SET-related Cell Division Autoantigen-1 (CDA1) Arrests Cell Growth*

We used an autoimmune serum from a patient with discoid lupus erythematosus to clone a cDNA of 2808 base pairs. Its open reading frame of 2079 base pairs encodes a predicted polypeptide of 693 amino acids named CDA1 (cell division autoantigen-1). CDA1 has a predicted molecular mass of 79,430 Daltons and a pI of 4.26. The size of the cDNA is consistent with its estimated mRNA size. CDA1 comprises an N-terminal proline-rich domain, a central basic domain, and a C-terminal bipartite acidic domain. It has four putative nuclear localization signals and potential sites for phosphorylation by cAMP and cGMP-dependent kinases, protein kinase C, thymidine kinase, casein kinase II, and cyclin-dependent kinases (CDKs). CDA1 is phosphorylated in HeLa cells and by cyclin D1/CDK4, cyclin A/CDK2, and cyclin B/CDK1 in vitro. Its basic and acidic domains contain regions homologous to almost the entire human leukemia-associated SET protein. The same basic region is also homologous to nucleosome assembly proteins, testis TSPY protein, and an uncharacterized brain protein. CDA1 is present in the nuclear fraction of HeLa cells and localizes to the nucleus and nucleolus in HeLa cells transfected with CDA1 or its N terminus containing all four nuclear localization signals. Its acidic C terminus localizes mainly to the cytoplasm. CDA1 levels are low in serum-starved cells, increasing dramatically with serum stimulation. Expression of the CDA1 transgene, but not its N terminus, arrests HeLa cell growth, colony numbers, cell density, and bromodeoxyuridine uptake in a dose-dependent manner. The ability of CDA1 to arrest cell growth is abolished by mutation of the two CDK consensus phosphorylation sites. We propose that CDA1 is a negative regulator of cell growth and that its activity is regulated by its expression level and phosphorylation.

Circulating autoantibodies to antigens in the nucleus are associated with systemic autoimmune diseases and are useful diagnostic markers for these diseases (reviewed in Ref. 1). These autoantibodies typically react with highly conserved epitopes of biologically important molecules and have proven highly useful as probes for the molecular cloning and functional characterization of their cognate autoantigens. For instance, human autoantibodies played a pivotal role in the discovery of molecules implicated in the splicing of pre-mRNA and in the identification of proliferating cell nuclear antigen as an auxiliary protein of DNA polymerase δ (1). We have ourselves successfully used this approach to identify novel proteins implicated in ion and protein transport (2–7).

Discoid lupus erythematosus is primarily a cutaneous subset of systemic lupus erythematosus associated with low titer autoantibodies to the nucleus in up to 40% of patients (8). We have shown previously that serum from a patient with discoid lupus erythematosus contains autoantibodies to the glycolytic protein enolase (9) and mitotic chromosomal antigens (10). Here, using serum from the same patient with discoid lupus erythematosus for immunoscreening a human cDNA library, we have isolated a novel nuclear antigen related to SET, a leukemia-associated protein. The transgenic expression of the novel nuclear antigen arrests cell growth. Mutation of its two consensus CDK1 phosphorylation sites abolishes its ability to arrest cell growth. CDA1 is a phosphoprotein that can be phosphorylated by CDKs in vitro. We named the molecule CDA1 (for cell division autoantigen-1).

EXPERIMENTAL PROCEDURES

Autoimmune Serum—The serum was from a patient with discoid lupus erythematosus identified at the Immunology Laboratories of Gribble’s Pathology (Melbourne, Australia).

Molecular Cloning and Sequence Analysis—DNA expression libraries of human testis (CLONTECH, catalog number HL 101b) and HeLa cells (Stratagene, catalog number 937216) were used. The human testis library was screened with the autoimmune serum as described (4). Positive plaques were detected with horse-radish peroxidase-labeled rabbit anti-human immunoglobulin (Dako A/S, Denmark) and enhanced chemiluminescence (DuPont). 5’ RACE products were generated on HeLa mRNA using 5’ RACE (Life Technologies, Inc., catalog number 18374-058) and primers based on the sequence of clone hT6 or hTsl-9 (Fig. 1A). Sublibraries for PCR screening were prepared by plating 50,000 plaque-forming units/plate of the cDNA library and harvesting the phages in 10 ml of SM buffer (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 6 mM MgSO4, and 0.01% [w/v] gelatin). Each sublibrary was tested for clones containing the 5’ end of the target cDNA by PCR using primers located at the very 5’ end of known cDNA sequences. PCR-positive sublibraries were replated and screened using 32P-labeled DNA probes. DNA sequencing was carried out as described (4). The predicted amino acid sequence was analyzed for hydrophilicity according

* This work was supported by a grant from the National Health and Medical Research Council of Australia and a Kathleen Cuningham grant from the National Breast Cancer Foundation. 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.

