CDCA4 Is an E2F Transcription Factor Family-induced Nuclear Factor That Regulates E2F-dependent Transcriptional Activation and Cell Proliferation*

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The TRIP-Br1/p34SEI-1 family proteins participate in cell cycle progression by coactivating E2F1- or p53-dependent transcriptional activation. Here, we report the identification of human CDCA4 (also known as SEI-3/Hepp) as a novel target gene of transcription factor E2F and as a repressor of E2F-dependent transcriptional activation. Analysis of CDCA4 promoter constructs showed that an E2F-responsive sequence in the vicinity of the transcription initiation site is necessary for the E2F1–4-induced activation of CDCA4 gene transcription. Chromatin immunoprecipitation analysis demonstrated that E2F1 and E2F4 bound to an E2F-responsive sequence of the human CDCA4 gene. Like TRIP-Br1/p34SEI-1 and TRIP-Br2 (SEI-2), the transactivation domain of CDCA4 was mapped within C-terminal acidic region 175–241. The transactivation function of the CDCA4 protein was inhibited by E2F1–4 and DP2, but not by E2F5–8. Inhibition of CDCA4 transactivation activity by E2F1 partially interfered with retinoblastoma protein overexpression. Conversely, CDCA4 suppressed E2F1–3-induced reporter activity. CDCA4 (but not acidic region-deleted CDCA4) suppressed E2F1-regulated gene promoter activity. These findings suggest that the CDCA4 protein functions as a suppressor at the E2F-responsive promoter. Small interfering RNA-mediated knockdown of CDCA4 expression in cancer cells resulted in up-regulation of cell growth rates and DNA synthesis. The CDCA4 protein was detected in several human cells and was induced as cells entered the G1/S phase of the cell cycle. Taken together, our results suggest that CDCA4 participates in the regulation of cell proliferation, mainly through the E2F/retinoblastoma protein pathway.

The E2F family of transcription factors integrates cellular signals and coordinates cell proliferation (1, 2). Studies in recent years have identified E2Fs as important transcriptional regulators of the expression of many genes involved not only in DNA replication and cell cycle progression, but also in DNA damage repair, apoptosis, and cell differentiation and development (3, 4). Among the E2F family members, E2F1–5 possess a transcriptional activation domain at the C terminus and can induce transcription from target promoters together with dimerization partner DP1 or DP2 (5, 6). In contrast, E2F6 lacks a transcriptional activation domain and has been shown to compete for E2F-binding sites on promoters and to repress their activity (7). The E2F family is often subdivided into activator E2Fs (E2F1–3) and repressor E2Fs (E2F4–6) based on the pattern of their interactions with retinoblastoma tumor suppressor pocket-binding protein (pRb)2 family members (8, 9). The pRb proteins interact directly with the E2F C-terminal activation domain and silence it (10, 11). E2F1–3 can all bind exclusively to pRb, but the pRb relatives p107 and p130 interact specifically with repressor E2Fs. Among repressor E2Fs, E2F4 uniquely interacts with the pRb, p107, and p130 proteins (9). E2F7 and E2F8, very recently identified as members of the E2F family, share unique structural features, including the absence of dimerization, pRb binding, and transcriptional activation domains (12–17).

E2F1 binds DNA as a heterodimer with the DP1 or DP2 protein and achieves activation through a C-terminal activation domain (10, 18), which has been shown to interact directly with coactivators such as TATA-binding protein and MDM2 (19, 20). Many transcription factors recruit histone acetyltransferase and histone deacetylase (HDAC) activities. The appearance of histone acetyltransferases, including cAMP-responsive element-binding protein-binding protein (CBP), p300, p300/ CBP-associated factor (PCAF), and Tip60, correlates with the timing of induction of E2F-dependent transcription (21–23). Indeed, p300 and CBP interact with the activation domain of

2 The abbreviations used are: pRb, retinoblastoma tumor suppressor pocket-binding protein; HDAC, histone deacetylase; CBP, cAMP-responsive element-binding protein-binding protein; PHD, plant homeodomain; siRNA, small interfering RNA; DDB, DNA-binding domain; RT, reverse transcription; TAD, transactivation domain; GST, glutathione S-transferase; GFP, green fluorescent protein; HA, hemagglutinin; RNAi, RNA interference; FBS, fetal bovine serum; BrdUrd, 5-bromo-2-deoxyuridine; PBS, phosphate-buffered saline; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; ChIP, chromatin immunoprecipitation; BisTris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol; MES, 4-morpholineethanesulfonic acid.

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CDCA4 Regulates E2F Transcriptional Activity

E2F1 and stimulate E2F1-mediated activation as coactivators (24, 25). On the other hand, HDAC1–3 activity is implicated in the pRB-mediated repression of E2F-regulated promoters in the G1 phase (26–28). To achieve cell cycle arrest into the G1 phase, pRB also requires the activity of Brg1/Brm, the two known human homologs of the yeast nucleosome-remodeling complex SWI2/SNF2 (29). Prohibitin and TopBP1 are known as Brg1/Brm-dependent E2F1-mediated repressors (30). TopBP1, a DNA replication initiator/DNA repair protein, is induced by E2F1 and recruits Brg1/Brm to repress E2F1-induced apoptosis (31).

Among transcriptional regulator proteins, TRIP-Br1/CDCA4 (SEI-1) (hereafter referred to as TRIP-Br1; transcriptional regulator interacting with PHD bromodomain 1) belongs to a novel family of proteins that share the N-terminal SERTA (for SEI-1, RBT1, and TARA) motif (32). TRIP-Br1 was originally reported to activate cyclin D1–Cd4 by antagonizing the inhibitory effect of p16INK4a during the late G1 phase of the cell cycle (33). The SERTA motif has been shown to be responsible for the interaction between TRIP-Br1 and Cd4. Interestingly, TRIP-Br1 and TRIP-Br2 (SEI-2) make a direct functional contact with E2F1/DP1, stimulating E2F1 transcriptional activity (34). RBT1 (replication protein A-binding transactivator 1), a SERTA motif-containing protein, has also been characterized as a transcriptional coactivator, but uniquely binds the second subunit of replication protein A (35). Recently, in addition to TRIP-Br1 and TRIP-Br2, SEI-3 (hereafter referred to as CDCA4, a HUGO Gene Nomenclature Committee-approved gene symbol; characterized previously as murine Hepp (36)) was characterized as a coactivator of p53-dependent transcriptional activation possibly through interacting with CBP or the ING (inhibitor of growth) family of chromatin-associated proteins, although the growth inhibition induced by overexpression of those proteins was p53-independent (37).

As an approach toward a better understanding of the full extent of gene expression under the control of the E2F/pRb pathway in cell proliferation, we characterized a series of DNA replication initiators (38, 39), including members of the novel CDCA (cell division cycle-associated) family of genes as candidate genes, using DNA microarray experiments (3). The genes of the CDCA family are characterized by the association of their expression patterns with those of known cell cycle genes such as CDC2, CDC7, and cyclins (40). We focused initially on human CDCA4 to determine whether the CDCA4 protein is functionally related to TRIP-Br1 and TRIP-Br2. Interestingly, CDCA4 repressed E2F1-induced transcriptional activation, although TRIP-Br1 and TRIP-Br2 coactivated it. Small interfering RNA (siRNA)-mediated gene knockdown of CDCA4 resulted in an increase in cell proliferation independent of the p53 status. We suggest a model in which CDCA4 functions as a critical modulator of cell proliferation and serves as a negative feedback regulator of activator E2Fs by inhibiting E2F-dependent transcriptional activation.

EXPERIMENTAL PROCEDURES

Bioinformatics—Bioinformatics analysis was performed as described previously (41). The TRANSFAC program (motif. genome.jp/) was used to determine the transcription factor-binding elements (42). The amino acid sequences of human TRIP-Br1 family proteins were aligned using the ClustalW Version 1.83 program (clustalw.genome.jp/) (43). The domain structure of the CDCA4 protein was searched using the Pfam program (pfam.wustl.edu/) and BLAST algorithms (www.ncbi.nlm.nih.gov/) (44).

Construction of Plasmids—Human CDCA4 promoter fragments were generated by PCR from genomic DNA and ligated into the KpnI-digested pGL3-Basic vector (Promega Corp.). PCR primers were designed to amplify 1125-bp (−1097+28; pGL3-ABCD), 747-bp (−747+28; pGL3-BCD), 709-bp (−681+28; pGL3-D), and 594-bp (−681−88; pGL3-delD) fragments of the CDCA4 promoter sequences, which are numbered relative to the transcription initiation site at position +1 described in the NCBI UniGene Database (Genome View). The GenBank accession number of the genomic clone used for the design of the PCR primers was AL512356. The forward (F) and reverse (R) PCR primers used were as follows: −1097F, 5′-CAGCCACTTTG- GCCAGCC-3′; −847F, 5′-TTGCCCACTATTCTCTC-3′; −747F, 5′-TACCTCCAGGTTCGCCGAC-3′; −681F, 5′-CTCAGGGATTGATCTGAGGAT-3′; −88R, 5′-CTCTCCGGCCGCTGCGGCAGC-3′; and +28R, 5′-TCCAAAGGGTGCGACGCT-3′. Kpl1 sites were added to the forward and reverse primers to facilitate subcloning. Motif D (5′-TGTGGCGC-3′) was mutated (5′-TGTGAGC-3′) for pGL3-mutD.

