Cyclin L2, a Novel RNA Polymerase II-associated Cyclin, Is Involved in Pre-mRNA Splicing and Induces Apoptosis of Human Hepatocellular Carcinoma Cells*

We report the cloning and functional characterization of human cyclin L2, a novel member of the cyclin family. Human cyclin L2 shares significant homology to cyclin L1, K, T1, T2, and C, which are involved in transcriptional regulation via phosphorylation of the C-terminal domain of RNA polymerase II. The cyclin L2 protein contains an N-terminal "cyclin box" and C-terminal dipeptide repeats of alternating arginines and serines, a hallmark of the SR family of splicing factors. A new isoform and the mouse homologue of human cyclin L2 have also been cloned in this study. Human cyclin L2 is expressed ubiquitously in normal human tissues and tumor cells. We show here that cyclin L2 co-localizes with splicing factors SC-35 and 9G8 within nuclear speckles and that it associates with hyperphosphorylated, but not hypophosphorylated, RNA polymerase II and CDK p110 PITSLRE kinase via its N-terminal cyclin domains. It can also associate with the SC-35 and 9G8 through its RS repeat region. Recombinant cyclin L2 protein can stimulate in vitro mRNA splicing. Overexpression of human cyclin L2 suppresses the growth of human hepatocellular carcinoma SMMC 7721 cells both in vitro and in vivo, inducing cellular apoptosis. This process involves up-regulation of p53 and Bax and decreased expression of Bcl-2. The data suggest that cyclin L2 represents a new member of the cyclin family, which might regulate the transcription and RNA processing of certain apoptosis-related factors, resulting in tumor cell growth inhibition and apoptosis.

Cyclins are key regulatory proteins that complex with and activate cyclin-dependent kinase (CDK) subunits (1–4), playing pivotal roles in the regulation of cell cycle progression (5).

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† Both authors contributed equally to this work.

‡ To whom correspondence should be addressed: Institute of Immunology, Second Military Medical University, Shanghai 200433, People's Republic of China. E-mail: caoxt@public3.sta.net.cn.

§ The abbreviations used are: CDK, cyclin-dependent kinase; CTD, C-terminal domain; RNAPII, hyperphosphorylated RNA polymerase II; RNAPIIAs, hypophosphorylated RNA polymerase IIa; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; GST, glutathione S-transferase; GFP, green fluorescent protein; BMSCs, bone marrow stromal cells; RT, reverse transcriptase; PI, propidium iodide.

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Novel RNAPII-associated Cyclin Induces Tumor Cell Apoptosis

(CCNL2), first identified by large scale random sequencing of a cDNA library of human bone marrow stromal cells (BMSCs). Human cyclin L2 is also a cyclin box and RS repeat region-containing protein and localizes within the nuclear speckles, co-localizing with splicing factors SC-35 and 9G8. We demonstrate that human cyclin L2 associates with RNAPIIIs, CDK p110 PITSLRE kinase (CDK1), SC-35, and 9G8. In addition, recent work has shown that cyclin L2 protein co-localizes in vitro and in situ with splicing. Furthermore, overexpression of exogenous cyclin L2 can suppress the growth of human hepatocellular carcinoma SMMC 7721 cells both in vitro and in vivo, inducing cellular apoptosis, possibly by up-regulating expression of p53 and Bax and down-regulating Bcl-2. These results suggest that cyclin L2 may be a functional cyclin that associates with transcriptional regulation and participates in regulating the pre-mRNA splicing process. The mechanism by which cyclin L2 suppresses the growth and induces apoptosis of tumor cells requires further investigation.

EXPERIMENTAL PROCEDURES

Mice and Cell Lines—Female BALB/c nu/nu athymic mice (BK Experimental Animal Co., Shanghai, China), 6–8 weeks of age, were maintained in specific pathogen-free environment. All cell lines, in-cluding HL-60, K-562, U937, KG-1, Reh, Daudi, NAMALWA, MOLT-4, Hut 78, Jurkat, Raji, HT-29, CAOV-3, HeLa, PC-3, MC-F7, U251, and the mouse melanoma cell line B16, were obtained from the ATCC, except for NB4 (a kind gift from Prof. Guanli Sun, Institute of Hematology, Ruijin Hospital, Shanghai, China) and the human hepatocellular carcinoma cell line SMMC 7721 (13). All cell lines were maintained in RPMI 1640 medium supplemented with 2 mM glutamine, penicillin (100 units/ml), streptomycin (100 μg/ml), and 10% (v/v) heat-inactivated fetal calf serum (Hyclone) in a 5% CO2 incubator at 37 °C.

Molecular Cloning of Full-length Human Cyclin L2 and Its Mouse Homologue—The main EST of human cyclin L2 was directly isolated from a human BMSC cDNA library by random sequencing as described previously (14). Briefly, BMSCs were generated from the bone marrow aspirates of an adult patient. Red blood cells were depleted, and the monocytes were plated in 6-well plates (1 × 106 cells/well) in RPMI 1640 supplemented with 10% fetal calf serum, 10% horse serum, 50 μM 2-mercaptoethanol, 10 mM HEPES, 2 mM glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin. After 24 h, floating cells were removed, and an adherent stromal cell layer was established. On the 14th day, stromal cells were activated with 10 μg/ml lipopolysaccharide (Sigma) and 100 ng/ml phorbol 12-myristate 13-acetate (Sigma) for 8 h and then collected for cDNA library construction. A plasmid cDNA library of pCMV-SPORT6.0 vector (Invitrogen) was constructed using a cDNA library of human bone marrow stromal cells (BMSCs). SPORT6.0 vector (Invitrogen) was constructed using a cDNA library of human bone marrow stromal cells (BMSCs). A plasmid cDNA (Sigma) and 100 ng/ml phorbol 12-myristate 13-acetate (Sigma) for 8 h were transfected into human hepatocellular carcinoma SMMC 7721 cells. Cells were lysed in cell lysis buffer (Cell Signaling). Anti-Myc immunoprecipitations were performed using mouse anti-Myc monoclonal antibody (Invitrogen) cross-linked to protein G-Sepharose beads (Santa Cruz Biotechnology) using dimethyl pimelimidate. Immunoprecipitation products or cell extracts were then separated by SDS-PAGE, transferred onto nitrocellulose membranes, and subjected to Western blot analysis. Relevant proteins were visualized using anti-PITSLRE kinase (Santa Cruz Biotechnology), anti-tyr-phosphorylated myc-6His tag was replaced by a FLAG sequence in the pCCNL2-wt vector to generate pCCNL2-FLAG. Antibody to recombinant human cyclin L2 (anti-CCNL2) was raised in rabbits against purified GST-CCNL2 fusion protein.