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The abbreviations used are: CDK, cyclin-dependent kinase; CDA1, cell division autoantigen-1; RACE, rapid amplification of cDNA ends; Pr, proline-rich; PCR, polymerase chain reaction; RT, reverse transcription; GST, glutathione S-transferase; PAGE, polyacrylamide gel electrophoresis; DM, double mutant; bp, base pairs; aa, amino acids; NLS, nuclear localization signal; BrdUrd, bromodeoxyuridine.

This paper is available on line at http://www.jbc.org

Vol. 276, No. 36, Issue of September 7, pp. 33665–33674, 2001
Printed in U.S.A.

Received for publication, August 23, 2000, and in revised form, May 29, 2001
Published, JBC Papers in Press, June 6, 2001, DOI 10.1074/jbc.M007681200

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to Kyte and Doolittle (11) and for secondary structure by the methods of Chou and Fasman (12–15) and Robson and co-workers (16, 17). 

Northern Blot Analysis—Human testis poly(A)⁺ RNA was from CLONTECH (catalog number 6535-1, lot number 6120921). Total HeLa RNA was prepared as described (19). mRNA was enriched using the Ambis kit 2.0 mRNA isolation system (Invitrogen). Northern blots of human testis and HeLa mRNA were carried out with [³²P]dCTP-labeled cDNA probes as described (19). Probe 1 is a PCR fragment of clone hT6 (nucleotides 1421–2280) and probes 2 (nucleotides 1595–2508) and 3 (nucleotides 741–1418) are PCR fragments of clone hTsl-9.

Affinity-purified Rabbit Antibodies to CDA1—Escherichia coli GST fusion proteins were produced in E. coli DH5α strain using pGEX vector (20) and purified using a glutathione-Sepharose 4B column (Amersham Pharmacia Biotech). Antibodies were raised in rabbits by immunization with purified GST-hT4 fusion protein (aa 552–695) as described (4) and affinity-purified by sequential passage through GST and GST-hT4 fusion protein columns. Bound antibodies were eluted from a GST-hT4 column in HCl-glycine, pH 2.5, supplemented with 1 mg/ml bovine serum albumin, neutralized to pH 7.0 with 2 x Tris and diazylated against PBS.

Serum Starvation and Stimulation—HeLa cells were serum-starved for 48 h or serum-starved for 48 h followed by stimulation with 10% fetal calf serum for 4 h and treated with thymidine or colcemid for 24 h to arrest cells at G2/S and M phases, respectively. M phase cells were removed by trypsinization and adherent cells were removed by scraping. CDA1 was detected by immunoblotting with anti-CDA1 antibodies. Cells were harvested and subjected to freeze/thawing, and the supernatants were removed by shake-off, and adherent cells were removed by scraping.

Stable tTa-HeLa cell lines containing trans- 

tTa-HeLa cells. To obtain stable cell lines, 40 μg of DNA of pTRE vector. A double mutant was constructed by digestion of both constructs with ClaI and XhoI and cloned into pCDNA3–2M, pCDNA3–2M containing Myc-tagged CDA1 or its N terminus was digested with SacII and XbaI and cloned into pTRE-tet (CLONTECH). The C terminus of CDA1 was amplified from hTsl-9, cloned into the pTRE-tet, and Myc-tagged at its N terminus. Purified pCDNA3–2M or pTRE-tet constructs were electroporated into HeLa or tTa-HeLa cells. To obtain stable cell lines, 40 μg of DNA of pTRE-tet constructs were co-transfected with pTK-Hyg plasmid in a ratio of 20:1, and cells were selected with hygromycin (200 μg/ml) in the presence of double mutations was confirmed by DNA sequencing. The expression of mutant constructs was confirmed by transient transfection followed by immunoblotting with an anti-Myc monoclonal antibody. A single Clai site is present between the two mutation sites and an XbaI site downstream of the multi-cloning sites of pTRE vector. A double mutant was constructed by digestion of both constructs with ClaI and XhoI followed by insertion of the T40A fragment into the XbaI site of the mutant sites were confirmed by DNA sequencing. The presence of double mutations was confirmed by NarI digestion.