To construct the Gal4 DNA-binding domain (DBD) and the CDCA4 fusion protein expression vector, reverse transcription (RT)-PCR was performed as described below. The PCR products were digested with BamHI and EcoRI and ligated into their respective sites in the pCMV-BD vector (Stratagene). The GenBank accession number of the cDNA clone used for the design of the PCR primers was NM_145701. The forward (F) and reverse (R) PCR primers used were as follows (with the BamHI sites in the forward and the EcoRI sites in the reverse primers underlined): BD1/747F, 5′-GTCGACAGCTCTGGCGGTCTTTG-CAAGGAGCAGTTGATCG-3′; BD2/37F, 5′-CCGGATCTTCCTGGCGGAGAGAG-3′; BD/75F, 5′-CCGGATCTCAGGGATGGCACTATTGACG-3′; BD/174R, 5′-CCGGATCTCTGGCGGAGAGAG-3′; DBD/37F, 5′-CTTCCGCTTCCGTCTAGAG-3′; BD/241R, 5′-CCGGATCTTTTTCAGGCTTTCTAGAG-3′.

The full-length transcription activation domain (TAD) of E2F1 was also cloned into the pCMV-BD vector. The GenBank accession number of the cDNA clone used for the design of the PCR primers was NM_005225. The PCR primers used were 5′-CCGGATCCGGCTTGGCGGGCCCGCT-3′, 5′-CCGGATCTTTAAGAGAGAGAGAGGCTTCCAGC-3′, and 5′-CCGGATCTTTAAGAGAGAGAGC-3′ (with the BamHI sites in the forward primers and the EcoRI sites in the reverse primers underlined).

The N-terminally FLAG-tagged CDCA4 and glutathione S-transferase (GST)-tagged CDCA4 expression vectors were constructed as follows. Briefly, PCR products were digested with

3 K. Yoshida, unpublished data.
BamHI and EcoRI and ligated into their respective sites in the pCMV-TAG2B (Stratagene) and pGEX-6P-1 (Amersham Biosciences) vectors, respectively. The forward and reverse PCR primers used were DBD/1F and DBD/241R, respectively. The enhanced green fluorescent protein (GFP)-CDCA4 fusion protein expression vector was constructed by PCR. The PCR products were digested with EcoRI and BamHI and ligated into their respective sites in the pEGFP-N2 vector (Clontech). The forward (F) and reverse (R) PCR primers used were as follows: 1F/GFP, 5'-CGGAATTCGCCGCATGTTTGACAGAGACTGAG-3'; 3F/EFP, 5'-CGGAATTCGCCGCATGTTTGGACAGAGACTGAG-3'; 174R/GFP, 5'-CGGAATTCGCCGCATGTTTGGACAGAGACTGAG-3'; and 241R/GFP, 5'-CGGAATTCGCCGCATGTTTGGACAGAGACTGAG-3'. The pcDNA3-HA-E2F1, pcDNA3-HA-E2F2, pcDNA3-HA-E2F3, pcDNA3-HA-E2F4, and pcDNA3-HA-E2F6 expression plasmids (where HA denotes hemagglutinin) were a gift from Dr. Joseph R. Nevins (Duke University Medical Center). The pCMV-HA-E2F5, pCMV-HA-TPBP, pCMV-HA-pRb, pCMV-HA-p107, and pCMV-HA-p130 expression plasmids were supplied by Dr. Junji Magae (Institute of Research and Innovation, Paris, France). The pTAG4A-p53 expression vector was a gift from Dr. Kazuhiko Yamane (Case Western Reserve University of Pennsylvania Medical Center), pBJ5-Brg1 by Dr. Wei-dong Wang (Ohio State University). The pRcCMV-pRb expression vector was a gift from Dr. Robert A. Weinberg (Whitehead Institute for Biomedical research, Massachusetts Institute of Technology). The pMT3-HA-mTRIP-Br1 and pMT3-HA-hTRIP-Br2 expression plasmids were a gift from Dr. Stephen I-Hong Hsu (National University of Singapore). The FLAG-tagged p300 and HA-tagged CBP expression plasmids were obtained from the BioResource Center (Tsukuba Institute, RIKEN). pF-Bmi1 was kindly provided by Dr. Jay L. Hess (University of Pennsylvania Medical Center), pB5-Br1 by Dr. Wei-dong Wang (National Institutes of Health, Baltimore), and pcDNA3-TopBP1-Myc by Dr. Junjie Chen (Mayo Clinic, Rochester, MN) and Dr. Kazuhiko Yamane (Case Western Reserve University, Cleveland, OH). The human Brm expression vector (4102) was a gift from Dr. Christian Muchardt (Institut Pasteur, Paris, France). The pTAG4A-p53 expression vector was described previously (45). As a mock transfection, pcDNA3 (Invitrogen) or pCMV-based vectors were used. The reporter plasmids pGL3-MCM7 (pGL3-0.46 kb) and pGL3-geminin (pGL3-4.98 kb) were described previously (38). The pE2F-TA-Luc and pTA-Luc expression vectors were purchased from Clontech.

**Cell Culture and RNA Interference (RNAi)—HeLa (RBC0007, BioResource Center) and A549 cells (TKG0184, Cell Resource Center for Biomedical Research, Institute of Development, Aging, and Cancer, Tohoku University) were cultured in Earle’s modified Eagle’s medium (Invitrogen) supplemented with 10% fetal bovine serum (FBS; Bioserum, Fukuyama, Hiroshima, Japan), 1% nonessential amino acids (Invitrogen), and antibiotics/antimycotics (Invitrogen). Saos-2 cells (TKG0469, Cell Resource Center for Biomedical Research) were cultured in McCoy’s 5A medium (Invitrogen) supplemented with 10% FBS and antibiotics/antimycotics. WI-38 cells (IFO05075, Health Science Research Resources Bank, Osaka, Japan) were cultured in Earle’s modified Eagle’s medium supplemented with 10% FBS and antibiotics/antimycotics. To measure the growth-dependent induction of human CDCA4 gene expression, Saos-2 cell growth was arrested in the G0 phase by incubation in the absence of FBS for 3 days, and the cells were reintroduced into the cell cycle by culture with 20% FBS. Cell lysates were recovered at 0, 14, and 24 h after serum stimulation.

To determine the number of viable cells in culture, the WST-1 assay (Roche Applied Science) and the Cell Titer-Glo luminescent cell viability assay (Promega Corp.), which are based on quantification of the ATP present, were performed in accordance with the manufacturers’ protocols. For RNAi, 2 x 10^5 cells were transfected with Lipofectamine 2000 (Invitrogen) following the manufacturer’s instructions. Cells were plated onto a 6-well plate prior to transfections. Briefly, 125 pmol of annealed siRNA duplex (Ambion, Inc.) and 2.5 μl of transfection reagent were incubated in 0.5 ml of Opti-MEM I reduced serum medium (Invitrogen) for 15 min to facilitate complex formation. The resulting mixture was added to cells cultured in 2 ml of modified Eagle’s medium. Twenty-four hours after the first siRNA transfection (day 1), cells were split 1:1 to maintain the cells in the log phase. Three hours after splitting, the second siRNA transfection was performed. The target sequences of the oligonucleotides used were as follows: CDCA4-1, 5’-GGUGUCACCAUGCUUUGUGCCt-3’ (sense) and 5’-GCACACGAAAAACACACCCt-3’ (antisense); and CDCA4-2, 5’-GCACACGAAAAACACACCCt-3’ (sense) and 5’-UCAAGUGACUGUUGAAAGCCt-3’ (antisense). Silencer negative control #1 siRNA (Ambion, Inc.) was used as a negative control. The cells were lysed after 24 h (day 2) and 72 or 96 h (day 3 or 4, respectively) following the second RNAi transfection to isolate total RNA and protein, respectively.

**5-Bromo-2’-deoxyuridine (BrdUrd) Incorporation Assay**—To determine DNA synthesis based on BrdUrd, labeling was performed (BrdUrd labeling and detection kit III, Roche Applied Science) in accordance with the manufacturer’s protocol. Control or CDCA4 siRNA was transfected into HeLa cells as described above. Twenty-four hours after the second siRNA transfection (day 2), 3 x 10^4 cells were plated onto a 96-well plate. The next day, cells were prelabeled with 10 μM BrdUrd for 14 h before treatment. Cells were washed twice with cold phosphate-buffered saline (PBS) and fixed in 70% ethanol and 0.5 M HCl for 30 min at −20 °C. Cells were then washed three times with PBS and treated with nuclease. After incubation for 30 min at 37 °C, the cells were washed three times with PBS, and 1% bovine serum albumin-containing PBS including peroxidase-conjugated anti-BrdUrd monoclonal antibody was added to each well. After incubation for 30 min at 37 °C, cells were washed three times with PBS. BrdUrd labeling was visualized using peroxidase substrate and was measured at 405 nm with a reference wavelength at 490 nm.