Mammalian Expression Vector Construction and Cell Transfection—The construction of pCCNL2-wt containing the full-length coding region of human cyclin L2 tagged by myc-6His was described above. Domain-negative mutants of human cyclin L2, including pCCNL2-ΔC (deletion of both cyclin domains) and pCCNL2-ARS (deletion of RS region), were generated by PCR mutations. Full-length cyclin L2 was cloned into pcDNA3.1/Myc-His(+) vector in a negative direction to give the anti-construct pCCNL2-AS. The myc-6His tag was replaced by a FLAG sequence in the pCCNL2-wt vector to generate pCCNL2-FLAG. pcDNA3.1/Myc-His(+) vector was linearized with NotI or BamHI, and the full-length coding sequence of cyclin L2 was ligated into pcDNA3.1/Myc-His(+) vector. pcDNA3.1/Myc-His(+) vector was transformed into L-cells and screened under 500 μg/ml G418 (Calbiochem) for ~3–5 weeks. Cell clones of stably transfected cells were obtained by the limited dilution method.

Immunoprecipitation and Western Blot—Cells were lysed in cell lysis buffer (Cell Signaling). Anti-Myc immunoprecipitations were performed using mouse anti-Myc monoclonal antibody (Invitrogen) cross-linked to protein G-Sepharose beads (Santa Cruz Biotechnology) using dimethyl pimelimidate. Immunoprecipitation products or cell extracts were then separated by SDS-PAGE, transferred onto nitrocellulose membranes, and subjected to Western blot analysis. Relevant proteins were visualized using anti-PITSLRE kinase (Santa Cruz Biotechnology), anti-tyr-phosphorylated myc-6His tag was replaced by a FLAG sequence in the pCCNL2-wt vector to generate pCCNL2-FLAG. Antibody to recombinant human cyclin L2 (anti-CCNL2) was raised in rabbits against purified GST-CCNL2 fusion protein.

Fluorescence Confocal Microscopy Analysis—pcDNA3.1/Myc-His(+) vector was linearized with NotI or BamHI, and the full-length coding sequence of cyclin L2 was ligated into pcDNA3.1/Myc-His(+) vector. pcDNA3.1/Myc-His(+) vector was transformed into L-cells and screened under 500 μg/ml G418 (Calbiochem) for ~3–5 weeks. Cell clones of stably transfected cells were obtained by the limited dilution method.
was eluted from the gel slice in elution buffer (0.5 M NH₄ acetate, 2 mM EDTA, 0.2% SDS) overnight at 37 °C. The eluted sample was precipitated with ethanol and stored as dry pellets at −80 °C. Splicing reactions were performed as described (16) and contained 80 mM potassium acetate, 20 mM creatine phosphate, 1 mM freshly prepared ATP, 1 unit/ml RNasin (Takara), 2 ng of labeled β-globin DNA precursor, 50 μg of HeLa nuclear extract (Promega), or 25 μg of cytoplasmic extract plus 250 ng of GST-CCNL2 fusion protein in a total volume of 20 μl. Reactions were incubated at 30 °C for 3 h, extracted with phenol/chloroform, and precipitated with ethanol. The reaction products were run on 8% denaturing polyacrylamide gels and were visualized by autoradiography.

In Vitro Analysis for Cell Growth—Cell proliferation of transiently or stably transfected SMMC 7721 cells was measured by MTT dye reduction assay (17). Briefly, cells were seeded into 96-well plates, and on the day of harvest, 10 μl of spent medium was replaced with an equal volume of fresh medium containing 10% MTT 5 mg/ml stock. Plates were incubated at 37 °C for 4 h; then 100 μl of MeSO (Sigma) was added to each well, and plates were shacked at room temperature for 10 min. Cellular viability was determined by measuring the absorbance of the converted dye at a wavelength of 570 nm.

Colony Forming Assay—Stably transfected SMMC 7721 or parental SMMC 7721 cells (5 × 10⁶/ml) were mixed with 1 ml of MethoCult methylcellulose-based medium (StemCell, British Columbia, Canada) and plated in 6-well plates according to the manufacturer's instructions. After 10 days of incubation at 37 °C in a humidified atmosphere containing 5% CO₂ in air, colonies (>50 cells) were counted using an inverted microscope.

Apoptosis Assay—Transiently transfected SMMC 7721 cells were washed, resuspended in staining buffer, and examined by ApoAlert Annexin V Apoptosis kit (BD Biosciences) and PI according to manufacturer's instruction. Stained cells were analyzed by FACS (FACS-calibur, BD Biosciences).