Phosphorylation of CDA1 by Cyclin/CDKs in Vitro—CDA1— or the CDK phosphorylation site double mutant of CDA1 (CDA1-DM) was immunoprecipitated from lysates generated from transfected HeLa cells. Immunoprecipitated CDA1 or CDA1-DM was incubated in a final volume of 30 μl consisting of 20 mM Hepes, pH 7.0, 1.0 mM dithiothreitol, 10 mM MgCl₂, 100 μM ATP, 10 μCi [³²P]ATP, 10 μM vanadate, 10 mM NaF, and 10 mM β-glycerophosphate for 60 min at 37 °C in the presence or absence of 70 units of cyclin/CDK (one unit of cyclin/CDK activity is defined as the amount of kinase activity capable of transferring 1 pmol phosphate/μg GST-Prb³⁷⁹–⁷⁹/min at 37 °C). Purified cyclin/CDK complexes were prepared from the baculovirus expression system as described previously (26). Reactions were terminated by the addition of EDTA to a final concentration of 16 mM. Samples were washed three times with TIPA buffer (20 mM Tris, pH 7.4, 300 mM NaCl, 2 mM EDTA, 1% (w/v) Triton X-100, 1% (w/v) sodium-deoxycholate, and 0.1% (w/v) SDS) supplemented with 1 mM dithiothreitol and protease inhibitors (10 μM leupeptin, 10 μM aprotinin, and 300 μM phenylmethylsulfonyl fluoride). After washing, the samples were resuspended in SDS buffer (20 mM Tris–HCl, pH 6.8, 10% (w/v) glycerol, 2% SDS, 5% β-mercaptoethanol), heated at 100 °C for 2 min, centrifuged, and electrophoresed on 10% SDS polyacrylamide gel. After electrophoresis, proteins were stained with 0.5% Coomassie Brilliant Blue R, dried under vacuum, and exposed to Kodak Biomax MR x-ray film for autoradiography. Phospho-amino acid analysis and tryptic phosphopeptide mapping of phosphorylated CDA1 or CDA1-DM was performed as described (27).

RESULTS

Molecular Cloning—Clones hT4 and hT6 were isolated from a human testis cDNA library using the autoimmune serum (Fig. 1A), β-galactosidase fusion proteins produced by the clones hT4 (Fig. 1B) and hT6 (data not shown) were specifically immunoreactive with the autoimmune serum, confirming that they encoded the serum-reactive antigen. 5' RACE1 and 5' RACE2 products were obtained using HeLa mRNA. The clones

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HL59 and hTsl-9 were isolated by the PCR screening of sublibraries. Full-length cDNA comprising 2808 bp is consistent with the 2.8-kilobase size of HeLa and human testis mRNA (Fig. 1C). Expressed sequence tags corresponding to various regions of CDA1 were identified in the database (data not shown).

Sequence Analysis—The presumptive initiation ATG (nucleotides 129–131) was preceded by a Kozak initiation motif (GTCGCCATGG) (21, 22) followed by a GC-rich region favorable for ribosome binding (80–87%) between nucleotides 1–500 and 80–87% between nucleotides 150–250) (28). The open reading frame of 2079 bp encodes a predicted polypeptide of 693 amino acids (Fig. 2) with a molecular mass of 79,430 daltons and a pI of 4.26. The predicted protein sequence suggests that CDA1 comprises a proline-rich (Pr) N-terminal domain (aa 4–121), a central basic domain (aa 180–418), and a C-terminal bipartite acidic domain (aa 420–536 and 564–675, respectively). The protein is predicted to be hydrophilic and to have a secondary structure of 92% helices, several short β-pleated sheets and reverse turns, and two regions of potential coiled-coil structure (aa 200–250 and aa 600–630). It contains four putative nuclear localization signals (NLSs), three putative phosphorylation sites for cAMP- and cGMP-dependent kinase (aa 13–16, RRLS; aa 201–204, KRES; aa 412–415, KRRK), four for protein kinase C (aa 12–14, TRR; aa 182–184, SSR; aa 185–188, SSR and/or SSR; aa 392–394 SGR), one for tyrosine kinase (aa 282–290, RRDEDIFRY), multiple sites for casein kinase II, and two potential sites for phosphorylation by CDK1 (29, 30) and CDK2 (31–33) (Fig. 2B). A region of the C-terminal most acidic domain and a portion of the central basic domain share 40% identity and 68% similarity with almost the entire amino acid sequence of SET (34) including the 54 residues in its acidic C-terminal tail (Fig. 3, A and B). The same central region of CDA1 is homologous to other proteins related to SET (Fig. 3C), namely testis-specific protein Y-encoded (TSPY, GenBank accession number U58705), 3-β-glutamylcarboxypeptidase (35), and a 3-β-glutamylcarboxypeptidase (36).
Homology of CDA1 to human SET and related proteins. A, a diagram of CDA1 structure and its relationship to SET. Hom. 1 and Hom. 2 are homologous regions of CDA1 and SET, respectively (40% identity, 68% similarity). B, profiles of acidic and basic residues of CDA1 compared with SET. C, amino acid sequences of the homologous region of CDA1 (aa 216–395) aligned for maximum homology by introducing gaps (dashed lines) with human SET, human TSPY, KIAA, an uncharacterized human brain protein, and human nucleosome assembly proteins NAP1L, NAP1L3, and NAP2. Residues identical to CDA1 are boxed, and conservatively replaced amino acids are boxed. Inserted NAP residues are represented by “xxxxxx.”

