphoamino acid were determined by using unlabeled standards. S, phosphoserine; T, phosphothreonine; Y, phosphotyrosine. (D) Kinase reactions were performed *in vitro* with GST-cyclin B–Cdc2(K33R) in the presence (lanes 2 to 5) or absence (lane 1) of 2 μg of bacterially produced GST-Myt1Hu. The concentration of ATP used in each reaction is indicated above each lane. Reactions were resolved by SDS-PAGE, and the phosphorylation status of Cdc2 was monitored by immunoblotting.

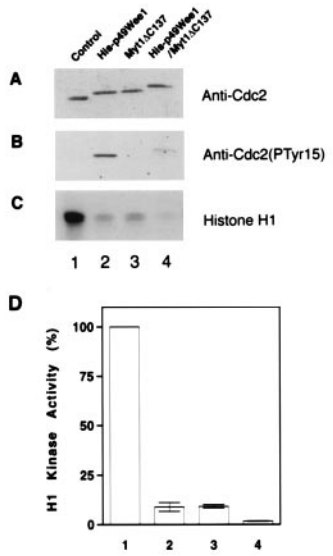

FIG. 6. Contribution of Thr 14 and Tyr 15 phosphorylation to the inhibition of Cdc2 kinase activity. GST-cyclin B–Cdc2 complexes bound to glutathione-agarose beads were incubated with buffer alone (lane 1), soluble His-p49^{wee1} (lane 2), soluble Myt1ΔC137 (lane 3), or soluble forms of His-p49^{wee1} and Myt1ΔC137 (lane 4) in the presence of an ATP-regenerating system *in vitro*. Part of the reaction was resolved directly by SDS-PAGE, and the status of Cdc2 phosphorylation was monitored by immunoblotting with a Cdc2 antibody (A) or anti-Cdc2(pTyr15) serum (B). The rest of the reaction was assayed for histone H1 kinase activity (C and D). Representative phosphorylation of histone H1 as monitored by autoradiography is shown in panel C, and a bar graph of relative histone H1 phosphorylation (mean ± standard deviation of three independent experiments) is shown in panel D. Data are displayed as a percentage of histone H1 phosphorylation mediated by Cdc2-cyclin B1 complexes incubated in buffer only (control).

Cdc2 in combination with different concentrations of Myt1Hu (Fig. 4A). The status of Thr 14 and Tyr 15 phosphorylation of Cdc2 was assessed by its electrophoretic mobility on SDS-gels (2). A retardation in the electrophoretic mobility of Cdc2 of one increment is due to the phosphorylation of either Thr 14 or Tyr 15. A retardation of two increments is due to phosphorylation at both sites. Coinfection of Cdc2-cyclin B1 with Myt1Hu resulted in the appearance of two new electrophoretic forms of Cdc2, indicative of Thr 14 and Tyr 15 phosphorylation (Fig. 4A, top panel, lanes 2 and 3). The slowest electrophoretic form reacted with an antibody specific for phosphotyrosine (middle panel, lanes 2 and 3). Interestingly, Myt1 quite readily phosphorylated Cdc2 when Thr 14 was intact and Tyr 15 was mutated (top panel, lanes 8 and 9) but did not efficiently phosphorylate Tyr 15 when Thr 14 was mutated to alanine (top panel, lanes 5 and 6). This result indicates a preference on the part of Myt1 for Thr 14 phosphorylation. A mutant of Cdc2 lacking both Thr 14 and Tyr 15 was not a substrate of Myt1 in insect cells (top panel, lanes 11 and 12). Interestingly, different electrophoretic forms of Myt1 were observed upon its synthesis in insect cells. The slowest electrophoretic forms of Myt1 were evident in cases where Cdc2 was either poorly phosphorylated (A14Y15 mutant) or not phosphorylated by Myt1(A14F15 mutant). In these situations, Cdc2 is active as a protein kinase. These results indicate that Myt1 serves as a substrate of Cdc2-cyclin B1 complexes when coproduced in insect cells. Evidence that the different electrophoretic forms of Myt1Hu, seen in both HeLa cells and overproducing insect cells, represent different phosphorylated forms of Myt1Hu is provided by the data shown in Fig. 4C. Myt1Hu immunoprecipitated from HeLa cells (lanes 1 to 3) and overproducing insect cells (lanes 4 to 6) was treated with phosphatase buffer alone (lanes 1 and