In Vivo Gene Transfer and Evaluation of Tumor Growth—nu/nu mice were subcutaneously inoculated with 3 × 10⁶ SMMC 7721 cells. When the tumors had grown to ~2.5 mm in diameter, mice were randomly divided into three groups of five tumor-bearing mice. In vivo electroporation was performed to deliver naked DNA into pre-established tumors (18, 19). Briefly, 20 μg of plasmid DNA in 30 μl of saline was injected into the tumors of tumor-bearing mice. One minute after injection, the site where the plasmid was injected was sandwiched in an electrode (BTX 533 2-needle array electrode) with poles 5 mm in diameter. Three electrical pulses of 20-ms duration at a voltage of 600 V were delivered using an ECM 830 Square Wave Electroporation System (BTX). Following the procedure, tumor growth of tumor-bearing mice was monitored every other day. The tumor volume was calculated as follows: \( V = 0.4 \times (a \times b^2) \) (\( V \) = volume, \( a \) = maximum tumor diameter, \( b \) = diameter at 90° to \( a \)) (19).

On the 32nd day following treatment, mice were killed. The tissues in the inoculated site of the tumor-bearing mice were dissected, fixed in 4% paraformaldehyde, and then embedded in paraffin. Deparaffinized 5-μm-thick specimens were subjected to hematoxylin and eosin staining and immunohistochemical ABC staining, respectively.

RESULTS

Identification and Sequence Analysis of Human Cyclin L2—An EST for a novel cyclin-like protein was originally isolated from human BMSC cDNA library by large scale random sequencing. The full-length sequence was assembled in silico by searching the NCBI GenBank™ data base, and finally obtained from human leukemia Raji cells. The 2319-bp full-length cDNA of the novel protein encoded a single 520-amino acid open reading frame with a theoretical molecular mass of 58.1 kDa and an isoelectric point of 10.29. There were two in-frame stop codons upstream of the open reading frame. A putative polyadenylation signal was located 26 bases upstream of the poly(A) stretch. BLAST search in the protein data base indicated that it most closely resembled human cyclin L1 (CCNL1, 65% identity and 78% similarity of overall protein sequences, Fig. 1A). High homologies were also found with other cyclins, including cyclin K (31% identity and 51% similarity), cyclin T1 (29% identity and 50% similarity), cyclin T2 (25% identity and 43% similarity), and cyclin C (30% identity and 48% similarity) (Fig. 1A). The predicted phylogenetic tree showed that it was more similar with human cyclin L1 in origin (Fig. 1B). Based on the sequence similarity with known cyclins, especially cyclin L1, the conservation of cyclin features, and its capacity of associating with RNAPol II and splicing factors (see below), the novel protein was designated as human cyclin L2 (CCNL2), according to the cyclin family nomenclature system recommended by HUGO.

The putative human cyclin L2 protein contained two cysteine domains, spanning amino acids 76–150 and 192–281. The cyclin domain is a helical domain present in cyclin proteins twice, as similar all-β folds. The predicted three-dimensional structure of human cyclin L2 generated by Swiss model did indicate that there was an obvious α-helix in the N terminus (data not shown) of human cyclin L2, which was in agreement with the common structural feature of the cyclin family. In addition to the highly conserved cyclin box region in the N terminus, there was also a conserved “serine/arginine-rich (RS)” repeat region in the C terminus of the human cyclin L2 protein (Fig. 1A), similar to that of cyclin L1 and some splice factor proteins known as SR protein. Although two transmembrane regions (amino acids 10–24 and 224–228) were predicted, no obvious signal peptide was found, suggesting that human cyclin L2 may be a non-secretory intracellular protein. A significant number of nuclear location signals indicated that human cyclin L2 might be a nuclear protein (Fig. 1A). The strong hydrophilicity in the C terminus of the human cyclin L2 was also consistent with features of cyclin L1 and cyclin K, both of which are important members of the transcriptional regulation cyclin family.

Identification of an Isoform and Mouse Homologue of Human Cyclin L2—During the cloning and sequencing of human cyclin L2 cDNA, a novel isoform was found in Raji cells. This new isoform of human cyclin L2 was designated as human cyclin L2s (CCNL2s) and submitted to NCBI GenBank™ (accession number AF1116620). The sequence of human cyclin L2s was identical to the 5’ region of human cyclin L2. The coding region cDNA of cyclin L2s was 684 bp in length, encoding a theoretical protein of 227 amino acids. There were two conservative cyclin domains in putative cyclin L2s protein, lacking the RS repeat region.

A mouse homologue of human cyclin L2 was identified from mouse melanoma B16 cells and designated as mouse cyclin L2. The mouse cyclin L2 cDNA was 1617 bp in length, encoding a putative protein of 518 amino acids with a calculated molecular mass of 58.0 kDa and an isoelectric point of 10.21. Mouse cyclin L2 protein showed 90% identity to and 93% similarity with human cyclin L2. There were also two cyclin domains and one SUA7 domain in mouse cyclin L2 protein, highly consistent with human cyclin L2. A BLAST search of the NCBI genome data base indicated that the mouse cyclin L2 gene is located in the low terminus of mouse chromosome 4. A previous report (20) identified a 224-amino acid protein, mouse ANIA-6B, that was the same as N terminus of mouse cyclin L2. Based on the protein and genome structural analysis of human and mouse cyclin L2, mouse ANIA-6B might be the alternatively spliced isoform of mouse cyclin L2. The sequence of mouse cyclin L2 is available in the GenBank™ data base under accession number AY337018.

Expression Pattern of Human Cyclin L2 mRNA—The expression of human cyclin L2 mRNA was detected by RT-PCR and Northern blot analysis. RT-PCR detected expression of human cyclin L2 in all human hematopoietic cell lines and solid tumor cell lines examined (Fig. 2, A and B), including U-937 (histiocytic lymphoma), NB4 and HL-60 (promyelocytic leukemia), KG-1 (acute myelogenous leukemia), K-562 (chronic myelogenous leukemia), MOLT-4, Jurkat, and Hut 78 (T lymphoblastical
leukemia), Daudi (B lymphoma) and NAMALWA (Burkitt’s lymphoma), Reh (acute lymphocytic leukemia), HeLa (cervix epithelioid carcinoma), U251 (glioma), CaoV-3 (ovary adenocarcinoma), PC-3 (prostate adenocarcinoma), HT-29 (colon adenocarcinoma), SMMC 7721 (hepatocellular carcinoma), and MCF-7 (breast adenocarcinoma). Relatively higher levels of human cyclin L2 mRNA expression were observed in K-562, Reh, CaoV-3, U251, and HeLa cells.

