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A Novel Human Gene Encoding HECT Domain and RCC1-like Repeats Interacts with Cyclins and Is Potentially Regulated by the Tumor Suppressor Proteins

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Cyclin E-Cdk2 is an evolutionary conserved cyclin-dependent kinase (CDK) complex that drives the G1 to S phase transition of the cell cycle. A novel cDNA encoding a HECT family protein also containing RCC1-like repeats was isolated by a yeast two-hybrid screening using both cyclin E and its inhibitor p21. The protein product of this cDNA, Ceb1, interacts with various cyclin subunits of CDKs in mammalian cells. Expression of Ceb1 is specifically detected in testis and ovary and is highly elevated when the functions of the tumor suppressor proteins, p53 and RB, are compromised by mutations or viral oncoproteins. The present results suggest that Ceb1 may play a critical role when its expression and the CDK activity are upregulated by inactivation of p53 and RB.

A serine/threonine-specific cyclin-dependent protein kinase (CDK), cyclin E-Cdk2, plays an essential role in the transition from G1 (Gap 1) to S (synthesis) phase of the cell cycle and also involves in centrosome duplication (1–3). While the target substrates of cyclin E-Cdk2 remain obscure, the control of the activity of the cyclin E-Cdk2 has been well defined (4). The Cip/Kip family binds and regulates the activity of cyclin E-CDK2 during the G1 phase (5). This family consists of $p21^{Cip1/CAP20/SDI1/WAF1}$ (p21), $p27^{Kip1}$ (p27), and $p57^{Kip2}$. p21 binds various cyclin-CDK complexes including cyclin E-Cdk2, forms a quaternary complex containing PCNA, and inhibits the activity of CDK in the tumor suppressor p53-dependent manner (5, 6). Other than its inhibitory role, p21 plays a stimulatory role in the assembly of CDK and targeting cyclin-CDK complex to the nucleus (5).

Several candidate substrates have been so far proposed for cyclin E-Cdk2. The protein product of the retinoblastoma tumor suppressor gene, RB, and related gene products, p107 and p130, are negative regulators of cell proliferation and potential substrates for cyclin E-Cdk2 at the G1 to S phase transition (7). Since several experiments, however, indicated that cyclin E-Cdk2 was indispensable for the S phase entry even in the absence of RB, it has been proposed that critical G1/S substrate(s) of cyclin E-Cdk2 other than RB might exist (8–10). A nuclear protein NPAT, which is encoded by a gene next to the ATM locus, is one of the most promising substrates of cyclin E-Cdk2, which might be directly linked to the S phase entry regulated by cyclin E-Cdk2 (11). The activity of cyclin E-Cdk2 is also required for the induction of M phase in frog egg extracts (12) and for centrosome duplication (2, 3), but the downstream substrates of cyclin E-Cdk2, which mediate these phenomena, remain largely unknown.

Through the interaction with cyclin E and p21, we identified a novel human gene encoding a HECT domain protein also containing RCC1-like repeats. In the present study the cDNA cloning and initial characterization of this gene and its protein product are reported.

MATERIALS AND METHODS

Plasmid construction. pBTM116-cycE/p21 was constructed as following. The full-length human cyclin E cDNA was amplified by

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PCR and subcloned in frame with LexA at the *Eco*RI-*Sal*I site of pBTM116. The resultant plasmid (pBTM116-cycE) was digested with *Pvu*II and ligated to the blunt-ended human p21-*Sph*I cDNA fragment (lacking the C-terminus PCNA interacting region; amino acids 137–164) that contained the ADH promoter. Plasmids harboring two different orientations of the p21 cDNA were obtained. pBTM116-cycE/p21 expresses p21 at a higher level than the other orientation and was used for the following screening. pBTM116-cyclin E/p27 was constructed, like pBTM116-cycE/p21, using pBTM116-cycE and pET21a-p27 (from Dr. J. Massague) encoding mouse p27. pBTM116-p21 was constructed by fusing the full length human p21 in frame with LexA at the *Sal*I site of pBTM116. To generate Myc-Ceb1-C, the Ceb1 cDNA encoding the carboxyl terminal 526 amino acids was amplified by PCR and ligated at the *Xho*I site of pCS2$^+$MT (13). pCS2$^+$MT Ceb1-full was made by subcloning the full-length Ceb1 cDNA at the *Xho*I site of pCS2$^+$MT. To generate the mammalian GST-Ceb1 expression vector, 1.6-kb *Bam*HI-*Not*I digested Ceb1 fragment from pGEX4T-1-Ceb1-C was subcloned at the *Bam*HI and *Not*I sites of pEBG. Construction of Myc-tagged cyclins and CDK inhibitors was made by cloning the full-length cDNA sequences into the pCS2$^+$MT at the *Xho*I-*Xba*I sites. Templates for PCR were following. Myc-cyclin A; pGEM4Z-cycA (pCycA) from Dr. J. Pines, Myc-cyclin B1; pGEM 4Z-cycB (pCycB) from Dr. J. Pines, Myc-cyclin D1; cyclin D in pBluescript-KS$^-$ from Dr. S. I. Reed, Myc-p21; pBluescript-hp21 from Dr. J. W. Harper, Myc-p27; pCS2$^+$p27 (14). Myc-cyclin E was described previously (14).