Immunoochemical Characterization—Two bacterial GST fusion proteins encoded by cDNA clones hT4 and hT6 both reacted with autoimmune serum (data not shown). Rabbit antibodies to GST-hT4 fusion protein was affinity purified by sequential passage through GST and GST-hT4 fusion protein columns (Fig. 4A). Affinity-purified antibodies to CDA1 reacted with human and monkey cells but not with dog, hamster, mouse, or chicken cells (Fig. 4B). The migration of CDA1 in SDS-PAGE (120 kDa) was slower than that predicted by its molecular mass (79.43 kDa). The apparent molecular mass of 120 kDa was also observed in HeLa cells transfected with Myc-tagged CDA1 (Figs. 6, 7, and 9). The migration of GST-hT4 and GST-hT6 fusion proteins in SDS-PAGE (52 kDa and 84 kDa) was also slower than that predicted by the molecular mass of the fusion proteins (44 kDa and 56 kDa, data not shown). Aberrant migration of proteins containing acidic amino acids has been reported previously (35).

CDA1 is present in the nuclear but not cytosol and membrane fractions of untransfected HeLa cells (Fig. 4C). Myc-tagged CDA1 or its N terminus containing all four putative NLSs localizes by immunofluorescence to the nucleus and nucleolus of HeLa cells transfected with these constructs (Fig. 4D). The same localization pattern was also observed in HeLa cells transfected with pEGFP-CDA1 (data not shown). In contrast, the nuclear fraction (lane 2) but not in cytosol (lane 3) and membrane (lane 4) fractions. D, the immunofluorescence of HeLa cells transfected with Myc-tagged CDA1 (a) or its N terminus reacted with anti-Myc antibody (b). Note the nucleus and nucleolus staining. HeLa cells transfected with the C terminus of CDA1 show predominant cytoplasmic staining (c).
Expression of CDA1 after Serum Starvation or Stimulation—To determine whether CDA1 levels vary with cell growth, HeLa cells were serum-starved for 48 h or starved for 48 h and then stimulated with 10% fetal calf serum for 4 h followed by treatment for 24 h with either 2 mM thymidine to block cells at G1/S and S phase or with 0.15 μg/ml colcemid to block cells at M phase. CDA1 was just detectable in serum-starved cells, increasing dramatically in stimulated cells peaking in S phase (Fig. 5).

Transgenic Expression of CDA1 Arrests Cell Growth—To further investigate the role of CDA1 on cell growth, we established stable HeLa cell lines transfected with CDA1 under the control of a “tetracyclin-off” responsive promoter. The withdrawal of doxycycline from the culture medium of stable cell lines transfected with CDA1 resulted in maximal expression of the CDA1 transgene after 3–4 days (Fig. 6A). Transgenic expression of CDA1 correlated with the complete arrest of cell growth over the entire 4 days of culture.