On human adult multiple tissue Northern blots, human cyclin L2 was expressed as a message of about 5.0 kb in all of the normal human tissues assayed. Relatively higher expression levels were seen in ovary, heart, liver, and pancreas, whereas much lower expression levels were seen in spleen, colon, peripheral blood leukocyte, lung, and skeletal muscle (Fig. 2C).

We also examined the expression of human cyclin L1 in the above cell and tissue templates. Interestingly, the results revealed differential expression of human cyclin L2 and L1. Unlike cyclin L2, cyclin L1 expression was more restricted in cultured cell lines. Cyclin L1 was not detected in K-562, U-937, and NAMALWA cells and in other solid tumor cells except for CaoV-3. Its expression was much lower than that of cyclin L2. On human multiple tissue cDNA panels, the expression patterns of both cyclins were also dissimilar (Fig. 2C). The differential distribution of closely related cyclins belonging to the same class may reflect a functional diversity, resulting from the existence of a finely tuned mechanism of the cell cycle and transcriptional regulation by different members of the multiple cyclin family.

Nuclear Cytolocalization of Human Cyclin L2—Because human cyclin L2 contained nuclear localization signals and was predicted to be a nuclear protein, we examined the subcellular localization of FLAG-tagged cyclin L2 to determine whether it localized within the nucleus. pCCNL2-FLAG was co-transfected into SMMC 7721 cells with pEGFP-Nuc (encoding a
The expression of CCNL2-FLAG protein was assayed by Western blot analysis with anti-FLAG antibody, and a 59-kDa protein was detected in the crude extract of pCCNL2-FLAG-transfected SMMC 7721 cells (data not shown), consistent with the calculated molecular weight of cyclin L2. In transfected SMMC 7721 cells, we observed both generalized nucleoplasmic and punctated nuclear staining (a "speckled" pattern of expression) of CCNL2-FLAG protein (Fig. 3B), which preferentially co-localized with the nuclear GFP signal (Fig. 3C). The result confirmed the structure prediction that cyclin L2 is a typical nuclear protein.

**Human Cyclin L2 Co-localizes with Splicing Factors SC-35 and 9G8**—The "speckled" nuclear localization pattern is characteristic of proteins associated with spliceosome complexes such as SC-35 (21, 22). The distinct RS repeat region in human cyclin L2 suggested that it might be found in the nuclear speckles co-localized or associated with other splicing components. pCCNL2-FLAG was transfected into SMMC 7721 cells, and splicing factors SC-35 and 9G8 were detected by indirect immunofluorescence. The FLAG-tagged CCNL2 protein was found in similar punctated nuclear speckled regions (Fig. 3, E and H) to both SC-35 (Fig. 3D) and 9G8 (Fig. 3G), which suggested that cyclin L2 largely localized to the same subnuclear regions as the splicing factors (Fig. 3, F and I). Curiously, the cyclin L2 and SC-35 signals did not appear to overlap completely. The SC-35 protein appeared to be more heavily concentrated than CCNL2-FLAG, although was 9G8 not. The fluorescence dye staining of CCNL2-FLAG and indirect immunofluorescence staining for SC-35 may not superimpose one another, perhaps indicating a different functional association of cyclin L2 with SC-35 and 9G8.

**Human Cyclin L2 Associates with RNA Polymerase II, Splicing Factors SC-35, 9G8, and CDK p110 PITSLRE**—The cell nucleus (particularly the nuclear speckles) is believed to contain compartments enriched for nuclear proteins involved in transcriptional regulation and mRNA processing. Human cyclins C, H, K, and the closest homologue of cyclin L2, cyclin L1, have been proposed to play roles in the regulation of basal transcription through their association with and activation of CDKs that phosphorylate the CTD of the large subunit of RNA polymerase II. To explore the possibility that human cyclin L2 may associate with RNA polymerase II or other CDKs and splicing factors and to delineate the domain(s) of cyclin L2 required for these interactions, several domain-negative constructs tagged by myc-6His were overexpressed in SMMC 7721 cells and immunoprecipitated with anti-Myc antibody cross-linked with protein G-Sepharose beads. As shown in Fig. 4A, anti-Myc antibody could detect expression of Myc-tagged CCNL2-wt (59 kDa), CCNL2-ΔCL (39 kDa, both cyclin domains deleted) and CCNL2-ΔRS (51 kDa, RS repeat region deleted). We then examined the association of cyclin L2 with RNA polymerases II and other CDKs and splicing factors.

**RNA polymerase II exists in two major forms: RNAPIIa and RNAPIIb.**

**Fig. 2. Expression pattern of human cyclin L2 mRNA.** RT-PCR with human cyclin L2- and cyclin L1-specific primers was performed on hematopoietic cell lines (A) and solid tumor cell lines (B). All the cells were similarly positive for β-actin (lower panel). C, Northern blot analysis (upper panel) and RT-PCR (lower panel) on multiple tissue Northern blots and multiple tissue cDNA panels, respectively, for tissue distribution of human cyclin L2. Northern blots were analyzed with a probe corresponding to a C-terminal fragment of human cyclin L2.