Tissue culture, transfection, and virus infection. HEK293, HeLa S3, HT1080, NHF-SV40-1 and 2, SaOS-2, U2-OS, WI-38, and WI-38(SV40) were maintained at 37°C in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (Hyclone). WI-38(SV40) and NHF-SV40-1 and 2 are SV40 transformed cell lines originated from WI-38 and NHF (Normal human fibroblasts). Transfection of animal cells was performed as described previously (14). Production and infection of HPV E6 and E7 (15), and SV40 T antigen retroviruses (16) were performed using LXSN-E6 and -E7 (from Dr. D. Galloway), and pZipSV40 (gift from Dr. P. S. Jat) as described (17). The recombinant adenovirus vector harboring wild-type p53 (Adp53), which was originally made by Dr. Hamada (Cancer Research Institute) and Dr. Miyazaki (Osaka University), contains the modified chicken b-actin promoter with the CMV-1E enhancer (CAG). Viral stocks were expanded in HEK293 cells; cells were harvested 48 h after infection and disrupted by four cycles of freezing and thawing. Cell debris were removed by two cesium chloride gradient ultracentrifugations. The virus titer was determined by plaque-forming activity in 293 cells. Concentrated virus was dialyzed with PBS containing 10% glycerol, aliquoted, and stored at −80°C until use. Wild-type and retrovirus-infected WI-38 cells expressing SV40 T antigen were infected with Adp53 at MOIs of 3. Twenty-four hours after infection, total RNAs were isolated from the infected cells and were subjected to Northern blotting.

Two-hybrid screening and cloning of the full-length Ceb1 cDNA. A LexA-based two-hybrid system was used to screen for proteins that can interact with cyclin E and p21 (18, 19). The "bait/co-bait" plasmid (pBTM116 cycE/p21) was used to express both human cyclin E (bait), which was fused to the DNA binding domain of LexA, and human p21 (co-bait) lacking PCNA-binding domain. A human B-cell cDNA library (20) in the pACT plasmid was screened, and two clones (pACT-Ceb1-C) were found to encode the carboxyl terminal 526 amino acids of Ceb1. The 3.4-kb full-length Ceb1 cDNA (accession number of DDBJ/EMBL/GenBank: AB027289) was identified by RT-PCR with total RNA from HEK293 cells. A pair of primer (5′-cgggatcctctgggcctgggaccccgcaa-3′/5′-ggctcgagcaagctggtcagccaaatc-3′) was chosen for isolating the 3.1-kb cDNA encoding the entire amino acids of Ceb1, based on the information obtained from 5′ rapid amplification on cDNA ends (RACE). Sequencing was performed to confirm that the clone possessed the identical 3′ nucleotide sequence to pACT-Ceb1-C and that the 5′ sequence was identical to those of the two independent cDNA clones obtained by 5′ RACE. The amplified cDNA was digested with *Bam*HI and *Xho*I and ligated to the mammalian expression vector pCS2$^+$ (13) to generate pCS2$^+$ Ceb1-full.

Antibody production and immunofluorescence staining. The GST-fusion plasmid encoding the carboxyl terminal amino acids of Ceb1 (amino acids 499–925) was transformed into *E. coli* DH10B to express a soluble GST-Ceb1 protein. The recombinant protein was purified with glutathione–Sepharose beads and used for immunization in rabbits, and the Ceb1 specific antibodies were affinity-purified with the GST-Ceb1 conjugated to AFFI-GEL15 (Bio-Rad) (21). Indirect immunofluorescence staining using anti-cyclin E and other antibodies was performed as described (8). Cells were observed using a Nikon photomicroscope, and the images were acquired through a CCD camera with FISH Imager (Appligene Oncor) and processed using a Macintosh computer.

Western blot, immunoprecipitation, and GST pull-down assay. Cells ($\sim 5 \times 10^6$) were lysed in 1 ml of a lysis buffer [50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.5% Nonidet P-40] containing 10 μg each of aprotinin, leupeptin, and pepstatin A per ml, and after centrifugation the supernatants were incubated with specific antibodies for 3 h and subsequently with protein G-sepharose (Pharmacia) for 30 min with frequent rotations. The immunoprecipitates were washed three times with the lysis buffer, collected by centrifugation, and suspended in 1× Laemmli sample buffer. For the pull down assay for the GST-fusion Ceb1, glutathione–Sepharose beads (Pharmacia) were used instead of antibodies and protein G–Sepharose. All procedures were done on ice. Western blot analysis was performed as described (8).

Chromosome mapping. DNA from BAC clone F787 (human Ceb1 genomic DNA) was labeled with digoxigenin dUTP by nick translation. Labeled probe was combined with sheared human DNA and hybridized to normal metaphase chromosomes derived from PHA stimulated peripheral blood lymphocytes in a solution containing 50% formamide, 10% dextran sulfate and 2× SSC. Specific hybridization signals for two color experiments was accomplished by incubating the slides in fluoresceinated antidigoxigenin antibodies and Texas red avidin followed by counterstaining with DAPI.

