Characterization of Cyclin L1 and L2 Interactions with CDK11 and Splicing Factors

**INFLUENCE OF CYCLIN L ISOFORMS ON SPLICE SITE SELECTION**

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Although it has been reported that cyclin L1α and L2α proteins interact with CDK11p110, the nature of the cyclin L transcripts, the formation of complexes between the five cyclin L and the three CDK11 protein isoforms, and the influence of these complexes on splicing have not been thoroughly investigated. Here we report that cyclin L1 and L2 genes generate 14 mRNA variants encoding six cyclin L proteins, one of which has not been described previously. Using cyclin L gene-specific antibodies, we demonstrate expression of multiple endogenous cyclin L proteins in human cell lines and mouse tissues. Moreover, we characterize interactions between CDK11p110, mitosis-specific CDK11p58, and apoptosis-specific CDK11p46 with both cyclin Lα and -β proteins and the co-elution of these proteins following size exclusion chromatography. We further establish that CDK11p110 and associated cyclin Lα/β proteins localize to splicing factor compartments and nucleoplasm and interact with serine/arginine-rich proteins. Importantly, we also determine the effect of CDK11-cyclin L complexes on pre-mRNA splicing. Pre-incubation of nuclear extracts with purified cyclin Lα and -β isoforms depletes the extract of in vitro splicing activity. Ectopic expression of cyclin L1α, L1β, L2α, or L2β or active CDK11p110 individually enhances intracellular intron splicing activity, whereas expression of CDK11p58/p46 or kinase-dead CDK11p110 represses splicing activity. Finally, we demonstrate that expression of cyclins Lα and -β and CDK11p110 strongly and differentially affects alternative splicing in vivo. Together, these data establish that CDK11p110 interacts physically and functionally with cyclin Lα and -β isoforms and SR proteins to regulate splicing.

It has become apparent over the past decade that several cyclin-dependent kinases (CDKs) and their cyclin regulatory partners participate in regulating mRNA production (1). Thus far, CDK7, CDK8, and CDK9 functions are ascribed to transcriptional initiation and elongation, and CDK12 (CrkRS) and CDK13 (CDC2L5) functions are related to pre-mRNA splicing (2–4). Interestingly, CDK11p110 plays roles in both transcription and splicing, suggesting that this CDK may link the two processes (5, 6). In addition, the CDK11p110 partner proteins cyclins L1 and L2 also influence splicing (7, 8). Two distinct genes, Cdc2L1 and Cdc2L2 (acronym for Cell division control 2 Like), encode the human p110 and p58 PITSLRE protein kinases (9–12). These kinases were renamed CDK11p110 and CDK11p58 when cyclins L1 and L2 were identified as regulatory subunits of CDK11p110 (13). Expression of the CDK11p110 isoforms is ubiquitous and constant throughout the cell cycle (11). In contrast, CDK11p58 is expressed and functions specifically in G2/M via an internal ribosome entry site (IRES) located within the CDK11p110 mRNA (14–17). During apoptosis, a third isoform, CDK11p46, is generated by caspase-dependent cleavage of CDK11p110 and CDK11p58, leaving the catalytic domain intact (18, 19).

A role for CDK11p110 in transcription and pre-mRNA splicing is supported by data from both this and other laboratories. We have shown that soluble nuclear extracts contain two macromolecular CDK11p110 protein complexes of 1–2 MDa and ~800 kDa. These complexes contain transcription-related proteins such as the largest subunit of RNA polymerase II, FACT (facilitates chromatin transcription), CK2, and general transcription factor IIF. We demonstrated the involvement of CDK11p110 in transcription more directly by showing that anti-CDK11p110 catalytic domain antibodies reduced RNA transcription from both TATA-like and GC-rich promoters in vitro (5). Recently, CDK11p58, in association with cyclin D3, was reported to negatively affect androgen receptor transcriptional activity.

The abbreviations used are: CDK, cyclin-dependent kinase; RNPS1, RNA-binding protein with serine-rich domain; CR2, casein kinase II; HFF, human foreskin fibroblast; RT, reverse transcription; IP, immunoprecipitation; IRES, internal ribosomal entry site; PFI, phytohemagglutinin; GST, glutathione S-transferase; WT, wild-type; NE, nuclear extract; PBS, peripheral blood cell; HA, hemagglutinin; mAb, monoclonal antibody; pAb, polyclonal antibody; Nif²⁻ NTa, nickel-nitrioltriacetic acid; PBS, phosphate-buffered saline; PCNA, proliferating cell nuclear antigen; SFC, splicing factor compartment; DN, Asp-to-Asn mutation; SR, serine/arginine-rich.