**Fig. 3. Cytolocalization and co-localization of human cyclin L2 protein in human hepatocellular carcinoma SMMC 7721 cells.** Cells were transiently transfected with pCCNL2-FLAG and/or nuclear localizing GFP expression vectors; double-color confocal microscopy analysis of human cyclin L2 (anti-FLAG, red), nuclear GFP signal (green), and indirect immunofluorescence staining of SC-35 and 9G8 (green) was performed 72 h after transfection. Confocal micrographs show SMMC 7721 cells expressing the CCNL2-FLAG protein (B, E, and H) with nuclear GFP expression (A), SC-35 (D), and 9G8 (G) stained in vivo, and the respective fluorescence signals overlaid (C, F, and I).
SMMC 7721 cells were transfected with splicing factors SC-35 and 9G8. Phosphorylated RNA polymerase II, CDK p110 PITSLRE, and RS, pCCNL2-AS, or mock vector. Proteins were immunoprecipitated from cell lysates using anti-Myc or control mouse or rabbit IgG and detected using anti-Myc antibody (A) and antibodies (B) indicated on the left side of each panel.

RNAPII, a hyperphosphorylated CTD of RNA polymerase II. Immunoprecipitation results showed that human CCNL2 associated with the hyperphosphorylated form, RNAPII, but not RNAPIIa (Fig. 4B). The hyperphosphorylation of RNA polymerase II correlates with its association with certain splicing factors involved in mRNA processing. Here we further demonstrated that cyclin L2 associates with both the two splicing factors (Fig. 4B), which was consistent with their co-localization to nuclear speckles.

Cyclins, together with their CDK partners, regulate the orderly progression of the cell cycle. We investigated whether human cyclin L2 could possibly interact with known CDKs. Because the CDK p110 PITSLRE (also called CDK11) has been identified as the CDK partner for human cyclin L1 (12) and human cyclin L2, it remains to be determined whether cyclin L2 can partner with other CDKs.

The results also showed that association with RNAPII and p110 PITSLRE required the presence of the N-terminal cyclin domains of human cyclin L2, because in cells overexpressing CCNL2-ΔCL, a truncated protein lacking both cyclin domains, no association of cyclin L2 with RNAPII or p110 PITSLRE was detected. In CCNL2-ARS-expressing cells, the levels of RNA-PAPI and p110 PITSLRE precipitated were approximately the same as those with CCNL2-wt, suggesting that RS repeats might not be necessary for RNAPII and p110 PITSLRE interactions. Notably, the interactions with SC-35 and 9G8 in CCNL2-ΔCL-expressing cells were similar to those with CCNL2-wt, whereas in CCNL2-ARS-expressing cells, interactions decreased. Although this indicates a requirement for only the RS repeat region, the possibility cannot be not excluded that indirect interactions between cyclin L2 with SC-35 and 9G8 may occur, perhaps linked by p110 PITSLRE via the cyclin domains of cyclin L2.

Together with the nuclear distribution, the findings indicate that human cyclin L2 might play an active role in the cell nucleus, with involvement in the RNA processing complex via association with hyperphosphorylated RNA polymerase II, the splicing factors SC-35 and 9G8, and the CDK p110 PITSLRE.

Human Cyclin L2 Stimulates Pre-mRNA Splicing—Because cyclin L2 both co-localizes and associates with the splicing factors SC-35 and 9G8, does it contribute to splicing function? We performed in vitro splicing assays using HeLa cell nuclear extract (50 µg), assaying the effect of anti-CCNL2 polyclonal sera on in vitro splicing of a β-globin RNA precursor. As shown in Fig. 5, the presence of preimmune sera or anti-GST antibody did not affect splicing, whereas anti-CCNL2 sera remarkably inhibited splicing activity, suggesting that human cyclin L2 is involved in pre-mRNA splicing. We also performed in vitro splicing assays using sub-optimal amounts of HeLa cell nuclear extract (25 µg), with the addition of GST or GST-CCNL2 recombinant protein. In the absence of additional protein, or when GST was added to the reaction, the splicing activity was greatly reduced compared with that obtained with 50 µg of HeLa cell nuclear extract. Addition of recombinant GST-CCNL2 protein (250 ng) strongly stimulated splicing activity (Fig. 5). Human cyclin L2 thus appears to play a positive role in pre-mRNA splicing.

Overexpression of Human Cyclin L2 Suppresses Growth of Human Hepatocellular Carcinoma Cells and Induces Cell Apoptosis in Vitro and in Vivo—Hepatocellular carcinoma accounts for 80–90% of liver cancers and is one of the most prevalent carcinomas worldwide, particularly in Africa and Asia. We wondered if overexpression of human cyclin L2 would affect the growth of human hepatocellular carcinoma SMMC 7721 cells, which may contribute to understanding the molecular mechanisms of the cellular malignancy phenotype and invasion and metastasis of hepatocellular carcinoma.

Proliferation of SMMC 7721 cells transfected with different cyclin L2 constructs was analyzed by MTT assay. As shown in Fig. 6A, the proliferation of pCCNL2-wt and pCCNL2-ARS transiently transfected SMMC 7721 cells was inhibited compared with that of mock vector-transfected or parental SMMC 7721 cells (p < 0.05), although transfection of pCCNL2-AS or pCCNL2-ΔCL had minor effects on cell proliferation. The via-
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In order to determine whether overexpression of cyclin L2 in human hepatocellular carcinoma could mediate therapeutic effects in vivo, human hepatocellular carcinoma-bearing nude mice were used as a model. Purified human cyclin L2 expression plasmid was delivered into the tumors by in vivo electroperoration. Immunohistochemical analysis of hepatocellular carcinoma tissue confirmed high expression of Myc-tagged human cyclin L2 protein in tissues from tumor-bearing nude mice intratumorally transfected with pCCNL2-wt (Fig. 7A, panel a), indicating that in vivo electroporation can effectively mediate human cyclin L2 gene transfer into tumors. We next investigated whether intratumoral gene transfer of human cyclin L2 could inhibit the growth of parental SMMC 7721 cells in nude mice. We found that, compared with mock DNA, intratumoral gene transfer of human cyclin L2 could significantly inhibit the growth of SMMC 7721 tumors in nude mice (Fig. 7B). These findings indicate that intratumoral transfer of the human cyclin L2 gene can inhibit tumor growth significantly in vivo, consistent with the results obtained in vitro.