To determine whether the total arrest of cell growth observed is a consequence of expression of any transgene, we compared the growth of cells transfected with CDA1 to that of stable cell lines transfected with its N terminus (Fig. 6B). We confirmed that expression of the CDA1 transgene for 5 days completely arrested cell growth over this entire period. This was collaborated by morphological assessment of cell density. DNA synthesis in S phase assessed by the BrdUrd incorporation rate showed a gradual decline over the first 3 days with complete arrest by days 4 and 5. However, the DNA content histograms showed normal cell cycle profiles. In contrast, expression of the N-terminal transgene did not arrest cell growth (Fig. 6B) nor did it affect cell density or BrdUrd uptake (data not shown). Over 90% of growth-arrested cells remained viable over this time. The expression of CDA1 and its N-terminal transgenes was confirmed by immunoblotting (data not shown).

To address whether the ability of CDA1 to arrest cell growth depends on expression levels of the CDA1 transgene, stable cell lines transfected with CDA1 were cultured for 2 weeks for colony outgrowth assay or for 5 days for cell growth assay with doxycycline in concentrations ranging from 0 to 5 ng/ml (Fig. 7). The incremental increase in CDA1 expression induced by decreasing doxycycline concentrations was accompanied by a corresponding incremental decrease in colony numbers, cell growth, cell density, and BrdUrd incorporation rate but with retention of normal cell cycle profiles. Colony outgrowth, cell

**Fig. 6.** CDA1 transgene expression arrests cell growth. A, HeLa cells (2 × 10^5) stably transfected with Myc-tagged CDA1 were cultured with or without 5 ng/ml doxycycline for 4 days. The cells were counted (a) and immunoblotted (50 μg protein/lane) with anti-Myc antibody (b). B, HeLa cells (4 × 10^5) stably transfected with CDA1 or its N terminus were cultured overnight with 5 ng/ml doxycycline and then in doxycycline-free medium for 5 days. a, cells were fixed and stained with crystal violet, and the optical density of cell-extracted dye was determined. b, cell viability assessed by Trypan blue exclusion (percentage of dead cells). c, phase-contrast microscopy shows cell density. d, BrdUrd incorporation and cell cycle profiles. Stably transfected HeLa cells were cultured without doxycycline for 5 days and labeled with BrdUrd. The dot plot shows cells incorporating BrdUrd (FL1-H) and DNA content (FL2-A). R4, percentage of cells with DNA content equivalent to S phase but not synthesizing DNA. The histogram of cell cycle profiles shows the cell number (counts) against DNA content (FL2-A).
growth, cell density, and BrdUrd incorporation rates were dramatically suppressed with maximal expression of the CDA1 transgene. The inhibition of cell growth was not accompanied by any change in cell viability. In contrast, expression of the N-terminal transgene did not inhibit colony numbers, cell growth, cell density, or BrdUrd incorporation rates (data not shown for colony growth, cell density, and BrdUrd incorporation).

Transgenic Expression of CDA1 Containing Mutant CDK Phosphorylation Sites (CDA1-DM) Fails to Arrest Cell Growth—To determine the role of the two CDK consensus phosphorylation sites for CDA1 activity, we generated a Myc-tagged CDA1 construct in which both consensus phosphorylation sites were mutated. The serine at position 20 and threonine at position 340 of wild-type CDA1 was replaced with alanine to generate the double mutant (CDA1-DM). A HeLa cell line, 2C6D3 stably expressing Myc-tagged CDA1-DM, was established using the same Tet-off system. In contrast to wild-type CDA1, overexpression of the double mutant failed to arrest HeLa cell growth determined by BrdUrd incorporation and DNA content (Fig. 8A). On days 4 and 5, after doxycycline was withdrawn from the medium to turn the transgene on, the cells were still actively up-taking BrdUrd and showed similar cell cycle profiles as those with the transgene turned off. Immunoblotting of cells on the same days showed overexpression of Myc-tagged CDA1-DM, whereas the cells with the transgene turned off had undetectable levels of transgene product (Fig. 8B). Immunofluorescence staining of this cell line using anti-Myc antibody showed identical staining patterns and intensities as a wild-type cell line (data not shown). NarI digestion of RT-PCR products confirmed the presence of the double mutation in transgene transcripts regulated by doxycycline (Fig. 8C).