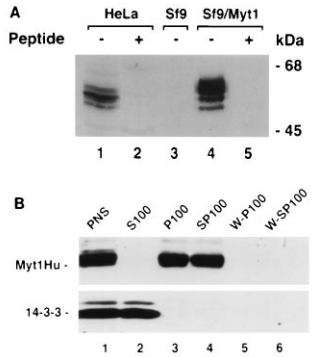

FIG. 7. Localization of Myt1Hu to membranes. (A) Proteins obtained from HeLa cells (100 μg; lanes 1 and 2), uninfected Sf9 insect cells (2.5 μg; lane 3), or Myt1Hu-producing Sf9 insect cells (2.5 μg; lanes 4 and 5) were resolved by SDS-PAGE on a 10% gel and transferred to nitrocellulose. Immunoblotting was performed with affinity-purified Myt1Hu antibody in either the absence (lanes 1, 3, and 4) or presence (lanes 2 and 5) of antigenic peptide. (B) Crude fractions of cytosol and cellular membranes were prepared from HeLa cells as described in Materials and Methods. PNS, 50 μg of postnuclear supernatant (lane 1); S100, 50 μg of cytosolic 100,000 × *g* supernatant (lane 2); P100, 100,000 × *g* membrane pellet washed with PBS (lane 3); SP100, 100,000 × *g* membrane pellet washed with PBS–0.5 M NaCl (lane 4); W-P100, PBS wash of P100 membrane pellet (lane 5); W-SP100, PBS–0.5 M NaCl wash of SP-100 membrane pellet (lane 6). Equal amounts of P100, SP100, W-P100, and W-SP100 fractions were analyzed (1/5 of total fraction volume). Fractions were resolved on an SDS–10% polyacrylamide gel, transferred to a nitrocellulose membrane, and analyzed by Western blotting using an antibody specific for Myt1Hu or 14-3-3.

4) or phosphatase buffer containing PP1 in either the absence (lanes 2 and 5) or presence (lanes 3 and 6) of microcystin. As seen in lanes 2 and 5, dephosphorylation by PP1 resulted in an enhancement in the electrophoretic mobility of Myt1Hu which was blocked by microcystin.

Labeling experiments were also performed *in vivo* to examine the effects of Myt1Hu on the phosphorylation status of wild-type and mutant forms of Cdc2. As seen in Fig. 4B, Cdc2 and each of the Cdc2 mutants were phosphorylated to low levels when they were synthesized in insect cells in the absence of Myt1 (bottom panel, lanes 1, 3, 5, and 7). We have previously demonstrated that this is due to the phosphorylation of threonine 161, presumably by an endogenous insect cell CAK (3, 41). In the presence of Myt1, enhanced phosphorylations of wild-type Cdc2 (bottom panel, lane 2), Cdc2A14Y15 (bottom panel, lane 4), and Cdc2T14F15 (bottom panel, lane 6) were evident, although mutation of threonine 14 to alanine impaired the ability of Myt1 to phosphorylate Cdc2 on tyrosine 15 *in vivo* (bottom panel, lane 4). No enhancement in phosphorylation of the double mutant (A14F15) was evident upon coproduction with Myt1Hu (bottom panel, lane 8), indicating that this mutant did not serve as a substrate for Myt1Hu *in vivo*. As seen in Fig. 4B (top and middle panels), different electrophoretic forms of Myt1 were evident upon its synthesis in insect cells with various Cdc2-cyclin B1 complexes. The slowest electrophoretic forms of Myt1 were evident in cases where Myt1 were coproduced with mutants of Cdc2 where threonine 14 had been changed to alanine (lanes 4 and 8). Because the A14 mutants are poorly phosphorylated by Myt1, they are not enzymatically inactivated when coproduced with Myt1 in insect cells. Thus, one interpretation of this result is that Myt1 serves as a substrate of active Cdc2-cyclin B1 complexes upon coproduction in insect cells. This interpretation is supported by the increased ^{32}P incorporation into Myt1 when Myt1 was coproduced with either Cdc2A14Y15 (middle panel, lane 4) or Cdc2A14F15 (middle panel, lane 8) compared with either wild-type Cdc2 (middle panel, lane 2) or Cdc2T14F15 (middle panel, lane 6). These results are also consistent with those shown in Fig. 4A.