RESULTS

Isolation of Cyclin E-p21 Binding Protein

To look for human proteins that interact with cyclin E-Cdk2, we screened a human cDNA library by a yeast two-hybrid method (18, 19). Cyclin E (bait) and a CDK inhibitor p21 (co-bait) were used for the screening. Cyclin E and p21 interact with each other in the yeast two-hybrid system (data not shown) and p21 is a component of the inactive cyclin E-Cdk2 complex in primary human fibroblasts (5). Therefore, cyclin E and p21 interact in yeast and form a complex (cyclin E-p21), and this becomes a target of binding for putative cyclin E-p21 interacting proteins. From a screening of $\sim 1 \times 10^7$ transformants, seven positive clones were obtained, and nucleotide sequence analysis revealed that three were human p21 and other four were grouped into two novel cDNAs. One of them, named Ceb1 (for cyclin *E*-binding protein), interacted with cyclin E-p21, but not with cyclin E, p21, or cyclin E-p27 (another CDK inhibitor) (Fig. 1A). The interaction in yeast between cyclin E-p21 and Ceb1 was, however, five times less than that observed between cyclin E and p21 in the beta-galactosidase assay (data not shown).

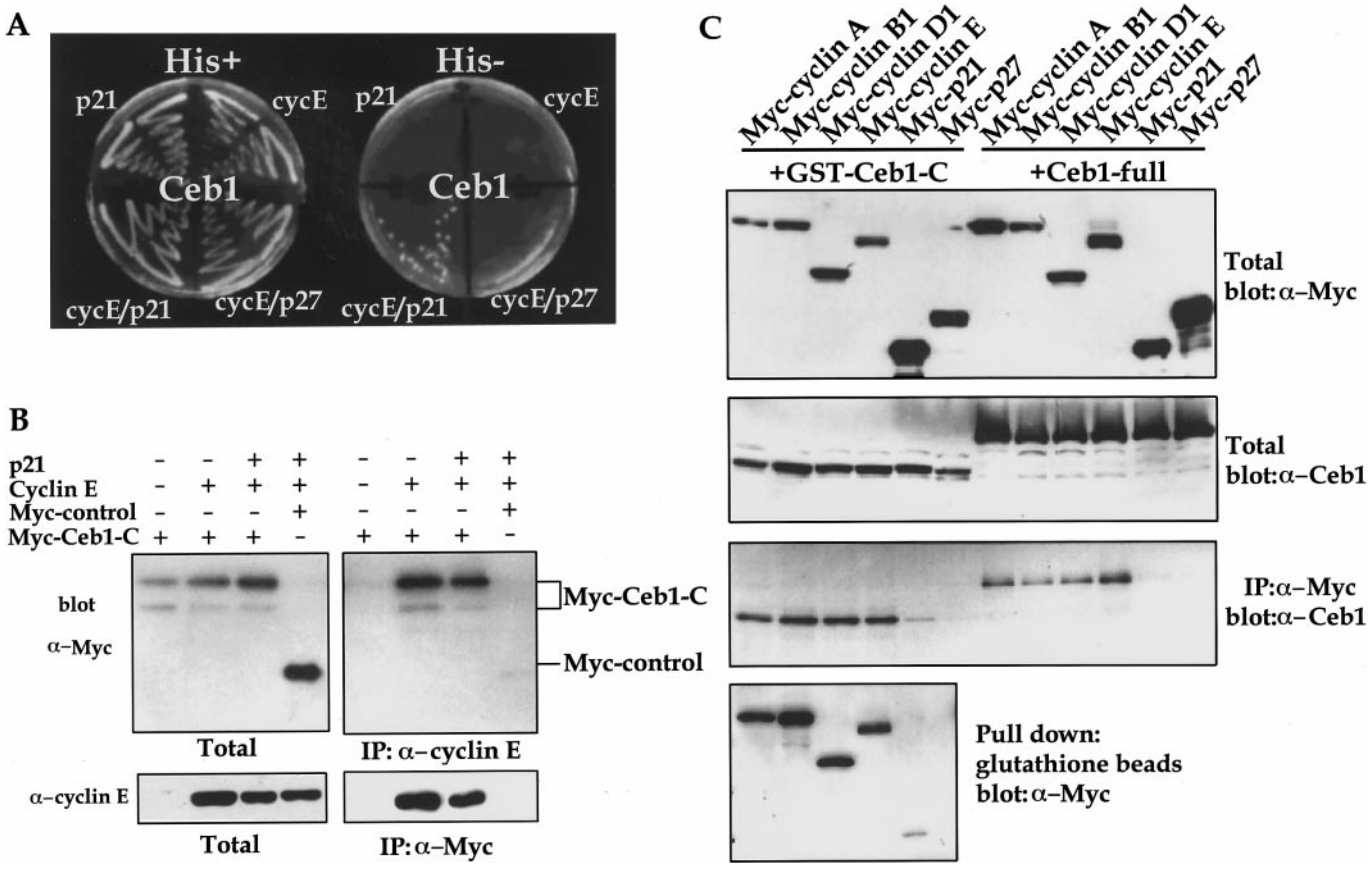

FIG. 1. Interaction of Ceb1 with cyclins. (A) Ceb1 interacts with cyclin E-p21 in yeast. Yeast L40 strain was transformed with the "prey" plasmid pACT-Ceb1-C and various "bait" plasmids (cyclin E, p21, cyclin E/p21 or cyclin E/p27), and the growth of the colonies of the transformants on the SD plates (lacking leucine and tryptophan) in the presence (His+) or absence (His−) of histidine was compared. Cyclin E, p21, cyclin E/p21 or cyclin E/p27 corresponds to L40 containing pBTM116-cyclin E and pACT-Ceb1-C, pBTM116-p21 and pACT-Ceb1-C, pBTM116-cyclin E/p21 and pACT-Ceb1-C, or pBTM116-cyclin E/p27 and pACT-Ceb1-C. (B) Association of Ceb1 with cyclin E in HEK293 cells. Cyclin E expression plasmid (3 μg) with the Myc-Ceb1-C (10 μg) or control plasmid Myc-control (10 μg) was cotransfected into HEK293 cells with or without p21 expression plasmid (3 μg), and the immunoprecipitation was performed with the lysates from the transfected cells and