The S20A and T340A mutations generated additional NarI sites not present in wild-type CDA1. The S20A RT-PCR product (381 bp) was amplified from transcripts of cells stably transfected with mutant CDA1 using a vector primer and a CDA1-specific primer that detected transgene transcripts only. The T340A RT-PCR product (667 bp) was similarly amplified using instead a pair of CDA1-specific primers that detected endogenous as well as transgene transcripts. RT-PCR products derived from cells with the transgene turned on showed an identical-sized band as PCR products derived from wild-type and mutant CDA1 plasmids using the same primers. NarI digestion of RT-PCR products derived from mutant CDA1 transcripts gave the same digestion patterns as PCR products generated from mutant CDA1 plasmids with expected sizes (213 and 169 bp for S20A; 512 and 155 bp for T340A). The PCR products generated from wild-type CDA1 were not digested. With the S20A RT-PCR reaction, no product was seen using transcripts from cells with the transgene turned off, indicating an absence of detectable transgene transcripts. With the T340A RT-PCR reaction, the product from transcripts derived from cells with the transgene off was of a lower yield and was not digested by NarI, suggesting that it was the RT-PCR product of endogenous wild-type CDA1.

CDA1 Is Phosphorylated in Vivo—To determine whether CDA1 is phosphorylated in vivo, HeLa cells transfected with Myc-tagged CDA1 were metabolically labeled with either [35S]methionine or 32P. A 35S- or 32P-labeled 120-kDa protein was specifically immunoprecipitated by an anti-Myc antibody (Fig. 9A). The 32P-labeled Myc-tagged N terminus of CDA1 containing putative phosphorylation sites was also specifically immunoprecipitated (data not shown).
CDA1 Is Phosphorylated in Vitro by Cyclin/CDKs—Because mutation of the two CDK phosphorylation sites of CDA1 abolished its growth-inhibitory activity, we sought to determine whether CDA1 is phosphorylated on these sites by cyclin/CDKs. We incubated CDA1 immunoprecipitated from HeLa cells with purified catalytically active cyclin/CDK complexes in \textit{in vitro} phosphorylation reactions. These studies revealed that CDA1 was phosphorylated in the absence of added cyclin/CDK, which was probably caused by the activity of a co-immunoprecipitating kinase. The level of CDA1 phosphorylation was increased by cyclin D1/CDK4, cyclin A/CDK2, and cyclin B/CDK1, but not by cyclin E/CDK2 (Fig. 9B). CDA1-DM was also phosphorylated in the absence of added cyclin/CDKs. However, the level of CDA1-DM phosphorylation was not increased by any of the added cyclin/CDKs, indicating that phosphorylation depended on the presence of the CDK phosphorylation site(s). This was confirmed by tryptic phosphopeptide mapping, which demonstrated that CDA1 phosphorylated in the absence of cyclin/CDKs displayed four major phosphopeptides, 1–4 (Fig. 9C). CDA1 phosphorylated with cyclin A/CDK2 significantly increased the phosphorylation of these phosphopeptides and generated three major new phosphopeptides, 5–7, resulting from the phosphorylation of either one or both of the CDK phosphorylation sites. The generation of several tryptic phosphopeptides from the phosphorylation of one or two sites is caused by the generation of partial digests by tryptic cleavage. To define which site(s) on CDA1 was phosphorylated by cyclin A/CDK2, we performed phosho-amino acid analysis of CDA1 phosphorylated either in the absence or presence of cyclin A/CDK2. These studies revealed that CDA1 was phosphorylated basally on threonine and to a greater extent on serine (Fig. 9D). The level of serine and threonine phosphorylation was increased by cyclin A/CDK2, confirming that both serine 20 and threonine 340 were phosphorylated by this kinase \textit{in vitro}.

DISCUSSION

We report the molecular characterization of a novel cell division nuclear autoantigen that we have named CDA1. CDA1 localization to the nucleus is supported by the following observations. First, the predicted amino acid sequence of CDA1 contains four putative NLSs. Second, CDA1 localizes to the nuclear fraction of HeLa cells. Third, Myc-tagged or enhanced green fluorescent protein-tagged CDA1 and its N-terminal segment containing all four nuclear localization signals localizes to the nucleus and nucleolus of transfected HeLa cells. The nuclear localization of CDA1 is supported further by observations of Ueki et al. (36), who used a nuclear transportation trap for isolating nuclear proteins. A partial cDNA clone isolated in this way (GenBank\textsuperscript{TM} accession number AB015345) and encoding aa 208–693 of CDA1 localizes to the nucleus in COS-7 cells. These latter observations indicate that the third and fourth NLSs contained in aa 208–693 of CDA1 are sufficient to target CDA1 to the nucleus. The role
of the first and second NLSs in targeting CDA1 to the nucleus or nucleolus remains unknown.