Phosphorylation of Cdc2 by Myt1Hu occurs in a cyclin-dependent manner. Both SpWee1 and Wee1Hu show a strong preference for the cyclin-bound form of Cdc2 (41, 42, 44). In the case of Wee1Hu, this dependency can be abrogated if its N terminus is deleted (44, 45). As seen in Fig. 5A, the catalytic domain of Wee1Hu did not discriminate between monomeric Cdc2 and cyclin B1-bound Cdc2 (lane 2). As expected, phosphoamino acid analysis of Cdc2 revealed phosphotyrosine (Fig. 5C). Although GST-Myt1Hu quite readily phosphorylated the cyclin-bound form of Cdc2, it did not detectably phosphorylate monomeric Cdc2 (lane 1). These results indicate a preference on the part of Myt1Hu for the cyclin-bound form of Cdc2. Phosphoamino acid analysis of Myt1Hu-phosphorylated Cdc2 revealed predominantly phosphothreonine and low levels of phosphotyrosine (Fig. 5B). The low level of detectable phosphotyrosine was likely due to the low levels of ATP used in the *in vitro* kinase assay (50 μM). As seen in Fig. 5D, phosphorylation of Cdc2 on Thr 14 (singly shifted form) by Myt1Hu was relatively insensitive to ATP concentrations in the range tested (0.05 to 2 mM). However, further phosphorylation of Cdc2 on Tyr 15 (slowest electrophoretic form) by Myt1Hu was not appreciably detected until 0.2 mM ATP, implying a higher apparent K_m for phosphorylation of tyrosine 15 than for phosphorylation of threonine 14.

Inhibition of Cdc2 kinase activity by Thr 14 and Tyr 15 phosphorylation. To determine the relative contributions of Thr 14 and Tyr 15 phosphorylation to the inhibition of Cdc2 kinase activity, kinase reactions were performed *in vitro* to generate Cdc2 that was stoichiometrically phosphorylated on either Thr 14 or Tyr 15 or on both Thr 14 and Tyr 15 (Fig. 6). Complexes of GST-cyclinB1–Cdc2 were purified from insect cells and were incubated in buffer alone (lane 1) or with the catalytic domain of Wee1Hu (His-p49^{wee1}, lane 2), the catalytic domain of Myt1Hu (Myt1ΔC137, lane 3), or both His-p49^{wee1} and Myt1ΔC137 (lane 4). Part of the reaction was resolved by SDS-PAGE to monitor for the electrophoretic mobility of Cdc2 (Fig. 6A) and its immunoreactivity with an antibody specific for the Tyr 15-phosphorylated form of Cdc2 [anti-Cdc2(pTyr15) (Fig. 6B)]. The remainder of the reaction was assayed for histone H1 kinase activity (Fig. 6C and D). Incubation of GST-cyclinB1–Cdc2 complexes with the Wee1 (Fig. 6A, lane 2) or Myt1 (Fig. 6A, lane 3) kinase resulted in a single shift in the electrophoretic mobility of Cdc2. In the case of Wee1, this reflected Tyr 15 phosphorylation, as indicated by the immunoreactivity of Cdc2 with an antibody specific for the Tyr 15-phosphorylated form of Cdc2 [anti-Cdc2(pTyr15) (Fig. 6B, lane 2)]. Cdc2 phosphorylated by Myt1 alone did not react with the Cdc2(pTyr15) antibody, indicating that Cdc2 was phosphorylated solely on Thr 14 (Fig. 6B, lane 3). Phosphorylation of Cdc2 by both Wee1 and Myt1 generated the slowest electrophoretic form of Cdc2, which also reacted with the Cdc2(pTyr15) antibody (Fig. 6B, lane 4). Compared with the Tyr 15-phosphorylated form of Cdc2 (Fig. 6B, lane 2), the slowest electrophoretic form of Cdc2 reacted less well with the Cdc2(pTyr15) antibody, possibly due to interference by phosphorylated Thr 14. Next, histone H1 kinase assays were performed to determine the relative contributions of Thr 14 and Tyr 15 phosphorylation to the activity of the Cdc2-cyclin B1 complex. As seen in Fig. 6C and D, phosphorylation of Cdc2 on either Thr 14 or Tyr 15 resulted in an activity that was only 10% of that of Cdc2 not phosphorylated on either site. The phosphorylation of Cdc2 on both Thr 14 and Tyr 15 resulted in an activity that was only 1% of that of Cdc2 not phosphorylated at either site.