anti-cyclin E antibodies against human cyclin E or anti-Myc mAb (9E10). The immunoprecipitates and total cell lysates were run on a 12% polyacrylamide gel and transferred to PVDF membranes. The blots were probed with either anti-cyclin E or anti-Myc. (C) Association of Ceb1 with various cyclins. Plasmid (5 μg) coding for glutathione-S-transferase (GST) fusion Ceb1 (GST-Ceb1-C) or the full-length Ceb1 (Ceb1-full) was cotransfected into HEK293 cells with plasmids (5 μg) encoding either Myc-tagged cyclins or Myc-tagged CDK inhibitors. The pull-down experiments were performed with either anti-Myc mAb and protein G–Sepharose or glutathione–Sepharose beads, and the protein samples on the beads were subjected to Western blot analysis as described in B.

Structure of Ceb1

The sequence analysis of the full length Ceb1 cDNA showed that Ceb1 encodes a E6-AP related 1024 amino acids protein which contains a HECT (homologous to *E*6-AP *c*-terminus) domain at its carboxyl terminus (Figs. 2A to 2C) (22, 23). The HECT domain of E6-AP catalyzes the E3 ubiquitin-protein ligase activity for the tumor suppressor protein p53 (24). Among other HECT family proteins the HECT domain of Ceb1 exhibits the highest similarity (41% identity) to that of the deduced amino acid sequence of human estimated sequence tag D25215/KIAA0032 (D25215) (Figs. 2B and 2C) (25). Besides HECT domain, both Ceb1 and D25215 possess RCC1-like domain (RLD) consisting of seven repetitive sequences (26–28), at their amino termini (Figs. 2A, 2B, and 2D). The RLD of Ceb1 shows higher similarity to that of D25215 (37%) than that of human RCC1 (21%) (Fig. 2D). No significant sequence similarity to other proteins was detected for the intermediate region between RLD and HECT domain.

Chromosomal location of human Ceb1 was assigned to 4q22.2–22.3 after analyzing the Ceb1 genomic DNA (Fig. 2E). Interestingly, D25215 has been mapped to 4q21 that is proximal but distinct from the Ceb1 locus (25), suggesting that genomic duplication led to this protein family.

Ceb1 Interacts with Cyclins

To confirm the interaction of Ceb1 and cyclin E-p21, cyclin E and p21 were coexpressed with myc-tagged