Endogenous CDA1 levels are low in resting serum-starved HeLa cells and dramatically elevated in serum-stimulated cells. These observations suggest a role for CDA1 in cell growth. The suggestion is supported by our findings in stable HeLa cell lines transfected with Myc-tagged CDA1 in which CDA1 levels can be regulated by a tetracyclin-off promoter. Maximal expression of the CDA1 transgene in cells cultured in the absence of doxycycline was observed after 3–5 days. CDA1 transgene expression was associated with dramatic arrest of cell growth and cell density over the 4–5 days of culture. DNA synthesis assessed by BrdUrd uptake showed a gradual decline in the first 3 days followed by a virtual complete arrest by days 4–5. The capacity of CDA1 to arrest cell growth was confirmed further in the stable HeLa cell lines in which we regulated CDA1 expression levels by culture in the presence of different doxycycline concentrations. An incremental decrease of HeLa colony numbers, cell growth, cell density, and BrdUrd uptake paralleled corresponding incremental increases in CDA1 expression, indicating that cell growth and DNA synthesis depends on the level of expression of the CDA1 transgene. Inhibitory effects of CDA1 were not accompanied by a change in cell viability or in cell cycle profiles. The latter results were surprising, because we had expected the cells in which DNA synthesis had been arrested to accumulate at the G1/S transition. One possible explanation for these results is that CDA1 may exert inhibitory effects on multiple stages of the cell cycle, acting as a negative regulator of cell cycle progression. The suggestion is consistent with the elevated levels of endogenous CDA1 observed in G1, S, and M phases of the cell cycle.

Stable transfectants of HeLa cells harboring the CDA1 N-terminal transgene lacking its acidic C-terminal tail did not arrest cell growth or DNA synthesis. These observations suggest that the inhibition of cell growth and DNA synthesis observed with the CDA1 transgene is not the consequence of expression of just any transgene. The observations also suggest that the inhibitory effects of CDA1 on cell growth and DNA synthesis requires its acidic C-terminal tail. The acidic C-terminal tail of CDA1 has ~40% identity and 68% similarity with the acidic C-terminal tail of the leukemia-associated protein SET. The central region of CDA1 shares the same level of identity and similarity with most of the remainder of the SET protein and is also homologous to the other SET-related proteins, namely human nucleosome assembly proteins (NAPs), TSPY testis protein, and an uncharacterized brain protein KIAA0721. NAPs also have single or multiple acidic regions,

Fig. 9. CDA1 is phosphorylated in HeLa Cells and by cyclin/CDKs in vitro. A, HeLa cells transfected with Myc-tagged CDA1 were metabolically labeled by [35S]methionine or 32P. CDA1 (120 kDa, arrow) labeled by 35S or 32P is immunoprecipitated by an anti-Myc antibody and not by mouse IgG1 isotype control antibody (control antibody). B, CDA1 (lanes 5–9) or the phosphorylation site double mutant of CDA1 (CDA1-DM) (lanes 10–14) was immunoprecipitated from HeLa cells and then phosphorylated either in the absence (control) or presence of purified catalytically active cyclin D1/CDK4 (D1/K4), cyclin E/CDK2 (E/K2), cyclin A/CDK2 (A/K2), or cyclin B/CDK1 (B/K1), as described under “Experimental Procedures.” The phosphorylated proteins were resolved by SDS-PAGE and visualized by autoradiography. The positions of the CDA1 and CDA1-DM is indicated with an arrow. Lanes 1–4 represent autophosphorylation of cyclin/CDKs in the absence of CDA1 or CDA1-DM. C, CDA1 was phosphorylated either in the absence or presence of cyclin A/CDK2 (A/K2) and subjected to tryptic phosphopeptide mapping as described under “Experimental Procedures.” The positions of the major phosphopeptides are indicated with arrows. The origin is labeled ORI. D, CDA1 was phosphorylated either in the absence or presence of cyclin A/CDK2, separated by SDS-PAGE, transferred to polyvinylidene difluoride, and then hydrolyzed with HCl. The phospho-amino acids were separated by two-dimensional electrophoresis and visualized by staining with ninhydrin and autoradiography. The positions of phosphoserine (P-Ser), phosphothreonine (P-Thr) and phosphotyrosine (P-Tyr) are indicated with arrows.
Although they are not located consistently in the C-terminal tail.