**Luciferase Reporter Assay**—For the reporter assay, 2 x 10^4 cells were transfected with FuGENE 6 (Roche Applied Science) following the manufacturer’s instructions. Briefly, 200 ng of expression plasmid, 200 ng of firefly luciferase reporter plasmid (pGL3-Basic or pE2F-TA-Luc), and 0.6 ng of Renilla luciferase reporter plasmid (pRL-TK, Promega Corp.) on a 24-well dish were used for each transfection. For TAD mapping, 200 ng of pCMV-BD construct, 200 ng of firefly luciferase reporter plasmid (pFR-Luc, Stratagene), and 0.6 ng of Renilla luciferase...
CDCA4 Regulates E2F Transcriptional Activity

reporter plasmid (pRL-TK) on 24-well dish were used for each transfection. The cells were harvested 24 or 48 h after transfection, and a luciferase assay was performed using the Dual-Luciferase reporter assay system (Promega Corp.) in accordance with the manufacturer’s protocol. The results were read with a luminescence microplate reader (Wallac 1420 ARVOx multi-label counter, PerkinElmer Life Sciences). Experiments were performed at least in triplicate. As a control for transfection efficiency, the firefly luciferase activity values were normalized to Renilla luciferase activity values. Data are presented as the means ± S.D. Statistical differences were analyzed using Student’s paired t test. A p value of <0.05 was considered to indicate a statistically significant difference.

RT-PCR—Total cellular RNA was extracted from the cultured cells using the RNeasy mini kit (Qiagen Inc.) following the manufacturer’s instructions. Control and E2F1 adenoviral vectors and virus were kindly provided by Dr. Kiyoshi Ohtani (Tokyo Medical and Dental University). The virus preparation and the virus infection procedure were as described (45, 46). The RT step was performed as recommended by Invitrogen. Briefly, 500 ng of extracted RNA, oligo(dT) primer, and 1× annealing buffer were diluted in 8 μl of RNase/DNase-free water, heated to 65 °C for 5 min, and then chilled on ice. For first-strand cDNA synthesis, a heat-denatured RNA solution, together with 2× first-strand reaction mixture and SuperScript III/RNaseOUT enzyme mixture, was added to make up 20 μl of the reaction mixture, followed by incubation at 50 °C for 5 min, heating to 85 °C for 5 min, and cooling on ice. To amplify CDCA4 cDNA, primers 1F/GFP and 241R/GFP were used. To check the mRNA abundances in 16 different human tissues, for amplification of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was included in the kit. PCR was then performed as follows: denaturation at 94 °C for 3 min, followed by 25–30 cycles at 94 °C for 15 s, 60 °C for 30 s, and 68 °C for 1 min. To amplify ORC1 (NM_004153) and CDC25A (NM_001789) cDNAs, the following primers were used: ORC1, 5’-CTGAGAGCCATCCTCGCAAGA-3’ and 5’-GCTGGGCA-TTGTTGCCATGTG-3’; and CDC25A, 5’-TAGTTGGGCTC-CACAGGATG-3’ and 5’-AAGTCTGCCCCAGCTCTTGT-3’. PCR was then performed as follows: denaturation at 94 °C for 3 min, followed by 27 cycles at 94 °C for 15 s, 60 °C for 30 s, and 68 °C for 1 min. As a control, a GAPDH primer set (R&D Systems) was used. The amplified products were separated on 1% agarose gels and visualized by ultraviolet transillumination.

Chromatin Immunoprecipitation (ChIP)—ChIP assays were performed using the EZ-Chip kit (Upstate) following the manufacturer’s instructions with some modification (38, 39). Briefly, 2 × 10⁶ A549 cells were used for each ChIP; asynchronously growing cells were treated with formaldehyde at a final concentration of 1% to create protein-DNA cross-links; and the cross-linked chromatin were then extracted, diluted with SDS lysis buffer containing protease inhibitor mixture, and sheared by sonication (Branson Sonifier II) on ice to fragments with an average length of ~1000 bp. After being precleared with protein G-agarose at 4 °C for 1 h, the chromatin was divided into equally sized samples for immunoprecipitation with 2–5 μg of anti-E2F1 (catalog no. sc-193), anti-E2F4 (catalog no. sc-866), or anti-E2F6 (catalog no. sc-22823) antibody (Santa Cruz Biotechnology, Inc.) or anti-FLAG polyclonal antibody (Sigma). After overnight incubation at 4 °C, preblocked protein G-agarose was added, and the resultant immunocomplexes were pelleted by centrifugation after 1 h of incubation at 4 °C and washed with the buffer supplied with the kit. The immunoprecipitates were resuspended in TE buffer (10 mM Tris-HCl (pH 7.6) and 1 mM EDTA), and the cross-links were reversed by incubation with proteinase K and RNase A. After phenol/chloroform extraction and ethanol precipitation, the pellets were resuspended in 50 μl of distilled water and analyzed by PCR. As an input control, 0.5% volume of chromatin was amplified. The PCR primers used to detect the presence of specific DNA sequences, including the predicted E2F consensus sequence (site D, −85/−78), were 5’-CGAGAGAGTC-CACAACCTCTG-3’ and +28R. The PCR primers corresponding to the human actin promoter (negative control) are described elsewhere (76). The amplified products were separated on 1.2% agarose gels and visualized by ultraviolet transillumination.

Generation of Anti-CDCA4 Antibody—For the preparation of GST-CDCA4, DNA fragments encoding full-length human CDCA4 cDNA were amplified by PCR with primers DBD/1F and DBD/241R as described above and cloned into pGEX-6P-1 at the BamHI and EcoRI sites. The resultant construct was transformed and expressed in Escherichia coli BL21(DE3)pLysS cells (Promega Corp.) as a GST fusion protein. The bacterium-harbouring construct was incubated in Luria-Bertani medium at 37 °C until A₆₀₀ = 0.6. After isopropyl β-D-thiogalactopyranoside induction at a final concentration of 0.5 mM, bacteria were further incubated at 37 °C for 4 h. The GST-tagged CDCA4 protein was lysed with BugBuster HT protein extraction reagent (Novagen) and electrophoresed on NuPAGE 4–12% Bis-Tris gel with MES running buffer (Invitrogen). The gel was stained with GelCode Blue (Pierce), and the resultant bands corresponding to GST-CDCA4 protein were recovered.

Anti-CDCA4 polyclonal antiserum was prepared by immunizing a rabbit with the GST-CDCA4 fusion protein comprising full-length CDCA4 fused to the C terminus of GST. Rabbits were immunized six times with 250 μg of immunogen emulsified with Freund’s complete or incomplete adjuvant (2-week intervals up to 3 months). A terminal bleed was collected by cardiac puncture 24 days after the final injection. Recovered antiserum (sixth immune serum) was affinity-purified, and the immunoglobulin fraction was concentrated with protein A beads. The sixth immune serum was diluted 500-fold and used for Western blotting.

Western Blotting and Immunoprecipitation—HeLa cells (2 × 10⁶) were transfected with 1–2 μg of various eukaryotic expression constructs mixed with Lipofectamine Plus reagent (Invitrogen) in accordance with the manufacturer’s instructions. Forty-eight hours after transfection, cells were harvested and lysed in radioimmuneprecipitation assay buffer (50 mM Tris-HCl (pH 7.6), 150 mM NaCl, 1% Nonidet P40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, 1 μg/ml pepstatin, and 1
µg/ml leupeptin) for 20 min on ice. The cell lysates were centrifuged, and the protein concentration was determined using the Bio-Rad protein assay kit. Before being subjected to SDS-PAGE, the reaction was stopped by the addition of lithium dodecyl sulfate sample buffer (Invitrogen) containing 100 mM dithiothreitol. After being heated at 70 °C for 10 min, equal amounts of cellular protein (20–50 µg) were electrophoresed on NuPAGE 4–12% Bis-Tris gel with MES running buffer and transferred to a Hybond ECL nitrocellulose membrane (Amersham Biosciences). The membrane was first blocked in PBS containing 0.1% Tween 20 and 5% nonfat dried milk and then incubated with the following antibodies: anti-Gal4 DBD (catalog no. sc-510, Santa Cruz Biotechnology, Inc.), anti-GAPDH (Ambion, Inc.), anti-HA (catalog no. H9658, Sigma), and anti-Myc (Invitrogen) monoclonal antibodies and anti-E2F1, anti-GFP (catalog no. 632459, Clontech), and anti-cyclin A (catalog no. sc-751, Santa Cruz Biotechnology, Inc.) polyclonal antibodies, followed by alkaline phosphatase-conjugated anti-mouse or anti-rabbit immunoglobulin (Promega Corp.). Western Blue stabilized substrate (Promega Corp.) was used to detect the signals in accordance with the manufacturer’s protocol. An INSTA-Blot membrane containing nine different human cell line lysates (10 µg/lane) was purchased from Imgenex (catalog no. IMB-105), and immunoblotting was performed following the manufacturer’s instructions.