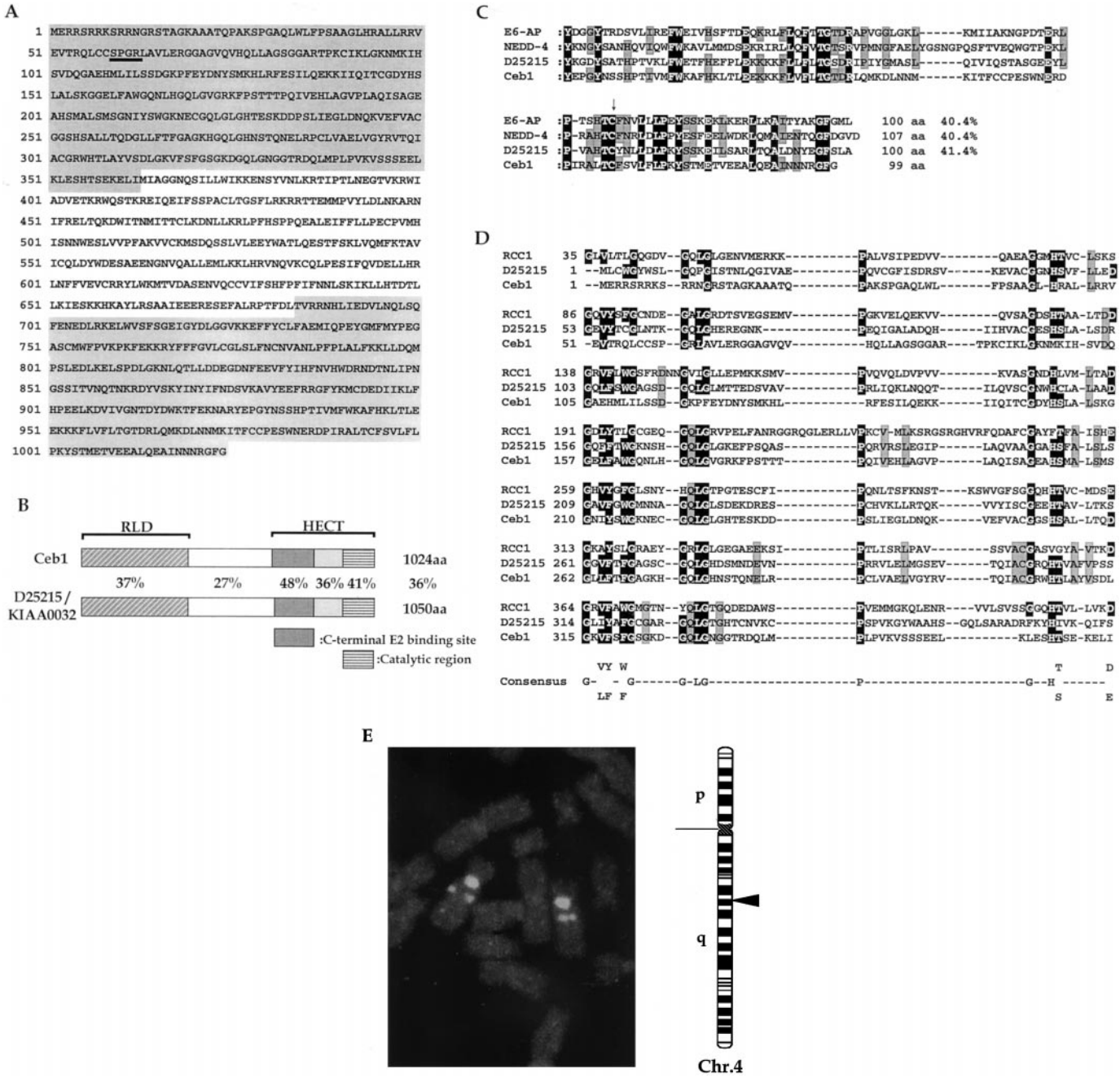

FIG. 2. Structure of Ceb1. (A) Complete amino acid sequences of Ceb1. The amino acid sequence deduced from the full-length Ceb1 cDNA is shown as single letter amino acid codes. Regions corresponding to the RLD and the HECT domain are shaded, respectively. The canonical CDK phosphorylation site (SPGR) in the RLD is underlined. (B) Structural motifs of human Ceb1 are depicted with those of a putative human protein encoded by D25215. Regions correspond to RLD, HECT, and E2 binding site and the catalytic regions in the HECT domain are indicated and overall homology (%) between the two proteins and homology within each region are also shown in the figure. (C) Alignment of the HECT domains from Ceb1 and HECT family proteins. The catalytic regions of human E6-AP, human NEDD4 protein, and D25215 are aligned with that of Ceb1 (22, 23, 46). The conserved cysteine residues involved in ubiquitin transfer are indicated by an arrow. Residues are shaded black if all proteins are identical and gray if three proteins are identical. (D) Comparison of the RLD from Ceb1 and other RLD-containing proteins. The predicted amino acid sequence of the RLD from Ceb1 is aligned with those from human RCC1 and D25215. Residues are shaded black if they correspond to the consensus amino acids residues of the RCC1-like repeats (26, 27) and residues other than the consensus residues are shaded gray if three proteins are identical. (E) Chromosomal location of Ceb1. The experiment was conducted in which a biotin labeled probe that is specific for the centromere of chromosome 4 (red) was co-hybridized with Ceb1 genomic DNA (green). DNA was stained by DAPI (blue). The position of Ceb1 (4q22.2-22.3) on depicted human chromosome 4 is shown by an arrowhead.

carboxyl terminal half of Ceb1 to assay for association in human embryonic kidney HEK293 cells. Surprisingly, cyclin E specifically interacted with the Ceb1 even without p21 as opposed to the result of yeast two-hybrid analysis (Figs. 1B and 1C). Likewise, the full length Ceb1 and the carboxyl terminal half of Ceb1 were assayed for their interaction with various myc-tagged cyclins, and both were found to interact with all tested cyclins as efficiently as with cyclin E (Fig. 1C). Unlike cyclins, CDK inhibitors, p21 and p27, showed either weak or no interaction with Ceb1 under the same condition. These results suggest that Ceb1 mainly interacts with cyclins and that coexpression of p21 with cyclin E in yeast may stabilize the expression of cyclin E (Fig. 1A) (29). We examined the cyclin-binding site of Ceb1 and found that at least two cyclin-binding sites are present within amino acids 499–925 (data not shown). Furthermore, we could not detect the association of endogenous Ceb1 and cyclins probably because Ceb1 is a less abundant protein even in highly expressed cell lines.

Expression of Ceb1 Is Specifically Detected in Reproductive Tissues and Is Elevated When the Activities of the Tumor Suppressors, p53 and RB, Are Compromised

The Ceb1 mRNA was specifically detected in testis and to a lesser degree in ovary among various tissues (Fig. 3C), but was either low levels or undetectable in human diploid lung fibroblasts WI-38 and tumor lines HT1080 and U2-OS (Fig. 3A), that possess functional tumor suppressors, p53 and RB. In contrast, augmented Ceb1 mRNA was detected in both p53 and RB-defective tumor lines including HEK293, HeLa S3, SaOS-2, SV40 transformed WI-38 [WI-38(SV40)], and SV40 transformed normal human fibroblasts (NHF-SV40-1 and -2) (Figs. 3A and 3D). Furthermore, immunoblot analysis using affinity purified anti-Ceb1 antibodies detected a 117-k Da protein in the total extracts from human cell lines expressing high level Ceb1 mRNA but not from those expressing low level or no Ceb1 mRNA (Fig. 3B). In contrast, D25215 is expressed at almost identical levels in every cell line, and p21, whose expression is largely p53-dependent (5, 6), showed roughly the inverse pattern of the Ceb1 expression (Fig. 3A).