Pathological observation revealed more morphologically necrotic or apoptotic changes in the pCCNL2-wt-transfected group (including pyknosis, densely stained nuclei, cytoplasm or nucleus granularity, ringing or crescenting, and cytoplasm bubbling) (Fig. 7C, panel a) than in the control groups. No obvious inflammatory infiltration was found. This suggests that delivery of human cyclin L2 cDNA into human hepatocellular hepatoma can suppress tumor growth in vivo.

Overexpression of Human Cyclin L2 Up-regulates the Expression of p53 and Bax and Down-regulates the Expression of Bcl-2—Overexpression of human cyclin L2 induced the apoptosis of SMMC 7721 cells in vitro. Cellular apoptosis involves complex molecular cascades, and dysfunction of a variety of genes may lead to the onset and progression of apoptosis. We analyzed the expression of the apoptosis-related protein p53, anti-apoptotic protein Bcl-2, and pro-apoptotic protein Bax by Western blot and RT-PCR analysis. As shown in Fig. 8A, transient expression of CCNL2-wt and CCNL2-ARS induced an increase in p53 and Bax protein expression (3- and 4-fold, respectively) in SMMC 7721 cells 72 h post-transfection compared with pCCNL2-AS-, pCCNL2-ΔCL-, and mock vector-transfected, or parental SMMC 7721 cells. Significantly up-regulated p53 and Bax mRNA expression was also detected by RT-PCR (Fig. 8B). Conversely, Bcl-2 expression was down-regulated in SMMC 7721 cells overexpressing human cyclin L2. These results demonstrate that overexpression of human cyclin L2 modulates the expression of critical apoptotic factors, leading to cell apoptosis. It would be interesting to determine whether other apoptotic factors may also have a relationship with apoptosis induced by overexpression of human cyclin L2.

**DISCUSSION**

Known cyclins include more than 20 types, from A to M and T, whose abundance oscillates with different cell phases. The first cyclins were identified and named due to their critical function in the regulation of the cell cycle (23). Known cyclins include cyclins C, H, T1, T2, K, and L1, which have proposed roles in transcriptional regulation, and cyclin G1, G2, and I, which appear to have distinct roles from either cell cycle or transcriptional regulation (24). In this study, we describe the identification and characterization of a novel cyclin family member, cyclin L2. Human cyclin L2 is a novel cyclin box- and RS repeat region-containing protein that localizes within the nuclear speckles, co-localized with splicing factors SC-35 and
Among the known human cyclin proteins, cyclin L2 is most closely related to the C-type cyclins, such as cyclins L1, K, T1, T2, and C, particularly in the cyclin box region. C-type cyclins and their CDK partners are defined by their capacity to phosphorylate the CTD of RNA polymerase II, a step required for transcriptional elongation and RNA processing. It has been reported that cyclin H/CDK7 (25, 26), cyclin C/CDK8 (27), and cyclin K/CDK9 (28–30) promote transcriptional elongation and affect RNA processing indirectly through phosphorylation of the CTD. Phosphorylation of the CTD by cyclin T/CDK9 is shown to be essential for the transition from initiation of transcription to effective elongation of nascent mRNA transcripts (31). Based on sequence homology and the experimental observation that human cyclin L2 immunoprecipitates are associated with the hyperphosphorylated form of RNA polymerase II, it is very likely that CTD is also a target of cyclin L2/CDK in vivo.

The various aspects of RNA elongation and processing, including capping, splicing, and polyadenylation of nascent transcribed RNA, are crucial for the proper expression and regulation of gene expression. Understanding the mechanisms underlying these processes can provide insights into the control of gene expression and the potential therapeutic targets for various diseases.

**Fig. 7.** In vivo evaluation of tumor growth and pathological observations. A, immunohistochemical detection of Myc-tagged human cyclin L2 protein in hepatocellular carcinoma tissue of nude mice intratumorally transfected with pcDNA-cyclinL2 (×400). a, pCCNL2-wt-transfected group; b, mock vector-transfected group. B, growth of hepatocellular carcinoma SMMC 7721 cells in nude mice was inhibited by intratumoral delivery of human cyclin L2 naked DNA. Statistical analysis was performed by Student’s *t* test, assisted by BioMedCalc software. 1, *p* < 0.05 versus the pCCNL2-wt delivery group; 2, *p* > 0.05 versus the mock vector delivery group. C, pathological observation by hematoxylin and eosin staining of human hepatocellular carcinoma tissue of nude mice (×400). On the 32nd day following treatment mice were killed and dissected. Hematoxylin and eosin staining was performed on tumor tissues. a, pCCNL2-wt-transfected group; b, mock vector-transfected group.