For immunoprecipitation of GFP-tagged CDCA4, 1 × 10^17 cells was lysed with 10 mM Tris-HCl (pH 7.8), 1% Nonidet P-40, 150 mM NaCl, 1 mM EDTA, and 10 µg/ml aprotinin and mixed with 30 µl of protein G-Sepharose (Amersham Biosciences) and anti-GFP polyclonal antibody overnight at 4 °C with gentle rotation. Alternatively, FLAG-tagged CDCA4 was lysed with lysis buffer containing 50 mM Tris-HCl (pH 7.4), 1% Triton X-100, 150 mM NaCl, 1 mM EDTA, and protease inhibitor mixture (catalog no. P8340, Sigma) and immunoprecipitated with anti-FLAG monoclonal antibody-agarose gel (catalog no. A2220, Sigma). Beads were washed four times, pelleted, and boiled in lithium dodecyl sulfate sample buffer, and the immunoprecipitates were subjected to SDS-PAGE. Subsequent to transfer onto a membrane, the blot was incubated with anti-GFP (catalog no. 632380, Clontech), anti-E2F1 (catalog no. sc-239, Santa Cruz Biotechnology, Inc.), or anti-cyclin A (catalog no. sc-251, Santa Cruz Biotechnology, Inc.) monoclonal antibody or anti-CDCA4 polyclonal antibody.

Fluorescence Microscopy—To analyze the subcellular localization of CDCA4 and its derivatives, a GFP fusion-based method was employed. The cells were transfected as described above. After 48 h, transfected cells were washed twice with cold PBS and then incubated in 4% paraformaldehyde-containing PBS at room temperature for 10 min. The cells were photographed with a Leica DM IRB fluorescence microscope.

RESULTS

Identification of CDCA4 as a Novel Transcription Factor E2F-regulated Gene—To unveil the genes whose expression is regulated by the transcription factor E2F1, we established a human cell line in which the expression of E2F1 was induced by the addition of doxycycline. Total RNA extracted from the cells before and after the induction was subjected to DNA microar-ray analysis, and the list of candidate genes was revealed. Among the identified genes, we decided to focus on the analysis of CDCA4 gene regulation and function because it is plausibly involved in cell cycle regulation (40). This notion prompted us to examine whether CDCA4 is a bona fide transcript induced by transcription factor E2F1. First of all, we checked the change in endogenous CDCA4 mRNA expression in another experimental system. For this purpose, HeLa cells were infected with an adenovirus encoding E2F1, and the isolated total RNA was subjected to RT-PCR analysis. Exogenously overexpressed E2F1 (but not the control adenovirus) increased endogenous CDCA4 mRNA expression, whereas the GAPDH mRNA expression level remained unchanged (Fig. 1A).

Next, we examined the expression of CDCA4 mRNA in human tissues. Previous work demonstrated the mRNA expression level of Cdca4 in mouse tissues, but not in human tissues (36, 37). Murine Cdca4 (Hepp) was identified as a gene expressed specifically in hematopoietic progenitor cells as opposed to hematopoietic stem cells (36). More recently, it has been reported that Cdca4 is expressed in a spatiotemporally restricted pattern during mouse embryogenesis (47). These results suggest that CDCA4 may be involved in specific cell differentiation and development as well as cell cycle regulation. To further investigate the expression pattern of CDCA4 in human tissues and organs, RT-PCR was performed. The human CDCA4 mRNA was expressed at higher levels in the pancreas, thymus, testis, spleen, liver, placenta, and leukocytes, and at relatively low levels in the lung, kidney, prostate, ovary, small intestine, and colon (Fig. 1B). In contrast, CDCA4 mRNA was slightly or not detected in the brain, skeletal muscle, and heart, whereas GAPDH mRNA was ubiquitously expressed in all tissues examined (Fig. 1B).

Identification of the cis-Elements and E2F Regulatory Elements of the CDCA4 Promoter—To substantiate the E2F-dependent transcriptional regulation of the CDCA4 gene, we set out to identify E2F regulatory elements of the CDCA4 promoter. First, we searched the proximal region of the transcription start site for potential cis-elements using TRANSFAC soft-
ware (Version 4.0, cutoff 85). The promoter region of human CDCA4 was found to contain four putative E2F-binding motifs, named A, B, C, and D, at positions −1076/−1069 (motif A; opposite the direction of transcription), −803/−796 (motif B), −703/−696 (motif C), and −85/−78 (motif D; opposite the direction of transcription) relative to the transcription initiation site (position +1) (Fig. 2A). The predicted E2F-binding motif D is surrounded by a GC-rich sequence and CAAT box. The presence of multiple E2F- and Sp1-binding motifs has also been recognized in many other growth-regulated genes such as E2F1 and geminin (38, 48).

To assess the basal promoter activity, we generated a series of human CDCA4 promoter-luciferase constructs containing various regions of CDCA4 promoter sequence and inserted them into a luciferase reporter construct (designated pGL3-ABCD, pGL3-BCD, pGL3-CD, pGL3-D, and pGL3-delD) (Fig. 2A). As shown in Fig. 2B, the pGL3-ABCD reporter construct, which contains the CDCA4 promoter with the 5′-end at position...
−1097 and the 3′-end at position +28 relative to the transcription initiation site, showed an ~90-fold increase in activity in HeLa cells (as determined by measuring the relative luciferase activities) compared with that in the control luciferase vector (pGL3-Basic, taken as 1). To determine in detail the cis-elements essential for the basal promoter activity of CDCA4, deletion reporter constructs lacking the 5′- or 3′-end of pGL3-ABC were assayed. All of the CDCA4 promoter constructs exhibited almost equal promoter activity compared with full-length pGL3-ABC (Fig. 2B). These results suggest that the putative E2F-binding motif and the proximal Sp1 site may not play critical roles in maintaining basal human CDCA4 promoter activity.

Next, we attempted to determine the E2F-responsive site in the CDCA4 promoter region. As shown in Fig. 2C, exogenous coexpression of E2F1 together with various CDCA4-reporter plasmid constructs including the deletion of E2F-responsive sites A–C caused an increase of up to 5-fold, whereas pGL3-Basic, pGL3-delD, and pGL3-mutD showed no increase in promoter activity compared with the pcDNA3 control vector. These results suggest that the E2F-binding motif D plays a critical role in E2F1-mediated human CDCA4 promoter activity. We sought evidence showing that members of the E2F family transcriptionally regulate the CDCA4 gene. Exogenous coexpression of E2F1–4 caused an increase of up to 3–6-fold in pGL3-D (but not pGL3-delD) reporter activity, whereas coexpression of E2F4 and E2F5 was associated with no increase in promoter activity compared with that in the pcDNA3 control vector (Fig. 2D). Furthermore, the luciferase reporter construct pGL3-mutD showed no change when cotransfected with the E2F1–6 expression vectors. For comparison of the expression levels of transfected plasmids, we performed Western blotting. A representative experiment showed that HA-tagged E2F1, E2F4, and E2F6 were almost equally detected after 48 h of transfection (Fig. 2E).

To substantiate the contention that E2F1 can bind to the endogenous human CDCA4 promoter, ChIP assays were performed using antibodies against E2F1, E2F4, and E2F6. The chromatin immunoprecipitated by the antibodies was then amplified by PCR with primers specific for the CDCA4 or control actin promoter (see Fig. 2A for primer location). DNA fragments containing E2F-responsive motif D in CDCA4 were specifically detected in the anti-E2F1 and anti-E2F4 antibody-immunoprecipitated fractions, whereas no genomic DNA fragment was detected when anti-E2F6 antibody or an irrelevant antibody (anti-FLAG) was added in place of the anti-E2F1 and anti-E2F4 antibodies (Fig. 2F). No association with actin was detected with any antibodies used. These results indicate that E2F1 and E2F4, which represent activator and suppressor E2Fs, respectively, are associated with the human CDCA4 promoter under physiological conditions.

CDCA4 Protein Possesses a Potent TAD in the C-terminal Region, and This Activity Is Inhibited by Transcription Factors E2F1–4—On the basis of the alignment of human TRIP-Br family members, their domain was well conserved (Fig. 3A). Human CDCA4 exhibits a substantial degree of conservation (27% identity and 41% similarity) with TRIP-Br1, including three characteristic sequence motifs, including the cyclin A-binding, heptad repeat (the SERTA motif), and acidic (including the PHD zinc finger- and bromodomain-binding) regions (Fig. 3A) (34, 37). Phylogenetic analysis of human TRIP-Br family members based on the ClustalW algorithm revealed that the human CDCA4 protein is the most remotely related to other proteins among TRIP-Br family proteins (Fig. 3B).