Three experimental approaches were carried out to determine if p53 and/or RB control the expression of the Ceb1 gene. First, WI-38 cells were infected with various retroviruses expressing viral oncoproteins that disable the function of p53 and RB (Fig. 3E) (15, 16). The Ceb1 mRNA was elevated up to 20-fold by the expression of SV40 large T antigen that disables both p53 and RB function, and 5-fold by the expression of a p53-specific antagonist, human papillomavirus (HPV) type 16 E6, or RB-specific antagonist, HPV type 16 E7. Secondly, MDAH041 cells, which lacks wild-type p53 but retains functional RB, showed an intermediate Ceb1 expression level (Fig. 3D), and conversely, infection of the wild-type p53-adenovirus in WI-38 expressing SV40 T antigen resulted in 3-fold reduction of Ceb1 mRNA and 5-fold elevation of p21 message (Fig. 3F). Lastly, WI-38 cells were treated with histone deacetylase inhibitors, trichostatin A and butyrate (30), which relieve the inhibition of gene expression mediated through the interaction between RB and a histone deacetylase (31). The Ceb1 mRNA in WI-38 cells treated with these inhibitors was elevated (3-fold) within 24–48 h (Fig. 3G). These results indicate that the expression of Ceb1 is indeed elevated when the function of p53 and RB are compromised. However, senescent cultures (PD60; 60 population doublings) of normal human fibroblasts NHF, whose p53 and RB functions are frequently activated (32), showed a relatively high level of Ceb1 mRNA (more than 3-fold) compared to the younger cultures (PD17) (Fig. 3D), thereby indicating that a regulatory pathway other than p53 and RB might also exist.

Ceb1 Is Mainly Located in the Cytoplasm

Subcellular location of Ceb1 was examined by indirect immunofluorescence using the Ceb1-specific antibodies (Fig. 4). Cells moderately expressing transfected Ceb1 showed exclusive cytoplasmic staining (Fig. 4A), and endogenous Ceb1 in WI-38(SV40) cells was also detected in the cytoplasm, though perinucleic localization was more evident (Fig. 4C).

DISCUSSION

We have described the isolation and characterization of human Ceb1 gene encoding RCC1-like repeats and a HECT domain. This structural feature of Ceb1 suggests that Ceb1 is involved in the regulation of intracellular transport of macromolecules, the cell cycle control, and the regulation of protein stability. Moreover, interaction of Ceb1 with cyclin E and other cyclins suggests that Ceb1 is a potential substrate or a regulator of CDKs including cyclin E-Cdk2.

The human gene encoding a nuclear chromatin-binding protein RCC1 was originally cloned by its ability to complement the temperature sensitivity of the hamster cell line tsBN2, which shows various cell-cycle phenotypes including the G1-phase arrest and premature initiation of mitosis (26, 28). Biochemical analysis of RCC1 subsequently revealed that RCC1 catalyzed a guanine nucleotide-exchange reaction for a nuclear Ras-like small GTPase protein Ran/TC4 (33). The GTP-bound state of Ran (Ran-GTP), through its effector proteins which largely mediate Ran's function, is critical for nucleo-cytoplasmic transport of macromolecules, the cell cycle regulation, and formation of mi-