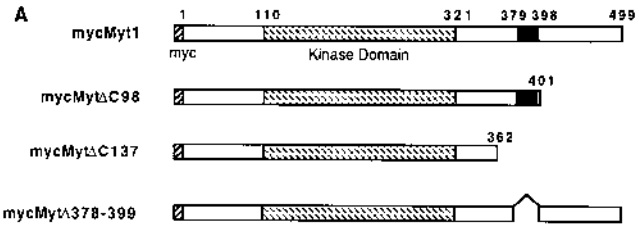

FIG. 8. A 20-amino-acid hydrophobic region is essential for the membrane targeting of Myt1Hu. (A) Schematic representation of various deletion mutants of Myc epitope-tagged Myt1Hu used in the transient transfection assays in panel B. Numbers denote amino acid residues of Myt1Hu. The catalytic domain of Myt1Hu is indicated by a shaded box, and the putative membrane-targeting domain is represented by a filled box. The N-terminal Myc epitope tag is also shown. (B) Indirect immunofluorescence of HeLa cells transfected with pcDNA mycMyt1Hu (a and b), pcDNAmycMytΔC98 (c and d), pcDNAmycMytΔC137 (e and f), or pcDNAmycMytΔ378-399 (g and h). Cells were stained with Cy3-conjugated secondary antibody and visualized by indirect immunofluorescence (a, c, e, and g) or phase contrast (b, d, f, and h), using a conventional fluorescence microscope.

Targeting of Myt1Hu to the ER and Golgi complex. Previous cell fractionation experiments indicated that in human cells, the kinase that phosphorylates Cdc2 on Thr 14 is membrane associated (27a). To localize endogenous Myt1Hu, an affinity-purified peptide antibody was generated and used in immunoblotting experiments. As seen in Fig. 7A, the antibody recognized Myt1Hu in HeLa cell lysates (lane 1) and in overproducing insect cells (lane 4). Immunoblotting of Myt1Hu