**Fig. 8.** Effect of human cyclin L2 overexpression on the expression of apoptosis-related proteins in SMMC 7721 cells. A, Western blot analysis of protein expression of p53, Bax, and Bcl-2. B, RT-PCR analysis of mRNA expression of p53, Bax, and Bcl-2. SMMC 7721 cells transfected with indicated vectors were harvested 72 h post-transfection.
scripts, are tightly coupled by protein factors physically associating with the CTD of RNA polymerase II (32). Human cyclin L2 possesses RS-repeat domains, a characteristic feature of SR protein/spliceosome components, and associates and co-localizes with the known splicing factors SC-35 and 9G8. RS domain proteins have been discovered in several factors associated with transcription components, including the SR-like CDK-associated factors (33) and in a 3' cleavage factor, cleavage factor-I 68-kDa subunit (34), suggesting that communication between splicing and both transcription and 3'-processing might in some cases be mediated by interactions involving RS domain proteins (35, 36). We have confirmed the stimulatory effect of cyclin L2 in pre-mRNA splicing. This activity may be mediated by interaction through its cyclin domains with RNA polymerase II and CDK p110 PITSLRE, or direct or indirect interaction via cyclin domain/RS repeat region with SC-35 and 9G8, as an RNA elongation/processing unit, in the nuclear speckles. Of note, while this manuscript was in revision, de Graaf et al. (37) reported cyclin L1 and cyclin L2 as interaction partners and substrates of Dyrk1A (dual specificity tyrosine phosphorylation-regulated kinase 1A), a nuclear speckle-locating protein kinase with transcription factors and splicing factors as its substrates. Because human cyclin L2 and cyclin L1 are expressed differentially at different levels in different cells and tissues, it is possible that these two closely related cyclins contribute to tissue-specific regulation of splicing.

The PITSLRE protein kinases are parts of the large family of p34cd2-related kinases whose functions appear to be linked with progression of the cell cycle and the signal transduction of apoptosis and oncogenesis. PITSLRE CDK also interacts selectively with RNAPII but not with RNAPII, and PITSLRE proteins also contain many serine/arginine dipeptide repeats, characteristic of spliceosome components. The p110 PITSLRE has been shown to participate in a signaling pathway that may regulate the processing of RNA transcription. It interacts with the RNA-binding protein RPS1, an activator of mRNA splicing, RNA polymerase II, and multiple transcriptional elongation factors, regulating some aspects of RNA splicing and/or transcription in proliferating cells (38–40). Due to its identification as a partner for human cyclin L1 (12) and its mouse homologue cyclin AN-6A (20), it is referred to as CDK11. We show that human cyclin L2 can associate with p110 PITSLRE in a cyclin domain-dependent manner, suggesting that it is also likely to be a partner CDK for cyclin L2.

Our work here also revealed that overexpression of human cyclin L2 suppressed the growth of hepatocellular carcinoma SMMC 7721 cells both in vitro and in vivo, and induced cellular apoptosis in vitro. Cyclin L2 is ubiquitously expressed throughout the body, and in a variety of human cell lines, including SMMC 7721 cells. Under normal conditions, tumor cells such as SMMC 7721 cells may have anti-apoptotic mechanisms in place which antagonize the apoptosis-inducing effects of cyclin L2. However, when cyclin L2 is overexpressed, the apoptosis-inducing effects of cyclin L2 may counteract these anti-apoptotic mechanisms, resulting in an imbalance between apoptosis and cell survival and cell cycle regulation, if any. Previous studies have implicated other cyclins in apoptosis and growth inhibition of tumor cells. A potential p53-binding site is present in intron 1 of the cyclin K gene, and colony formation assays indicate that overexpression of cyclin K suppresses growth of the glioblastoma cell lines U373MG and T98G and colon carcinoma SW480 cells (41). At high levels, cyclin G1, a p53-responsive gene induced in alternative reading frame-arrested cells, also induces a G1-phase arrest in mammalian cells that coincides with p53 activation. Interestingly, growth inhibition by cyclin G1 does not require p53 but instead exhibits partial retinoblastoma protein dependence (42). A recent study by cDNA microarray and RT-PCR indicates that cyclin L1 is commonly overexpressed in well differentiated but not in poorly differentiated primary head and neck tumors, compared with corresponding correlating normal tissues (43). Other RS-repeat-containing proteins such as Hs-BTF (44) can also act as apoptosis-promoting proteins that interact with Bcl-2-related proteins. To gain insight into the mechanism of cyclin L2-induced apoptosis in SMMC 7721 cells, we assessed the expression of the apoptosis-related proteins p53, Bax, and Bcl-2. In our study, following transient expression of cyclin L2 in SMMC 7721 cells, p53 and Bax expression was observed to increase significantly, and down-regulation of Bcl-2 expression could also be observed. Previous studies have demonstrated that overexpression of p53 can transactivate Bax expression and inhibit Bcl-2 expression, resulting in apoptotic cell death (45). Combined with other observations, including its association with RNAPII, CDK p110 PITSLRE, and splicing factors, and its stimulatory effect in pre-mRNA splicing, our results suggest that overexpression of cyclin L2 might regulate the RNA transcription and processing of certain apoptosis-related factors, possibly through a p53-dependent increase in Bax and reduction in Bcl-2 pathway signaling, leading to tumor cell growth inhibition.

In conclusion, we report here the cloning of human and mouse cyclin L2, the association of human cyclin L2 with p110 PITSLRE and the splicing factors SC-35 and 9G8, and the potential involvement of human cyclin L2 in the stimulation of pre-mRNA splicing. Growth inhibition and apoptosis-inducing effects of human cyclin L2 on tumor cells was also observed both in vitro and in vivo. Further experiments are required to elucidate the roles of the novel molecule, human cyclin L2, in transcriptional regulation and cell growth suppression and the related mechanisms.