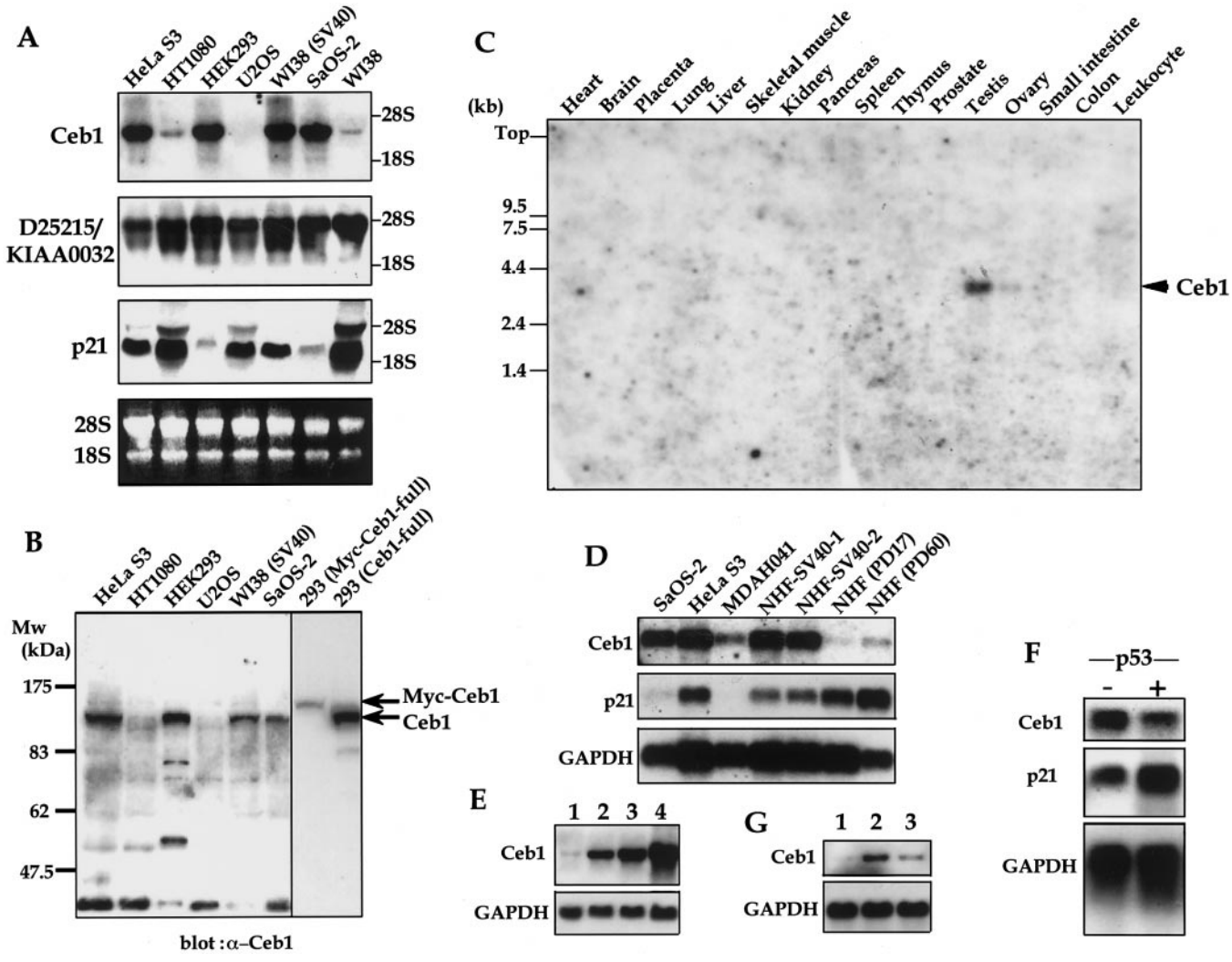

FIG. 3. Expression of Ceb1. (A) Expression of Ceb1 mRNA in various human tissue culture lines. Total RNAs (15 μg each lane) was run on a 1.0% formalin-agarose gel and transferred to a nylon membrane. Hybridization was performed with *in vitro* transcribed Ceb1, D25215, and p21 riboprobes. Ribosomal RNA stained with ethidium bromide is shown as a control. (B) Expression of Ceb1 protein in various human tissue culture lines. Total proteins (100 μg each) from indicated human cells were run on 8% SDS-polyacrylamide gel and subjected to Western blot analysis. Ceb1 protein was detected by affinity-purified anti-Ceb1 antibodies and enhanced chemiluminescence. HEK293 cell lysates (100 ng) expressing Myc-tagged or untagged full-length Ceb1 were used as the positive controls. (C) Expression of Ceb1 mRNA in human tissues. Northern blot analysis was performed with Ceb1 as the probe and a nylon membrane containing 2 μg of size-fractionated poly(A)$^+$ RNA from a variety of human tissue sources (Clontech Laboratories). (D) Northern blot analysis was performed as described in A. Expression of Ceb1 in various human cells was compared with that of p21 (p53-inducible) and GAPDH (control). (E) WI-38 cells were infected with control (lane 1), HPV E6 (lane 2), HPV E7 (lane 3), or SV40 T antigen (lane 4) retrovirus, and after two weeks of selection in DMEM medium containing 400 μg/ml G418, cells were collected for isolation of RNA. Northern blot analysis was performed with total RNAs (10 μg/lane) using Ceb1 and GAPDH (control) as the probes. (F) Infection of p53-adenovirus down-regulates the expression of Ceb1 in WI-38 cells expressing SV40 large T. WI-38 cells expressing SV40 T antigen retrovirus were infected with the wild-type p53 adenovirus (+) or control virus (−) and two days after infection cells were collected to isolate RNA. Northern blot analysis was performed using 10 μg of total RNAs, and Ceb1, p21, and GAPDH riboprobes. (G) WI-38 cells were treated with histone deacetylase inhibitor trichostatin A for 48 h (lane 2) or butyrate for 24 h (lane 3), and collected to isolate RNA. Northern blot analysis was performed as in A. Lane 1 corresponds to untreated WI-38.

totic spindle (26, 28, 34–37). The GDP-bound state of Ran (Ran-GDP) has been reported to inhibit cyclin B-Cdc2 protein kinase activation in frog egg lysates (38, 39), indicating that RCC1 may act as an upstream regulator of cyclin B-Cdc2.

Several RCC1-like genes have been found in vertebrates, which are implicated in genetic diseases, transcriptional regulation, and Golgi function (25, 40–43). It has been shown that the RLD of a Golgi protein p532 catalyzes the guanine nucleotide-exchange reaction for a small GTPase protein other than Ran (43), thereby suggesting that RLD is a general guanine nucleotide-exchange factor motif for small GTPase proteins. It is therefore likely that the RLD of Ceb1 also catalyzes the