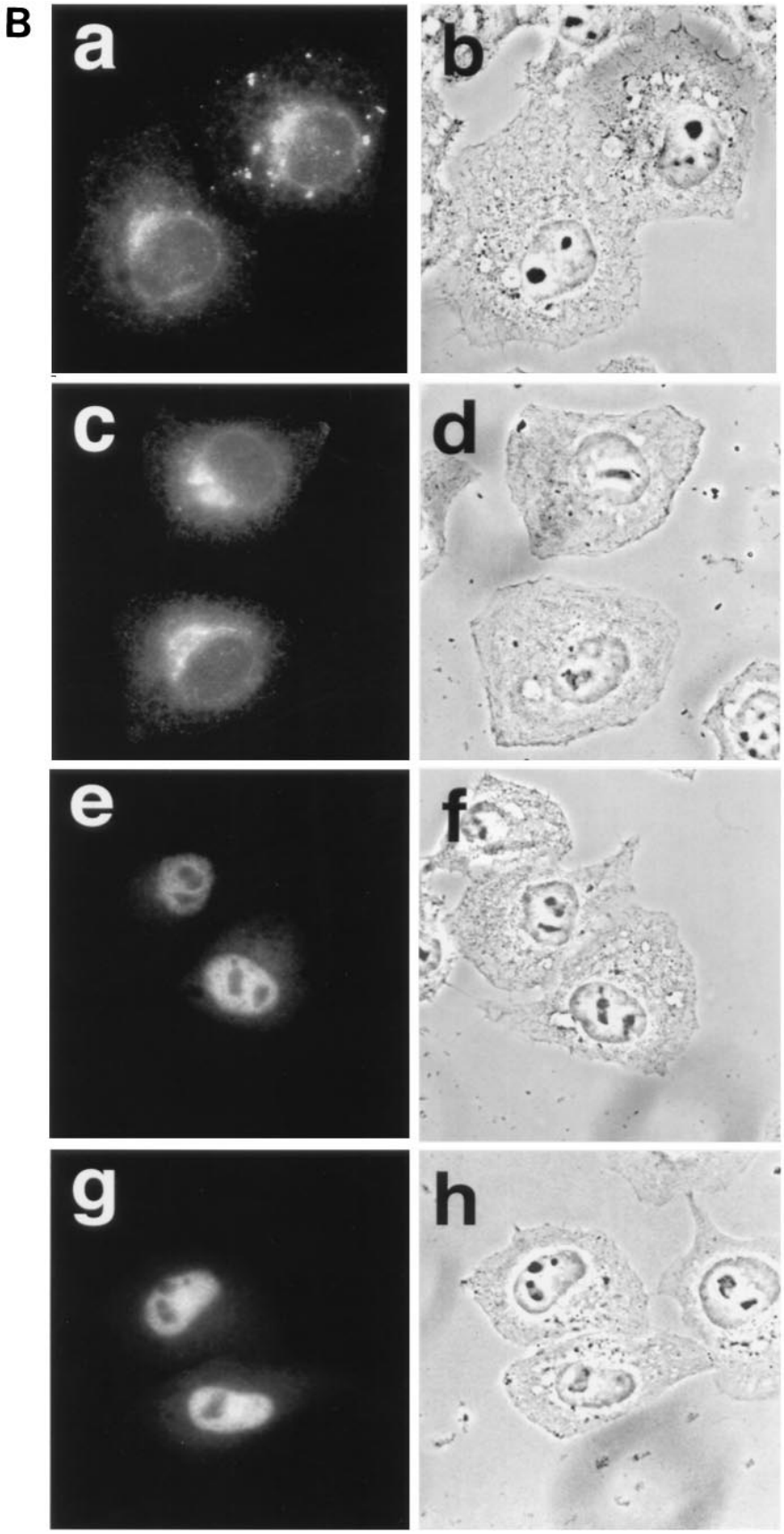

FIG. 8—*Continued.*

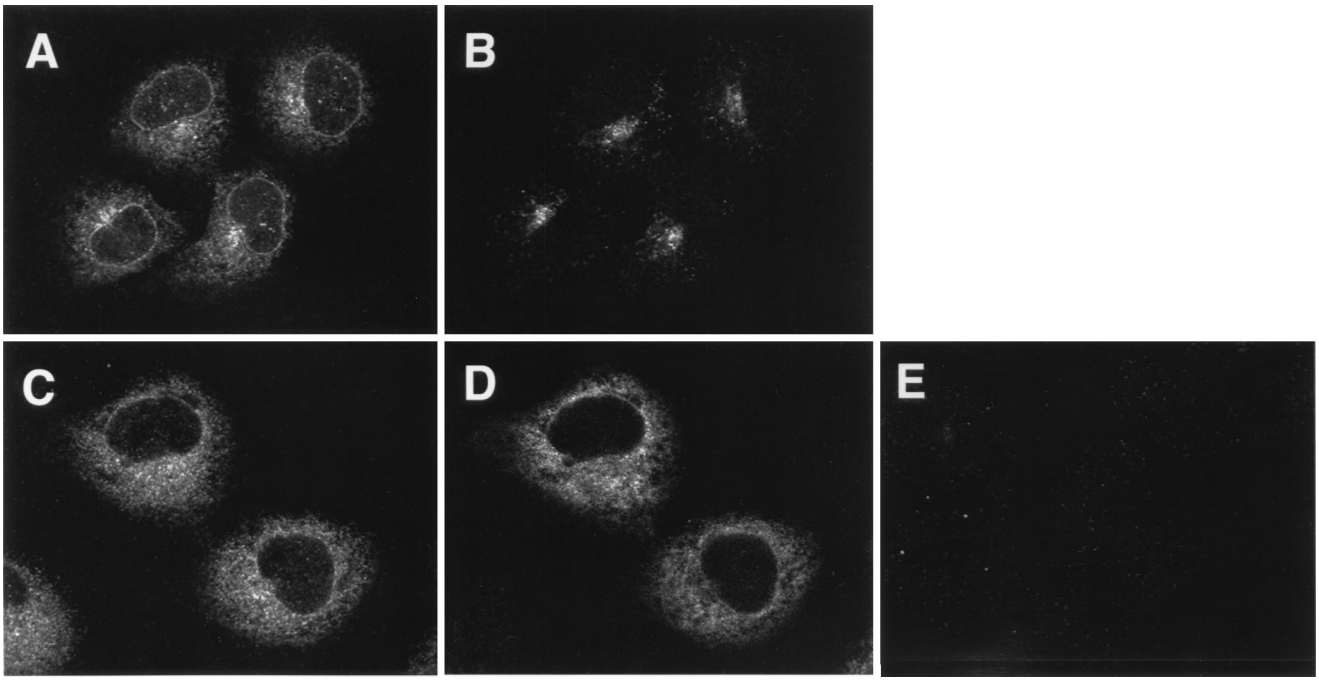

FIG. 9. Myt1Hu localizes to the ER and Golgi complex in HeLa cells. Indirect double immunofluorescent staining of HeLa cells was performed with affinity-purified anti-Myt1Hu polyclonal antibody and Cy3-conjugated donkey anti-rabbit secondary antibody (for detection of endogenous Myt1Hu [A]) and anti-γ-adaptin monoclonal antibody and FITC-conjugated donkey anti-mouse secondary antibodies (for detection of γ-adaptin [B]). Alternatively, HeLa cells were transfected with plasmids encoding PTP1B under the control of the cytomegalovirus promoter. Eighteen hours after transfection, cells were fixed and indirect double immunofluorescent staining of HeLa cells was performed. Visualization of endogenous Myt1 (C) was as described for panel A, and PTP1B was visualized by using an anti-PTP1B monoclonal antibody and FITC-conjugated donkey anti-mouse secondary antibodies (D). Myt1Hu antibody was incubated with excess peptide immunogen prior to indirect immunofluorescence (E). Fluorescence was detected by confocal microscopy.