Recently, CDCA4 has been shown to possess transactivation activity; however, the functional region has not been identified (37). For this purpose, the TAD of CDCA4 was mapped by deletion analysis. We prepared expression vectors for the Gal4 DBD fused to the various regions of the CDCA4 protein (Fig. 3C). These expression constructs were transfected into HeLa cells, and the expression levels and protein sizes were monitored by Western blotting using anti-Gal4 DBD antibody (Fig. 3D, left panel). Next, these constructs were cotransfected with the pFR-Luc reporter plasmid containing five copies of the Gal4 DBD-binding element, and luciferase activity was checked. Both full-length CDCA4 (DBD/1–241) and cyclin A-binding region-deleted CDCA4 (DBD/37–241) stimulated reporter activity by ~24- and 75-fold, respectively, compared with the Gal4 DBD alone (Fig. 3C). Further deletion of the N terminus of CDCA4 including the heptad repeat region (DBD/75–241) strongly enhanced reporter gene activation by ~4513-fold, indicating that the heptad repeat serves as a suppressive region within the CDCA4 protein. E2F1 consists of an N-terminal cyclin A-Cdk-binding domain, a DBD, a dimerization domain, and a C-terminal TAD (6). As illustrated in Fig. 3C (lower), we fused full-length E2F1 or its C-terminal TAD to DBD to compare the transactivation properties of CDCA4 and E2F1. The activity seen for DBD/75–241 derived from the CDCA4 protein was compatible with that for the C-terminal TAD (residues 286–437, including the indicated boxed region and the pRb-binding domain) of E2F1 fused to DBD (DBD/E2F1-TAD) (~6787-fold) (Fig. 3C). Full-length E2F1 fused to DBD enhanced reporter activity, but to a lesser extent (~58-fold). The expression level of E2F1 fused to DBD was checked by Western blotting (Fig. 3D, right panel). The result was similar to that obtained with CDCA4, where transactivation by the C-terminal region of CDCA4 was stronger than that by full-length CDCA4. The C-terminal acidic region of CDCA4 (DBD/175–241) was enough to potently enhance reporter activity (~475-fold) (Fig. 3C). In contrast, deletion of the C-terminal 67 amino acids of CDCA4 (DBD/1–174) resulted in a significant decrease in transcriptional activity to the basal level (~1-fold), indicating that the C-terminal acidic region is responsible for transactivation of CDCA4. The N-terminal of the CDCA4 protein, more specifically the SERTA domain, was predicted to have a nuclear localization signal (37, 47). As described below, N- or C-terminally deleted CDCA4 fused to GFP (73–241/GFP or 1–174/GFP, respectively) exclusively localized to the nucleus, indicating that deletion constructs could function in the nucleus as transcriptional coregulators.

E2F1 has been shown to stimulate TRIP-Br1 and TRIP-Br2 transactivation in a mammalian one-hybrid assay (34). To investigate whether this could be true for CDCA4, we examined the effect of E2F1 expression on the transactivation function of CDCA4 in a luciferase reporter assay. HeLa cells were cotransfected with the pFR-Luc reporter plasmid and
CDCA4 Regulates E2F Transcriptional Activity

A. The amino acid sequences of the human TRIP-Br family proteins, including TRIP-Br1 (NM_013376), TRIP-Br2 (NM_014755), RBT1 (NM_013368), and CDCA4 (NM_145701), are shown together with the characteristic domains. Identical residues are indicated with asterisks, and similar residues are indicated with single or double dots. Characteristic domains, including the cyclin A-binding, heptad repeat, and acidic regions, are underlined. B. Shown are the results from phylogenetic analysis of human TRIP-Br family members. ClustalW multiple sequence alignment was performed to construct a neighbor-joining tree.

C. Shown is a schematic of various Gal4 DBD/CDCA4 deletion mutants. The characteristic domains of CDCA4, including the cyclin A-binding, heptad repeat, and acidic regions, are indicated. DBD/full-length E2F1 (DBD/E2F1-full) and the N-terminal half-region-deleted version (DBD/E2F1-TAD) were used as controls. The characteristic domains of E2F1, including the DBD (DNA-binding), dimerization domain, and TAD, are indicated. Transcriptional activation of the CDCA4 protein was recruited to a heterogeneous promoter and was suppressed by E2F1. Cells were transiently transfected with the indicated CDCA4 protein derivatives fused in-frame to the Gal4 DBD, the luciferase minimal reporter plasmid pFR-Luc bearing five copies of the Gal4 DBD-binding sequence followed by the TATA box, and pRL-TK as an internal control. As a positive control, full-length E2F1 and its TAD were fused to the Gal4 DBD (activity is shown by the gray bars). -Fold activation refers to firefly luciferase activity normalized to Renilla luciferase activity and is expressed relative to the firefly activity observed with transfection of the Gal4 DBD alone (taken as 1). Values represent the means ± S.D. of three or four independent experiments. When the indicated constructs were cotransfected with the expression vector for E2F1, the transactivation activities induced by the DBD/CDCA4 chimeric protein decreased to the basal level (indicated in parentheses). The dashed lines omit the gap (500–4000-fold activation) between the longer and shorter bars.

D. Western blot analysis of 50 μg of total cell lysate from each transfection was performed with mouse anti-Gal4 DBD monoclonal antibody. Molecular mass markers are shown on the left.

E. The E2F1–4 proteins inactivate CDCA4-induced transactivation. HeLa cells were transiently transfected with 200 ng of the indicated expression constructs together with DBD or DBD/1–241 and pFR-Luc plus pRL-TK. -Fold activation refers to firefly luciferase activity normalized to Renilla luciferase activity and is expressed relative to the firefly activity observed with transfection of the Gal4 DBD (with pCMV; taken as 1). Values represent the means ± S.D. of three or four independent experiments. G, Western blot analysis was performed with the cell lysates transfected with pcDNA3 (control), FLAG-tagged p300, HA-tagged TRIP-Br1 and TRIP-Br2, and Myc-tagged TopBP1. Molecular mass markers are shown on the left. Arrows mark the positions of the proteins used in F.
DBD alone or various DBD/CDCA4 chimeric expression constructs. Unexpectedly, coexpression of E2F1 completely suppressed the enhanced expression of the luciferase reporter produced by DBD/1–241, DBD/37–241, and DBD/175–241 to the basal level (Fig. 3C). Similar to these phenomena, DBD/75–241, which exhibited most potent transcriptional activity, decreased to ~1.4% of its full activity (Fig. 3C). Given these findings, we also tested whether other E2F family members could inhibit CDCA4-mediated transcriptional activity. HeLa cells were cotransfected with the pFR-Luc reporter plasmid and DBD alone or the DBD/1–241 expression construct together with E2F1–8, DP2, p53, Bmi1, Brg1, or Brm. Interestingly, E2F2–4 as well as E2F1 completely suppressed the enhanced expression of the luciferase reporter produced by DBD/1–241 (~20-fold) to the basal level (Fig. 3E). In contrast to E2F1–4, E2F5–8 had no effect on the transactivation of CDCA4. DP2 has significant homology to E2Fs, sharing the dimerization and DNA-binding domains. Indeed, DP2 partially suppressed the transactivation of CDCA4. p53 or Bmi1, one of the best characterized members of the mammalian Polycomb protein, was used for the assay, but had no effect. In addition, Brg1 and Brm, which are responsible for the ATPase activity of the SWI/SNF chromatin-remodeling factor, showed no effect (Fig. 3E). No change in luciferase reporter activity was observed when cells were cotransfected with the vector plasmid (DBD alone) with or without the E2F1 expression vector (Fig. 3E).
CDCA4 Regulates E2F Transcriptional Activity

pRb has the ability to regulate cell cycle progression and to arrest cells in the G1 phase through interaction with E2F1–4, but not with E2F5–8 (26). To further delineate the mechanism, pRb family proteins were exogenously cotransfected with E2F1, pRb (but not p107 or p130) effectively reversed the suppressive effect of E2F1 on the transactivation function of CDCA4 (Fig. 3F), suggesting that pRb and E2F1 regulate CDCA4 function in a mutually opposing manner. CDCA4 has been shown to interact with the p300/CREB coactivator (37). To test whether the p300/CREB coactivator or another member of the TRIP-Br protein family affects transactivation by CDCA4, the reporter assay was carried out using expression vectors encoding CBP, p300, TRIP-Br1, and TRIP-Br2. As shown in Fig. 3F, neither the single expression of CBP, p300, TRIP-Br1, and TRIP-Br2 nor their coexpression with E2F1 affected transactivation by CDCA4 and E2F1-mediated inhibition of CDCA4 transactivation, at least in transient transfection experiments. TopBP1, an E2F1-binding and -inhibiting protein, also showed no effect on transactivation by CDCA4 and E2F1-mediated inhibition of CDCA4 transactivation (Fig. 3F). To check the expression levels of transfected plasmids, cell lysates were prepared after 48 h of transfection and Western blot analysis was performed. FLAG-tagged p300, HA-tagged TRIP-Br1 and TRIP-Br2, and Myc-tagged TopBP1 were detected in the cell lysates (Fig. 3G).