Human SET is a 32-kDa ubiquitous nuclear protein first identified fused to CAN in acute undifferentiated leukemia (34, 37). The fused set-can gene includes a large part of the acidic tail of SET. CAN has also been found fused with DEK in a subtype of acute myeloid leukemia (38), suggesting that CAN is an oncogene activated by fusion with SET or DEK. The only common motif of SET and DEK is their acidic domain. SET is a potent and specific inhibitor of protein phosphatase 2A, suggesting that leukemic effects of these proteins may be related to their interactions (40). SET has been rephatase 2A, suggesting that leukemic effects of these proteins may be related to their interactions (40). SET has been reported recently to associate directly with the CDK inhibitor p21Cip1 and to reverse p21-mediated inhibition of cyclinE/CDK2 activity. Based on these observations, it has been suggested that SET modulates p21Cip1-inhibitory activity and regulates G1/S transition by modulating cyclin E/CDK2 activity (42). SET has also been identified as the template-activating factor 1 required for adenovirus genome replication. The acidic tail of SET is required for this activity (43).

The predicted amino acid sequence of CDA1 contains putative phosphorylation sites for a variety of protein kinases including CDK1 and CDK2. Our demonstration that CDA1 is phosphorylated in HeLa cells suggests that one or more of these sites are phosphorylated, raising the possibility that CDA1 activity is regulated not only by expression level but also by phosphorylation. In this context, Xenopus SET has been shown to interact with B-type cyclins (44). Saccharomyces cerevisiae and Xenopus NAP1, a 60-kDa protein homologous to SET, also binds to B-type cyclins and is phosphorylated by the cyclin B/CDK1 complex. SET interacts with cyclin/CDKs that are active during the G1 to S phase transition, these studies suggest that CDA1 is phosphorylated in vivo, suggesting that these kinases may regulate CDA1 function in vivo. These observations suggest that in addition to the expression levels of CDA1, phosphorylation of these two sites by cyclin/CDKs may play important roles in regulating the function of this protein in cellular proliferation. Interestingly, cyclin D1/CDK4, cyclin A/CDK2, and cyclin B/CDK1 phosphorylated CDA1, whereas cyclin E/CDK2 did not. Because cyclin E/CDK2 is active during the G1 to S phase transition, these studies suggest that CDA1 may be differentially phosphorylated throughout the cell cycle on serine 20 and threonine 340 to regulate its function and in turn cellular proliferation.

In the absence of NAP1 in Saccharomyces cerevisiae, Clb2, a B-type cyclin, is unable to induce mitotic events or switch from polar to isotropic bud growth (45). Yeast cells lacking NAP1 also undergo a long delay at the short spindle stage with normal levels of Clb2/CDK1 kinase activity, suggesting that NAP1 is required for the regulation of microtubule dynamics and for Clb2/CDK1 kinase to amplify its own product. Saccharomyces cerevisiae NAP1 binds tightly to Gin4 kinase and is required for kinase activation by phosphorylation by Clb2 as cells enter mitosis. In turn, Gin4 kinase is required for NAP1 and Clb2 to promote progression through mitosis and for switching from polar to isotropic bud growth (46). NAP1 and NAP2 are phosphorylated in vivo at the G1/S boundary but not in S phase (47). Because CDA1 overexpression showed an activity opposite to that of SET and NAP, it is possible that phosphorylated CDA1 may inhibit activity of one or more CDKs that regulate cell cycle progression.

CDA1 contains an N-terminal Pr domain that is not present in SET or other related proteins. Pr domains are found in ligand domains of a number of regulatory proteins. These Pr domains bind to Src Homology 3 domains contained in a variety of intracellular and membrane-associated proteins and kinases (48–53) involved in signal transduction. The common amino acid sequence motif of Pr domains for Src Homology 3 domain binding is PXXP with neighboring residues forming patterns specific for individual Src Homology 3 domain of different proteins (54–57). CDA1 has three regions containing PXXP motifs, although no specific patterns are found for binding to known Src Homology 3 domains. The region also contains stretches of nine and five P residues. The observations suggest that CDA1 may interact, through its Pr domain, with unique cognate protein(s), the identification of which may provide more information on its function.

Acknowledgments—We thank Kulwant Sekhon for help with cell proliferation assays, Effie Smith for serum samples, and Wendy Pollock of Gribble’s Pathology for obtaining the serum samples.

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J. Biol. Chem. 2001, 276:33665-33674.
doi: 10.1074/jbc.M007681200 originally published online June 6, 2001

Access the most updated version of this article at doi: 10.1074/jbc.M007681200

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