was completely abolished by preincubation of the antibody with the synthetic peptide (lanes 2 and 5). The affinity-purified antibody was next used to determine the subcellular distribution of Myt1Hu. HeLa cells were fractionated into cytosol and membrane fractions by ultracentrifugation, and fractions were analyzed for the presence of Myt1Hu and the 14-3-3 family of proteins by immunoblotting. As seen in Fig. 7B, Myt1Hu partitioned with membranes (lane 3) whereas the 14-3-3 family of proteins partitioned with cytosol (lane 2), and high-salt treatment did not disrupt the membrane localization of Myt1Hu (lane 4).

To identify sequences in Myt1Hu responsible for membrane targeting, wild-type Myt1Hu and a series of Myt1 deletion mutants were transfected into HeLa cells and localized by indirect immunofluorescence using a monoclonal antibody specific to the Myc epitope tag (Fig. 8). Overexpression of Myt1Hu in HeLa cells revealed a reticular pattern of cytosolic staining that appeared more intense in the perinuclear region but also extended throughout the cytoplasm (Fig. 8B, panel a). Nuclear membrane but not plasma membrane staining was also observed. This pattern is characteristic of ER staining. In addition to the reticular staining pattern, most cells also showed an intensely stained region on one side of the nucleus, indicative of Golgi staining. Multivesicular bodies were observed in cells expressing high levels of Myt1Hu protein (panel a). A carboxy-terminal deletion of 98 amino acids of Myt1Hu had no effect on the subcellular distribution of Myt1Hu (Fig. 8B, panel c). However, a carboxy-terminal deletion of 137 amino acids resulted in Myt1 localizing primarily to the nucleus, although low levels of a diffuse cytoplasmic staining were also detected (Fig. 8B, panel e). These results suggested that sequences between amino acid 362 and 402 were required for membrane localization. Within this segment of 40 residues is a region with a predicted alpha-helical structure containing a stretch of 20 primarily hydrophobic or uncharged amino acids (GWALWQALLALLCWLWHGLA) bordered by arginine 378 and histidine 399. A similar region is conserved in Myt1Xe. A specific deletion of this region was made in Myt1Hu to determine whether it conferred a targeting function. As seen in Fig. 8B (panel g), deletion of amino acids 378 to 399 resulted in the localization of Myt1Hu primarily to the nucleus, demonstrating that amino acids 378 to 399 are necessary for conferring membrane targeting to Myt1Hu.

To localize endogenous Myt1Hu, indirect immunofluorescence was performed with the affinity-purified Myt1Hu-specific antibody. As shown in Fig. 9A, a pattern resembling ER and Golgi staining was observed for endogenous Myt1Hu. Identical results were obtained when cells were fixed with either paraformaldehyde, formaldehyde, or methanol. No staining was observed when the affinity-purified antibody was preincubated with excess peptide immunogen (Fig. 9E). As a marker for Golgi staining, a monoclonal antibody specific for the γ-adaptin protein was used in double-immunostaining experiments. As seen in Fig. 9B, confocal microscopy indicated colocalization between endogenous Myt1 and γ-adaptin. As a marker for ER staining, a monoclonal antibody specific for PTP1B was used. PTP1B localizes to the ER through a domain similar to that found in Myt1Hu (14). For these experiments, HeLa cells were transfected with plasmid encoding PTP1B and cells were costained with antibodies specific for Myt1Hu (Fig. 9C) and PTP1B (Fig. 9D). Colocalization of Myt1Hu with PTP1B is evident at the nuclear membrane and in reticular structures throughout the cytoplasm, supporting the conclusion that Myt1Hu is ER localized in human cells.

DISCUSSION

The major aims of this study were to isolate a cDNA encoding the protein kinase that phosphorylates Cdc2 on Thr 14 in human cells and to characterize its gene product. Here we report the cloning and characterization of Myt1Hu. Amino acid sequence comparisons indicate that Myt1Hu is most closely related to Myt1Xe (~46% sequence identity [35]) followed by other members of the Wee1 family of protein kinases (~15 to 20% sequence identity). Myt1Xe encodes a dual-specificity protein kinase that phosphorylates Cdc2 on both Thr 14 and Tyr 15 (35). All other members of the Wee1/Myt1 family of protein kinases phosphorylate Cdc2 exclusively on Tyr 15.