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REFERENCES

1. Wasch, R., and Cross, F. R. (2002) Nature 418, 556–562
2. Duman-Scheur, M., Weng, L., Xin, S., and Du, W. (2002) Nature 417, 299–304
3. Koeppe, D. M., Schaefer, L. K., Ye, X., Keyomarsi, K., Chu, C., Harper, J. W., and Elledge, S. J. (2001) Nat. Genet. 28, 173–177
4. Nguyen, V. T., Kiss, T., Michel, A. A., and Bensaude, O. (2001) Nature 414, 322–325
5. Miller, M. E., and Cross, F. R. (2001) J. Cell Sci. 114, 1811–1820
6. Bregman, B. D., Pessayre, R. G., and Kidd, V. J. (2000) Fronf. Biosci. 5, D244–D257
7. Chen, X. (2002) Dev. Cell 3, 518–519
8. Oegenschlaeger, T. (2002) J. Cell. Physiol. 190, 160–169
9. Liu, Z., Ueda, T., Miyazaki, T., Tanaka, N., Mine, S., Tanaka, Y., Taniguchi, T., Yamamura, H., and Minami, Y. (1998) Mol. Cell. Biol. 18, 3445–3454
10. Sanford, J. R., and Bruck, J. P. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 10184–10189
11. Tian, H., and Kole, R. (2001) J. Biol. Chem. 276, 33833–33839
12. Dicksonson, L. A., Edgar, A. J., Ebiny, J., and Gottegres, J. M. (2002) J. Biol. Chem. 277, 25465–25473
13. Chen, X. (2002) J. Cell Sci. 115, 1769–1776
14. Cao, X., and Maniatis, T. (1990) Nature 343, 417–421
15. Knauer, A. R., Maniatis, T., Ruskin, B., and Green, M. R. (1984) Cell 36, 993–1005
16. Reichert, V., and Moore, M. (2000) Nucleic Acids Res. 28, 416–423
17. Aleny, M. C., Schroder, D. A., Monks, A., Hursey, M. L., Czerwinski, M. J., Fine, D. L., Abbott, B. J., Mayo, J. G., Shoemaker, R. H., and Boyd, M. R. (1988) Cancer Res. 48, 589–601
18. Baba, M., Ishii, H., and Tatsuta, M. (2001) Gene Ther. 8, 1149–1156
19. Li, S., Xia, X., Zhang, Y., and Yuan, J. (2002) Gene Ther. 9, 390–397
20. Berke, D. J., Sugimoto, V., Zhu, P. P., Lavoie, B., Vincent, M., Krause, M., and Hyman, S. E. (2001) Neuron 32, 277–287
21. Fu, X. D., and Maniatis, T. (1990) Nature 343, 437–441
22. Spector, D. L. (1995) Annu. Rev. Cell Biol. 9, 265–315
23. Murray, A. W., and Marks, D. (2001) Nature 409, 444–446
24. Bates, S., Brown, S., and Vanden, K. H. (1996) Oncogene 13, 1105–1109
25. Larochelle, S., Chen, J., Knights, R., Pandur, J., Morcillo, E., Erjdenburg, H., Tempst, P., Suter, B., and Fisher, R. P. (2001) EMBO J. 20, 3749–3759
26. Andersen, G., Busso, D., Peterszman, A., Hwang, J. R., Wurtz, J. M., Ripp, R., Thierry, J. C., Egly, J. M., and Moras, D. (1997) *EMBO J.* 16, 958–967.
27. Tassan, J. P., Jaquenoud, M., Leopold, P., Schultz, S. J., and Nigg, E. A. (1995) *Proc. Natl. Acad. Sci. U. S. A.* 92, 8871–8875.
28. Edwards, M. C., Wong, C., and Elledge, S. J. (1998) *Mol. Cell. Biol.* 18, 4291–4300.
29. Lin, X., Taube, R., Fujinaga, K., and Peterlin, B. M. (2002) *J. Biol. Chem.* 277, 16873–16878.
30. Fu, T. J., Peng, J., Lee, G., Price, D. H., and Flores, O. (1999) *J. Biol. Chem.* 274, 34527–34530.
31. Peng, J., Zha, Y., MiJten, J. T., and Price, D. H. (1998) *Genes Dev.* 12, 755–762.
32. McCracken, S., Fong, N., Yankulov, K., Ballantyne, S., Pan, G., Greenblatt, J., Patterson, S. D., Wickens, M., and Bentley, D. L. (1997) *Nature* 385, 357–361.
33. Corden, J. L., and Patturajan, M. (1997) *Trends Biochem. Sci.* 22, 413–416.
34. Ruegsegger, U., Blank, D., and Keller, W. (1998) *Mol. Cell* 1, 243–253.
35. Blencowe, B. J., Bowman, J. A., McCracken, S., and Rosonina, E. (1999) *Biochem. Cell Biol.* 77, 277–291.
36. Hirose, Y., and Manley, J. L. (2000) *Genes Dev.* 14, 1415–1429.
37. de Graaf, R., Hekerman, P., Spelten, O., Herrmann, A., Packman, L. C., Bussow, K., Muller-Newen, G., and Becker, W. (2004) *J. Biol. Chem.* 279, 4612–4624.
38. Ariza, M. E., Broome-Powell, M., Lahti, J. M., Kidd, V. J., and Nelson, M. A. (1999) *J. Biol. Chem.* 274, 28505–28513.
39. Lahti, J. M., Xiang, J., Heath, L. S., Campana, D., and Kidd, V. J. (1995) *Mol. Cell. Biol.* 15, 1–11.
40. Zhang, S., Cai, M., Zhang, S., Xu, S., Chen, S., Chen, X., Chen, C., and Gu, J. (2002) *J. Biol. Chem.* 277, 35314–35322.
41. Mori, T., Anazawa, Y., Matsu, K., Fukuda, S., Nakamura, Y., and Arakawa, H. (2002) *Neoplasia* 4, 268–274.
42. Zhao, L., Samuels, T., Winkler, S., Kurgasnikar, C., Tompkins, V., Horne, M. C., and Quelle, D. E. (2003) *Mol. Cancer Res.* 1, 195–206.
43. Redon, R., Hussenet, T., Bour, G., Caulee, K., Jost, B., Muller, D., Abecassis, J., and du Mansir, S. (2002) *Cancer Res.* 62, 6211–6217.
44. Kasof, G. M., Goyal, L., and White, E. (1999) *Mol. Cell. Biol.* 19, 4390–4404.
45. Basu, A., and Haldar, S. (1998) *Mol. Hum. Reprod.* 4, 1099–1109.