CDCA4 Protein Suppresses Activator E2F-induced Transcriptional Activity—To further characterize the relationship between E2F1 and CDCA4, we assessed the effect of CDCA4 on transactivation induced by E2F1. For this, we employed pE2F-TA-Luc, in which the E2F consensus DNA-binding sequence is located upstream of the TATA box and luciferase gene. We used non-small cell lung cancer cells (A549) because the pRb pathway could be partially disrupted by human papillomavirus E7 expression in HeLa cells. As shown in Fig. 4A, little basal expression of the pTA-Luc reporter, which does not have any consensus sequences for E2F, was observed when the cells were cotransfected with the pcDNA3 vector plasmid or expression vectors for E2F1, pRb, and CDCA4-FLAG. In contrast, transfection with an E2F1 expression plasmid increased the basal reporter activity of pE2F-TA-Luc, and this was clearly suppressed by coexpression of CDCA4-FLAG or pRb. CDCA4-FLAG or pRb alone did not stimulate reporter activation (Fig. 4A). A recent study showed that CDCA4 enhances the transactivation function of p53, but not E2F1 (37). This is simply explained by the fact that only the stimulating effect of CDCA4 on the E2F reporter plasmid was examined without ectopic expression of E2F1. We also investigated the antagonistic effect of CDCA4 on E2F1 function under more physiological conditions. For this purpose, we used normal diploid fibroblast WI-38 cells. As shown in Fig. 4B, reporter activity induced by E2F1, E2F2, and E2F3 was completely suppressed with the coexpression of CDCA4. On the other hand, E2F4–6 could not induce pE2F-TA-Luc reporter activity under our assay conditions.

Because the CDCA4 protein regulates the transcriptional activation of E2F1, we studied which region of CDCA4 is responsible for the regulation of E2F1 activity. For this, C-terminally GFP-tagged regions of CDCA4 (1–241/GFP) were cotransfected with pTA-Luc (taken as 1). Values represent the means ± S.D. of three independent experiments. As shown in Fig. 4C, E2F4–6 could not induce pE2F-TA-Luc reporter activity. Because the CDCA4 protein regulates the transcriptional activation of E2F1, we studied which region of CDCA4 is responsible for the regulation of E2F1 activity. For this, C-terminally GFP-tagged regions of CDCA4 (1–241/GFP, 73–241/GFP, and 1–174/GFP) were cotransfected with pTA-Luc (taken as 1). Values represent the means ± S.D. of three independent experiments. A, as indicated, the indicated E2F expression plasmids were transfected with pcDNA3 alone (white bars), the indicated E2F expression plasmids alone (black bars), or the indicated E2F expression plasmids plus the CDCA4-FLAG expression plasmid (gray bars). Values are the means ± S.D. of three independent experiments. a, p < 0.05 versus pcDNA3; b, p < 0.05 versus E2F3.

FIGURE 4. Inhibition of activator E2F-induced transactivation by CDCA4. A, A549 cells were transfected with 200 ng of pTA-Luc (left panel) or pE2F-TA-Luc (right panel) reporter plasmid and 400 ng of the indicated expression plasmids. Reporter expression is presented as the -fold transactivation relative to the pcDNA3 control vector (taken as 1). Values represent the means ± S.D. of three or four independent experiments. B, WI-38 cells were transfected with 200 ng of pTA-Luc or pE2F-TA-Luc reporter plasmid and 400 ng of the indicated expression plasmids. Reporter expression is presented as the -fold transactivation relative to the pcDNA3 control vector (taken as 1). Values represent the means ± S.D. of three or four independent experiments. C, E2F2- and E2F3-induced pE2F-TA-Luc reporter activity was suppressed by CDCA4. The pE2F-TA-Luc reporter was cotransfected with pcDNA3 alone (white bars), the indicated E2F expression plasmids alone (black bars), or the indicated E2F expression plasmids plus the CDCA4-FLAG expression plasmid (gray bars). Values are the means ± S.D. of three independent experiments. a, p < 0.05 versus pcDNA3; b, p < 0.05 versus E2F3.
CDCA4 Regulates E2F Transcriptional Activity

A. GFP
1-241/GFP
73-241/GFP
1-174/GFP

B. Bright field
GFP
CDCA4
Cytoplasmic protein

C. kDa
GFP
1-241/GFP
73-241/GFP
1-174/GFP

D. pGL3-Basic
pGL3-Geminin
pGL3-MCM7

E. kDa
GFP
1-241/GFP

FIGURE 5. CDCA4 suppresses the transcriptional activity of E2F1. A, shown is a schematic of various CDCA4/GFP deletion mutants. The characteristic domains, including the cyclin A-binding, heptad repeat, and acidic regions, are indicated. B, shown is the subcellular localization of CDCA4/GFP transduced into HeLa cells. Forty-eight hours after transfection, cells were processed for fluorescence microscopy. All of the CDCA4/GFP deletion mutants were detected exclusively in the nucleus (middle panels). Localization of GFP (upper panels) and cytoplasmic protein (lower panels) is shown as a control. C, Western blot analysis of 50 μg of total cell lysate from each transfection was performed with rabbit anti-GFP polyclonal antibody. Arrows mark the positions of the CDCA4/GFP fusion proteins in A. The asterisk indicates the incompletely translated or post-translationally modified full-length CDCA4/GFP protein. The same band was also detected by the sixth immune serum raised by recombinant CDCA4 protein, as shown in Fig. 6 (right panels). D, the C-terminal acidic region, as well as the full-length CDCA4 protein, suppressed E2F1 transcriptional activation. HeLa cells were transiently transfected with 200 ng of the indicated pGL3-luciferase reporter plasmids (pGL3-Basic, pGL3-geminin, and pGL3-MCM7) and various combinations of pcDNA3, pcDNA3-E2F1, GFP, 1–241/GFP, 73–241/GFP, and 1–174/GFP together with pRL-TK. Fold activation refers to luciferase activity normalized to Renilla luciferase activity and is expressed relative to the activity observed with expression of pcDNA3 and GFP (taken as 1). Values are the means ± S.D. of three or four independent experiments. a.p < 0.05 versus pcDNA3 + GFP; b, p < 0.05 versus E2F1 + GFP. E, the inability of the CDCA4 protein to associate with E2F1 in vivo is demonstrated. HeLa cells were transiently transfected with the GFP or 1–241/GFP expression vector and lysed as described under “Experimental Procedures.” Lysates were immunoprecipitated (IP) with anti-GFP polyclonal antibody (pAb) and analyzed by Western blot (immunoblot (IB)) analysis with anti-GFP monoclonal antibody (mAb) to confirm self-immunoprecipitation (upper left panel) or with anti-E2F1 monoclonal antibody (upper middle panel). The arrowhead in the upper middle panel indicates the position of the immunoglobulin light chain (IgL). An aliquot of the whole cell lysate was analyzed by Western blot analysis with anti-E2F1 monoclonal antibody to confirm E2F1 input (upper right panel, arrowhead). CDCA4-FLAG or pCMV-TAG2B vector-transfected cells were lysed and immunoprecipitated on anti-FLAG monoclonal antibody-agarose. Immunecomplexes were detected with anti-CDCA4 polyclonal antibody (lower left panel) or anti-E2F1 polyclonal antibody (lower middle panel).

Each construct, reporter plasmids driven by the E2F-responsive promoters geminin and MCM7 were tested. Both geminin and MCM7 promoters contain E2F-binding sites, and deletion of these sites abolishes the responsiveness to E2F1 (38, 45). As shown in Fig. 5D, E2F1 stimulated the promoter activities of geminin and MCM7, but not pGL3-Basic, as described previously (38). The N-terminally deleted CDCA4 protein (73–241/GFP), as well as the full-length CDCA4 protein (1–241/GFP), clearly down-regulated the activity seen for the pGL3-geminin and pGL3-MCM7 promoter-luciferase reporters driven by E2F1, but showed no effect on the control luciferase plasmid (pGL3-Basic). In contrast, the C-terminally deleted CDCA4 protein (1–174/GFP) showed no effect on the activity obtained with the pGL3-geminin and pGL3-MCM7 promoter-luciferase reporters driven by E2F1 (Fig. 5D).