One common feature shared by SpWee1 and *Xenopus* and human Myt1 is the absence of a 19-amino-acid insertion that is present between subdomains VI and VII of other members of the Wee1/Myt1 protein kinase family (Fig. 1C). SpWee1 phosphorylates Cdc2 exclusively on Tyr 15 *in vitro*, yet paradoxically its gross overproduction in fission yeast results in detectable levels of Thr 14-phosphorylated Cdc2 (9, 13, 41, 42). In addition, SpWee1 autophosphorylates on both serine and tyrosine residues, and its catalytic domain is capable of phosphorylating the exogenous substrate enolase on both serine and tyrosine residues *in vitro* (13, 41, 42). SpWee1 is unique in this respect, as the human, *Xenopus*, *Drosophila*, and budding yeast Wee1-like kinases and the fission yeast Mik1 kinase are tyrosine-specific protein kinases (7, 8, 21, 26, 29, 34, 43). It will be interesting to determine whether the absence of the 19-amino-acid insertion is an important feature in conferring serine/threonine kinase activity to SpWee1, Myt1Xe, and Myt1Hu.

Throughout interphase in human cells, Cdc2-cyclin B complexes accumulate in the cytoplasm and the cyclin-bound form of Cdc2 is phosphorylated on both Thr 14 and Tyr 15. Phosphorylation on Thr 14 and Tyr 15 appears to be the primary mechanism of maintaining Cdc2-cyclin B complexes in an inactive state during the S and G_2 phases of the cell cycle. Interestingly, Wee1Hu is a nuclear protein (6, 20, 30). Thus, kinases other than Wee1 kinase must be present in the cytoplasm to catalyze the phosphorylation of Cdc2 on Thr 14 and Tyr 15. Previous biochemical studies of *Xenopus* egg extracts indicated the presence of a dual-specificity membrane-associated kinase capable of phosphorylating Cdc2 on both Thr 14 and Tyr 15 (2, 24). A cDNA encoding a kinase with these properties has been reported (35). This kinase, Myt1Xe, phosphorylates Cdc2 on both Thr 14 and Tyr 15 and is membrane localized. Antibodies specific for Myt1Xe were capable of immunodepleting the majority of the Thr 14 kinase activity from *Xenopus* egg extracts, indicating that it is the predominant Thr 14 kinase in these extracts. The purification of a Thr 14-specific protein kinase from bovine thymus has recently been reported (28). This purified kinase (designated Cdk T14 kinase) exhibits properties distinct from those of Myt1Hu in that it is a soluble cytosolic protein and it phosphorylates Cdks in a cyclin-independent manner.

Myt1Xe does not appear to undergo any autophosphorylation and is capable of phosphorylating Cdc2 on Thr 14 and Tyr 15 independently of one another (35). In contrast, Myt1Hu autophosphorylates on serine and tyrosine residues and shows a clear preference for Thr 14 phosphorylation over Tyr 15 phosphorylation. Mutants of Cdc2 substituting either alanine for Thr 14 or phenylalanine for Tyr 15 are both good substrates for Myt1Xe *in vitro*. In contrast, Myt1Hu quite readily phosphorylated Cdc2 on Thr 14 when Tyr 15 was mutated to phenylalanine but only poorly phosphorylated Cdc2 on Tyr 15 when Thr 14 was mutated to alanine. In addition, Myt1Hu exhibited a much lower apparent K_m for ATP when phosphorylating Thr 14 compared with Tyr 15 (Fig. 5D). Finally, whereas antiphosphotyrosine antibodies reacted with the slowest electrophoretic form of Cdc2, no detectable immunoreactivity was observed with the singly shifted form of Cdc2 (Fig. 4A). Taken together, these results indicate an ordered reaction with Thr 14 phosphorylation preceeding Tyr 15 phosphorylation.