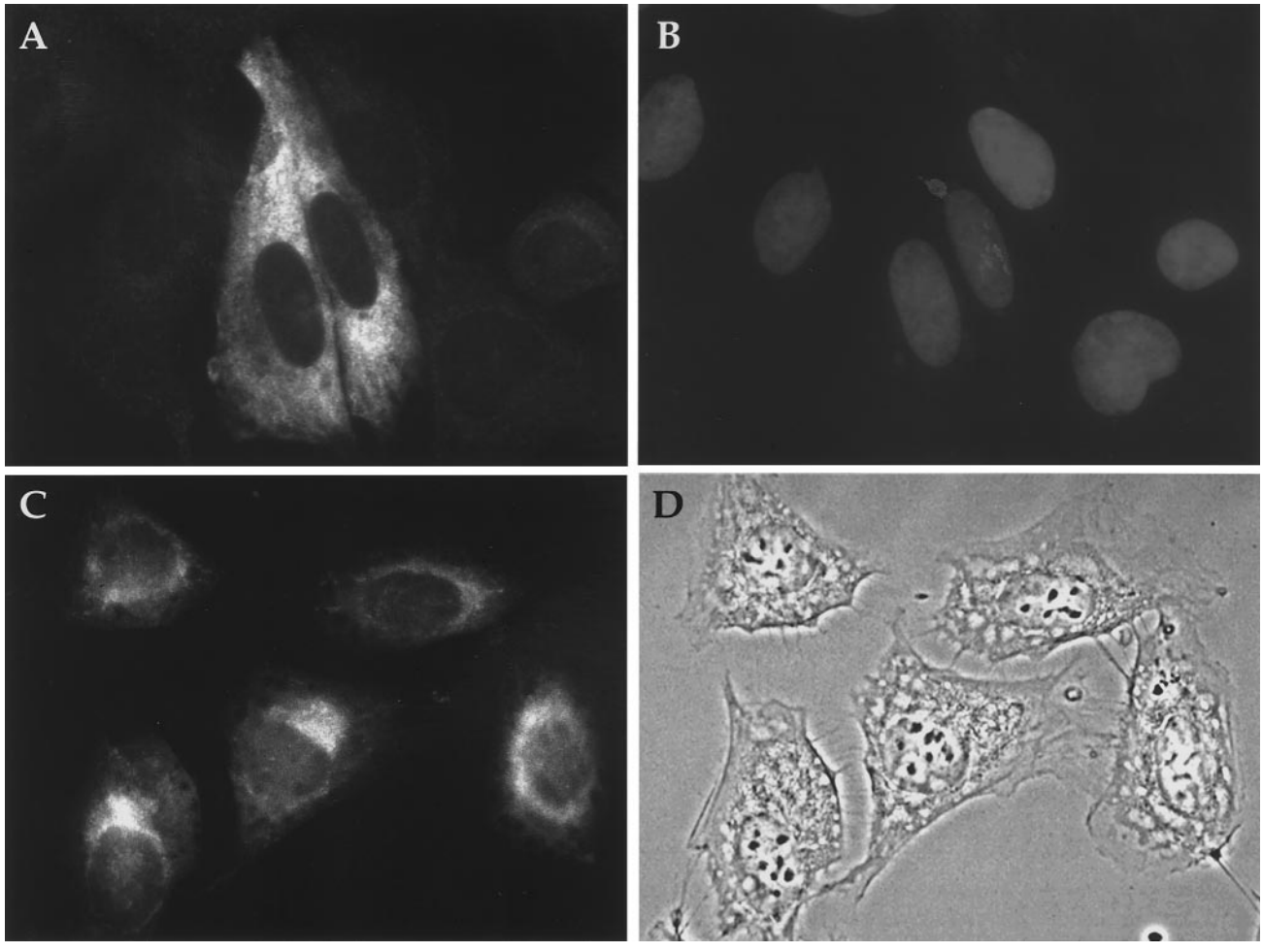

FIG. 4. Subcellular location of Ceb1. SaOS-2 cells were transfected with pCS2$^+$ Ceb1-full and 40 h after transfection the cells were stained with affinity-purified anti-Ceb1 antibodies and Texas-red-conjugated anti-rabbit IgG antibodies. Immunofluorescence images of the transfected cells stained with anti-Ceb1 (A) and Hoechst 33258 (DNA) staining of A (B) are shown. Location of endogenous Ceb1 in WI-38(SV40) is also shown: immunofluorescence image of cells stained with anti-Ceb1 (C), phase contrast image of C (D).

guanine nucleotide-exchange reaction for a small GTPase protein.

Ceb1 possesses HECT domain in addition to RLD, but so far we could not detect any increment of ubiquitinylation and degradation of cyclin E or p21 in various cells transfected with Ceb1 expression plasmid (data not shown). There might be a functional coupling between HECT domain and RLD because other RCC1-like proteins encoded by human p532 and mouse and human *rjs* also possess HECT domain at their carboxyl termini (41, 43), like Ceb1 and D25215.

Testis and ovary specific expression of Ceb1 suggests that Ceb1 has a specialized role in these organs. In support of this idea, cyclin E is expressed at high levels in testis (44, 45), and in a cell-free system using frog egg lysates, cyclin E-Cdk2 is required for the induction of mitotic state (12). Furthermore, the present results indicate that the expression of Ceb1 appears to be regulated by tumor suppressor proteins, RB and p53. It is interesting to speculate that Ceb1 regulates or mediates the activity of CDKs because Ceb1 is highly induced when both RB and p53 were inactivated and the CDK activity is subsequently elevated (5, 7, 32). Identification of Ceb1 interacting proteins may help revealing the function of Ceb1 and its relationship with cyclins and the tumor suppressors.

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