Because it has been reported that TRIP-Br1 interacts with E2F1/DP1 (34), we examined whether CDCA4 also interacts directly with E2F1/DP1. To clarify this, we performed immunoprecipitation studies with anti-GFP polyclonal antibody. The cells were overexpressed with GFP or GFP-tagged CDCA4 (1–241/GFP). Immunoblot analysis using anti-GFP monoclonal antibody showed that the immunoprecipitation worked. Unexpectedly, CDCA4 showed no association with E2F1 (Fig. 5E, upper panels). In addition, CDCA4-FLAG-transfected cells were immunoprecipitated by anti-FLAG monoclonal antibody-agarose, and immunocomplexes were then detected by anti-E2F1 polyclonal antibody. Again, there was no association...
CDCA4 Regulates E2F Transcriptional Activity

A. siRNA

B. Cell number (10^5)

C. Cell number (10^5)

D. Relative Luminescence (ATP)

E. Absorbance (OD 490 nm)

F. Western Blot

G. Western Blot

H. Western Blot

FIGURE 6. RNAi of CDCA4 affects the cell proliferation and expression of E2F1-regulated genes. A, the CDCA4 mRNA level was determined after RNAi targeting of CDCA4. HeLa or A549 cells were transfected with the control or siRNA oligonucleotide targeting CDCA4, and the expression of CDCA4 was analyzed by RT-PCR 24 h after the second RNAi. The mRNA expression levels of E2F1-regulated genes, including ORC1 and CDCA25A, were detected by RT-PCR. B, HeLa cells were transfected with control or CDCA4 siRNA, and the amounts of ATP were measured. C, the GAPDH mRNA level was determined after RNAi treatment, incorporated BrdUrd was detected with peroxidase-conjugated anti-BrdUrd monoclonal antibody. The y axis shows the A_{450 nm} values, indicating synthesis of DNA. The average number from six wells was plotted. Error bars indicate S.D. D, HeLa cells were treated as described for B, except that the amounts of ATP were measured. E, the GAPDH mRNA level was determined after RNAi treatment, incorporated BrdUrd was detected with peroxidase-conjugated anti-BrdUrd monoclonal antibody. The y axis shows the A_{450 nm} values, indicating synthesis of DNA. The average number from six wells was plotted. Error bars indicate S.D. E, the CDCA4 protein in a human cancer cell line was analyzed by Western blotting. Fifty micrograms of cell lysates prepared from HeLa, A549, and Saos-2 cells were loaded for each lane. The gray arrowhead indicates the position of the CDCA4 protein, which was not detected in the membrane probed with the preimmune serum. Ten micrograms of cell lysates prepared from Saos-2 cells were cultured without FBS for 3 days and then were re-entered into the cell cycle by the re-addition of 20% FBS. At the indicated time points after FBS treatment, cell lysates were recovered, and immunoblotting was performed with the indicated antibodies.

between CDCA4 and E2F1 (Fig. 5E, lower panels). Cyclin A was also not co-immunoprecipitated with CDCA4 data not shown. These results imply that CDCA4 is associated indirectly with E2F1/DPI possibly through pRb, p300/CBP, HDACs, or unknown proteins and thus represses E2F1 transcriptional activity.

CDCA4 Regulates Cell Proliferation Mainly through E2F Activity—A previous study demonstrated that overexpression of the CDCA4 protein results in inhibition of cell growth via a p53-independent mechanism (37). In contrast, stable or transient overexpression of murine CDCA4 in HeLa cells showed no obvious effect on cell proliferation and cell cycle progression (47). Gene knockdown mediated by siRNA is the most up-to-date technology to address this discrepancy. For this purpose, we knocked down endogenous CDCA4 expression in HeLa cells, in which p53 activity is subtle because of human papillomavirus E6-mediated degradation of p53 (50), and in A549 cells, in which p53 is intact. Two siRNA oligonucleotides against different parts of the CDCA4 mRNA were used to guard against off-target activity. In HeLa and A549 cells, siRNA against CDCA4 efficiently reduced CDCA4 mRNA after 24 h of consecutive siRNA transfection (Fig. 6A). We also checked the expression levels of E2F1-regulated genes in CDCA4 RNAi-treated cells compared with control RNAi-treated cells (Fig. 6A), suggesting that the loss of CDCA4 contributes to liberating E2F1 transcriptional activity. The GAPDH mRNA was detected equally in both control and CDCA4 siRNA-treated cells.

To better understand the CDCA4 function in greater detail, we examined whether or not CDCA4 RNAi can modulate cell proliferation.
proliferation. We found that CDCA4 RNAi increased the percentage of HeLa and A549 cell proliferation by up to ~200% (Fig. 6, B and C). In addition, cells treated with two different CDCA4 siRNA oligonucleotides produced more ATP than cells treated with control siRNA in HeLa cells (Fig. 6D). To determine whether this difference came from DNA synthesis activity, BrdUrd incorporation was measured. Significantly, BrdUrd enzyme-linked immunosorbent assay revealed an increased uptake of BrdUrd into cellular DNA in HeLa cells treated with CDCA4 siRNA (Fig. 6E). These results suggest that knockdown of CDCA4 in cancer cells promotes cell proliferation independent of the p53 status.

Next, we examined the endogenous CDCA4 protein level in siRNA-transfected cells. Exogenously expressed DBD/1–241 and CDCA4-FLAG were specifically detected by the sixth immune serum against recombinant CDCA4 protein, but not by preimmune serum (Fig. 6F, lanes 1–3 versus 4–6). Endogenous CDCA4 protein (indicated by the gray arrowhead) was detected by the sixth immune serum as having migrated almost to the same position as CDCA4-FLAG (indicated by the black arrowhead) (Fig. 6F, lanes 4 and 5 versus lane 6). The CDCA4 protein was barely detected after 72 h of treatment with CDCA4 siRNA (Fig. 6F, lane 7), indicating that siRNA-mediated gene knockdown was successful. In full-length CDCA4-transfected cells, a wrong sized band (indicated by the asterisk) was detected in addition to the right sized band (indicated by the black arrowhead) (Fig. 6F, lanes 8 and 9 versus lanes 10 and 11). The same situation was also observed in Fig. 5C, when anti-GFP polyclonal antibody was used. This is probably due to the degraded form or incompletely translated protein.

The CDCA4 protein was equally expressed in HeLa, A549, and Saos-2 cells (Fig. 6G, upper panels). In addition, the CDCA4 protein was detected at almost the same level in human cells derived from different tissues and organs, including Jurkat (T cell leukemia), Daudi (B lymphoblast), 293 (transformed primary embryonic kidney), Rh30 (rhabdomyosarcoma), A375 (melanoma), T98G (glioblastoma), HCT116 (colon cancer), and Hep2 (laryngeal carcinoma) cells (Fig. 6G, lower panels). As we demonstrated previously, in genes whose transcriptional regulation is controlled by transcription factor E2F1, the mRNA level is induced upon cells entering the G1/S phase of the cell cycle (38, 39). To substantiate this evidence, Saos-2 cells were arrested by serum-free medium for 3 days and then re-entered into the cell cycle with the re-addition of 20% FBS. CDCA4 and cyclin A proteins clearly accumulated as cells re-entered the cell cycle, whereas the GAPDH protein levels were constant (Fig. 6H).

DISCUSSION

CDCA4 was originally identified as murine Hepp, which is expressed preferentially in hematopoietic progenitors during mice embryonic hematopoiesis, although Cdc44 (Hepp) has not been functionally characterized (36). Recently, CDCA4 was reported as SEI-3, which participates in the regulation of p53-dependent transcriptional activity; however, its precise functions are still elusive especially in the context of cell cycle regulation (37). For example, although overexpression of the CDCA4 protein results in inhibition of cell growth, the growth arrest is mediated by a p53-independent signaling pathway (37). The fact that both TRIP-Br1 and TRIP-Br2, both closely related to CDCA4, interact with E2F1 as coactivators raises the possibility that CDCA4 may also serve as a coactivator of E2F1, and this could be one of the reasons why CDCA4 regulates cell proliferation in a non-p53-dependent manner. In this study, we have demonstrated that expression of CDCA4 is extremely well correlated with that of E2F1–E2F4, but not E2F5 and E2F6, in human somatic cells. Many lines of evidence have indicated that E2F activity is critical for the G1/S transition and DNA replication. The cell cycle-dependent induction of the CDCA4 protein is manifested at the protein level. Moreover, CDCA4 forms a negative feedback loop in which CDCA4 is first induced by E2Fs and then inhibits at least E2F1–3-dependent transcriptional activity. It is well known that the transcription of E2F1 is subject to an E2F-dependent self-inhibitory loop (51). The negative effects of CDCA4 on E2F-mediated transcriptional activation were unexpected, given that it strongly promotes p53-mediated transcription and that TRIP-Br1 and TRIP-Br2 promote E2F1-mediated transcription (34, 37). These results indicate that CDCA4 has an important role in the regulation of cell cycle progression through sequential effects on the transcriptional activity of E2F-responsive promoters during the G1 and S phases.