Like other members of the Wee1/Myt1 family of protein kinases, Myt1Hu shows a strong preference for the cyclin-bound form of Cdc2 and appears to be a substrate for Cdc2-cyclin B complexes, although the functional significance of this phosphorylation is unclear. The following evidence indicates that Myt1Hu is a dual-specificity protein kinase: recombinant Myt1Hu purified from Sf9 insect cells autophosphorylated on both serine and tyrosine residues (Fig. 3C); coproduction of Myt1Hu with Cdc2 and cyclin B1 in insect cells resulted in the phosphorylation of Cdc2 on both Thr 14 and Tyr 15 (Fig. 4A and B); and GST-Myt1Hu purified from bacteria phosphorylated the cyclin B1-bound form of Cdc2(K33R) on both Thr 14 and Tyr 15 *in vitro* (Fig. 5). However, Myt1Hu demonstrated a clear substrate preference for Thr 14, and conditions were developed to generate Cdc2 that was exclusively phosphorylated on Thr 14 *in vitro* (Fig. 5 and 6). These conditions were then used to determine the individual contributions made by Thr 14 and Tyr 15 phosphorylation to the inhibition of Cdc2-cyclin B kinase activity (Fig. 6). The phosphorylation of Cdc2 on either Thr 14 or Tyr 15 reduced the histone H1 kinase activity of Cdc2-cyclin B1 complexes approximately 10-fold. Phosphorylation of Cdc2 on both Thr 14 and Tyr 15 caused a further 10-fold reduction in kinase activity. Thus, phosphorylation of Cdc2 on both Thr 14 and Tyr 15 reduced the kinase activity of Cdc2-cyclin B1 complexes by 2 orders of magnitude.

Previous biochemical fractionation studies indicated that the Cdc2 Thr 14 kinase was membrane bound both in interphase extracts prepared from *Xenopus* eggs (2, 24, 35) and in human HeLa cells (27a). Crude fractionation studies indicated that Myt1Hu was associated with a membrane compartment in HeLa cells (Fig. 7B). Visualization of endogenous Myt1 by indirect immunofluorescence revealed a pattern indicative of ER and Golgi staining. In support of this finding, Myt1 colocalized with both γ-adaptin (a Golgi marker) and PTP1B (an ER protein). Both Myt1Xe and human Myt1Hu contain a potential transmembrane targeting domain C terminal to their catalytic domains. This region consists of a stretch of 20 amino acids, most of which are either hydrophobic or uncharged and which are bordered by charged residues on either side. The specific deletion of this region in Myt1Hu resulted in the localization of Myt1 to the nucleus, demonstrating that this domain is essential for the targeting of Myt1 to the the ER and Golgi complex. A similar observation was made for PTP1B. PTP1B localizes to the ER by its C-terminal 35 amino acids, and deletion of this region results in the location of PTP1B to the cytoplasm and nucleus (14). A physiological role for the redistribution of PTP1B in platelets by calpain-mediated C-terminal cleavage has been proposed (15). It will be interesting to examine the compartmentalization of Myt1Hu in mitosis when the Golgi apparatus and ER disassemble.

Cyclin B2 reportedly localizes to the Golgi complex (23). Thus, the targeting of cyclin B2-Cdc2 complexes to the Golgi complex would serve to colocalize them with Myt1 and facilitate the phosphorylation of Cdc2. Cyclin B1 has been localized to centrosomes, to a detergent-resistant compartment, and to microtubules (4, 23). How are the microtubule-associated Cdc2-cyclin B1 complexes regulated by Myt1Hu? The membranes of the ER exhibit remarkable spatial extension, are

continuous with the nuclear envelope, interact with the cytoskeleton, and are specialized for diverse functions. The polygon-shaped ER networks are formed by extension, branching, and fusion of tubules. Branching and extension of ER tubules require microtubules and microtubule motor proteins, and continuous interaction of the ER and microtubules is necessary to maintain the ER structure (57). Because of this, ER-localized Myt1 and Cdc2-cyclin B1 complexes are likely to be colocalized within the cell. Although topology studies have not been performed, we predict that the kinase domain of Myt1 is oriented toward the cytosolic face of the ER and Golgi complex, allowing access of the kinase domain to Cdc2-cyclin B complexes. If Myt1Hu regulates cytosolic Cdc2-cyclin B complexes, what is the role of the nuclear Wee1Hu kinase? It has been proposed that Wee1Hu regulates the timing of mitosis by ensuring that cyclin B-Cdc2 complexes are kept inactive when they translocate into the nucleus in late G_2 (20). However, Wee1 may function to regulate nuclear Cdks. Both Cdk2 and Cdk4 complexes have been shown to be phosphorylated on tyrosine (19, 56), although Wee1Hu does not appear to phosphorylate Cdk4 *in vitro* (59). In addition, a nuclear cyclin B3 family member has been identified in chickens, and its associated Cdc2 subunit may be regulated by nuclear Wee1 rather than by cytosolic Myt1 (16).

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