CDCA4 shares overall homology, as well as characteristic regions, including the SERTA motif, with TRIP-Br1, TRIP-Br2, and RBT1 (32). TRIP-Br1 and TRIP-Br2 have been shown to bind the DP1 subunit of E2F1/DP1 and to function as transcriptional coactivators and integrators of regulatory signals by recruiting the PHD zinc finger- and bromodomain-containing factors such as transcriptional corepressors KRIP-1 (transcriptional intermediary factor-1β) and transcriptional intermediary factor-1α (34). Furthermore, TRIP-Br proteins possess an intrinsic transactivation activity and interact with the coactivators p300 and CBP (49). p300/CBP proteins have been reported to be involved with a variety of sequence-specific transcription factors, including the E2F transcription factors (24, 52). Recently, TRIP-Br proteins have been shown to regulate the transcriptional activity of p53 tumor suppressor protein by cooperating synergistically with the ING family of chromatin-associated proteins (37). CDCA4 has also been shown to regulate the transactivation function of p53; however, no direct association between CDCA4 and p53 has been detected (37). Consistent with this observation, we were not able to demonstrate any physical interaction between CDCA4 and E2F1, even though CDCA4 and E2F1 seem to be mutually suppressive for their transcriptional activities. It would appear that the functional interaction of CDCA4 with E2F1 requires another bridging protein(s).

The transcriptional activity of E2F1 is regulated at multiple levels, mainly through its interaction with a variety of cellular proteins (1, 2). Indeed, pRB and prohibitin can bind and suppress the TAD of E2F1, whereas p300/CBP can bind and enhance the TAD of E2F1 (53, 54). The pRB family proteins have been shown to suppress E2F-mediated transcription through recruiting a variety of transcriptional corepressors, including HDAC1, DNA methyltransferase, Polycomb proteins, and a central component of the SWI/SNF chromatin-
CDCA4 Regulates E2F Transcriptional Activity

remodeling complex (Brg1/Brm) (27, 55–58). Among E2Fs, E2F1–4 binds to pRb, but not to p107 and p130 (2, 26). E2F1–4 (but not E2F5–8) potently decrease the transactivation of CDCA4. The suppressive effect of E2F1 on the CDCA4-induced transactivation is partially restored by pRb, but not by p107 and p130. It is likely that CDCA4 mediates the inhibitory effect of pRb against E2F1–4, albeit through an indirect interaction. Besides well known binding proteins such as pRb and p300/CBP, MDM2 and p53 have been suggested to bind to E2F1 or to affect its transcriptional activity indirectly (59).

Prohibitin and TopBP1 are known suppressors of E2F1 function. Prohibitin, a potential tumor suppressor protein, binds to pRb pocket family proteins and represses E2F1–5 transcriptional activity (60). Prohibitin recruits Brg1/Brm to E2F-responsive promoters, and it is recognized that this recruitment is required for the repression of E2F-mediated transcription by prohibitin (30). Although prohibitin associates with Brg1/Brm independently, prohibitin/Brg1/Brm-mediated transcriptional repression requires pRb (30). Interestingly, in a manner reminiscent of CDCA4, prohibitin was found to enhance p53-mediated transcriptional activity (61). In addition, TopBP1, a multiple BRCA1 C-terminal domain-containing protein involved in DNA replication and the DNA damage checkpoint, is induced by E2F and interacts with E2F1 during the G1/S transition of the cell cycle (62). TopBP1 specifically interacts with the N terminus of E2F1. Regulation of E2F1 by TopBP1 also requires the recruitment of Brg1/Brm to E2F1-responsive promoters (31). Notably, both Brm and p300/CBP possess the bromodomain. It is possible that the CDCA4 protein may exist as a multiprotein complex with E2F1/DP1, including bromodomain-containing cofactors or HDAC1, which has been shown to be involved in mediating pRb-mediated transcriptional repression and then suppressing the transcription of E2F1-regulated promoters (27, 56, 63). CDCA4 may interfere with E2F1 by occupying the E2F-binding consensus sequence, but in principle, CDCA4 would act as an adapter and then recruit corepressor molecules into the E2F1 regulatory DNA region.

pRb exerts its growth inhibitory function at least in part by regulating E2F1 activity (2). Phosphorylation of pRb by G1/S cyclin-Cdk complexes relieves active E2F1 binding to the promoter together with DP1 or DP2. Moreover, E2F1 possesses a binding site for cyclins in the N-terminal region, allowing E2F1 to mediate together with DP1 or DP2. Moreover, E2F1 possesses a binding site for cyclins in the N-terminal region, allowing E2F1 to mediate repression. In addition, E2F1 represses too the transcription of E2F-regulated promoters (27, 56, 63). CDCA4 may interfere with E2F1 by occupying the E2F-binding consensus sequence, but in principle, CDCA4 would act as an adapter and then recruit corepressor molecules into the E2F1 regulatory DNA region.

As summarized in Fig. 7, previous results, together with ours, indicate that TRIP-Br proteins play a dual role in the control of cell cycle progression through the transcriptional regulation of p53- and E2F-responsive genes. In this scenario, CDCA4 stands alone in a unique situation in which CDCA4 inhibits the transcriptional activation induced by activator E2Fs. Functionally, CDCA4 is a versatile factor, being a negative regulator in the regulation of E2F1 and a positive regulator in the regulation of p53. In such a case, the function of CDCA4 as an activator or a repressor could be specified by the presence of other proteins. Alternatively, CDCA4 may antagonize the interaction between TRIP-Br1/ TRIP-Br2 and other transcription factors on E2F-responsive promoters. The feedback control between E2F1 and CDCA4 is likely to be affected by cellular stimuli and could then define the status of the promoter occupancy of E2F1. E2F1 is known to be preferentially acetylated by PCAF (68), and PCAF acetyltransferase (histone acetyltransferase)-dependent E2F1 acetylation is required for the full activation of p73 transcription by E2F1 in response to DNA damage (69). This raises the possibility that CDCA4 could mediate the histone acetyltransferase (p300/CBP or PCAF) or HDAC1 recruitment to E2F1 in response to stimuli, including DNA damage.

TRIP-Br1 is consistently overexpressed in human squamous cell carcinomas of the head and neck (67). High level amplification of TRIP-Br1 at chromosome 19q13.1 is commonly detected in ovarian cancer (70). Chromosomal alterations in TRIP-Br1-transformed NIH3T3 cells are also detected (71). The E6 oncoprotein of human papillomavirus binds to and further increases the transcription stimulatory activity of TRIP-Br1 (72). These phenomena suggest that alterations in TRIP-Br1 could be a cause for promotion of cancer formation. A recent study has indicated that the physiological role for TRIP-Br proteins in coupling E2F seems to lie in the regulation of CDCA4.
of cyclin E expression during cell cycle progression to ensure the proper execution of DNA replication and the maintenance of genomic stability, the disruption of which could directly evolve into cellular abnormality (73). Abnormalities of chromosome 14 involving band q32.33, where human CDCA4 maps are among the most commonly observed cytogenetic alterations, have been observed in B cell malignancies (74). Whether or not these abnormalities, including translocations, affect CDCA4 expression still remains to be established. It is possible that the deregulation of CDCA4 could be a cause of and promote tumorigenesis. Whether this mechanism is impaired in human cancers, frequently associated with genetic impairments of the E2F1/pRb pathway, or could be exploited to improve the therapeutic index of genotoxic anticancer drugs will be the goal of our future investigations.

Several lines of evidence suggest that the p53 and pRb (including p16INK4a and p14ARF) tumor suppressor pathway is frequently deregulated in a wide range of human cancers (75). It is therefore important to identify critical target genes that affect cell proliferation in cancer cells, which would be a potentially attractive approach to cancer therapy. Thus, a functional interaction between CDCA4 and either E2F1 or p53 has a profound impact on early cell cycle progression, specifically in regulating the contrasting outcomes of cell cycle arrest and apoptosis. Our results suggest a critical role for CDCA4 in integrating and coordinating the functional interplay between the pathways of growth control mediated primarily by E2F1 and, to a lesser extent, by p53.

In summary, we have shown that CDCA4 expression is up-regulated by E2F1–4 and that, in turn, CDCA4 inhibits the transcriptional function of E2F1–3, probably via their interaction with pRb, chromatin-remodeling factors, or HDAC. Additional studies will assess whether a coexpressor such as HDAC1 or Brg1/Brm is needed in the CDCA4-mediated E2F1 regulation of transcription. The results described in this study strongly support the possibility that CDCA4 is placed in the pRb/E2F regulatory pathway. The ability of CDCA4 to differentially regulate the transcriptional activity of activator E2Fs and p53 places it in a unique situation in which it could integrate proliferative and apoptotic signals. It is quite possible that the levels and activity of CDCA4 could help determine the fate of cells while facing proliferative as well as apoptotic signals.

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CDCA4 Regulates E2F Transcriptional Activity

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