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The on-line version of this article (available at http://www.jbc.org) contains supplemental “Experimental Procedures,” additional reference, Table 1, and Data 1–6.

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activity, whereas CDK11<sup>p110</sup> positively affected transcription of numerous reporter genes (20). Similarly, CDK11 was identified as a positive regulator of hedgehog signaling in both fly and vertebrate cells (21).

We have also identified two splicing factors, RNPS1 (22) and 9G8 (6), as partners for CDK11<sup>p110</sup>. RNPS1 is an SR protein that functions as a general activator of splicing, promotes alternative splicing in a substrate-specific manner, and is a component of the exon-exon junction complex. RNPS1 co-immunoprecipitates with CDK11<sup>p110</sup>, and both RNPS1 and CDK11<sup>p110</sup> are phosphorylated by CK2 (23, 24). The general splicing factor 9G8, which also promotes the nucleocytoplasmic export of mRNA, co-immunoprecipitates with CDK11<sup>p110</sup> and is a CDK11<sup>p110</sup> substrate. A role for CDK11<sup>p110</sup> in pre-mRNA splicing was confirmed using conventional in vitro splicing assays that showed that immunodepletion of the CDK11<sup>p110</sup> kinase from nuclear extracts greatly reduced splicing activity (6).

The cyclin L1 (Ania-6a) gene is alternatively spliced to produce mRNAs encoding three putative proteins, cyclins L1<sub>1</sub>, L1<sub>β</sub>, and -γ (13). Cyclin L1<sub>α</sub>, the largest isoform, is an atypical 526-amino acid cyclin consisting of an N-terminal cyclin box and a C-terminal RS domain similar to that of SR proteins. Cyclin L1<sub>β</sub>, a 232-amino acid protein, contains the entire cyclin box but lacks the RS domain. Similarly, the 172-amino acid cyclin L1<sub>γ</sub> protein is a C-terminal truncated version of cyclin L1<sub>β</sub>. The cyclin L2 (Ania-6b) gene encodes two putative proteins, cyclins L2α and -β, that are structurally similar to cyclins L1<sub>α</sub> and L1<sub>β</sub> (13). The observation that cyclin L1<sub>α</sub> and L2<sub>α</sub> contain an RS domain and the finding that they both co-immunoprecipitate with CDK11<sup>p110</sup> are consistent with a role for CDK11<sup>p110</sup>-cyclin L1<sub>/2α</sub> complexes in pre-mRNA splicing. This hypothesis is supported by the fact that cyclin L1<sub>α</sub> is an immobile component of the splicing factor compartment (25) that is also associated with hyperphosphorylated RNA polymerase II (13). In addition, de Graaf et al. (26) identified cyclin L2α as a substrate of DYRK1A, a dual specificity protein kinase. Phosphorylation of several transcription factors and induces SR protein redistribution. Other support for a functional role of CDK11<sup>p110</sup>-cyclin L complexes in splicing includes data from our group revealing that CDK11<sup>p110</sup>, cyclin L1<sub>α</sub>, and 9G8 form a ternary complex and that 9G8 is phosphorylated by CDK11<sup>p110</sup> and data from others demonstrating that bacterially produced cyclins L1<sub>α</sub> and L2<sub>α</sub> stimulate splicing in an in vitro assay (7, 8).

Here, we characterize in detail the mRNA species encoding the various cyclin L1 and L2 isoforms. Using cyclin L1<sub>α</sub>- and L2<sub>α</sub>-specific antibodies as well as an antiserum capable of detecting all cyclin L proteins, we demonstrate expression of multiple endogenous cyclin L proteins in human cell lines and mouse tissues and show that both CDK11<sup>p110</sup> and CDK11<sup>p58</sup> co-immunoprecipitate with endogenous cyclin L<sub>1</sub> and L2<sub>α</sub> proteins. Using transient transfections, we also establish that cyclin L1<sub>/2α</sub> and -β proteins interact with CDK11<sup>p110</sup>, CDK11<sup>p58</sup>, and CDK11<sup>p46</sup> protein kinases and that all cyclin L proteins co-localize with CDK11<sup>p110</sup> in nuclear splicing factor compartments (SFCs) and in the nucleoplasm. Furthermore, recombinant cyclin L1<sub>/2α</sub> and -β proteins bind CDK11<sup>p110</sup> as well as SR proteins, and cyclins L1<sub>/2α</sub> and -β, CDK11<sup>p110</sup>, and SR proteins co-fractionate in complexes ranging in molecular mass from ~100 kDa to ~1 MDa. Finally, we performed a comprehensive analysis of the contribution of the individual cyclin L and CDK11 isoforms to splicing activity using both in vitro and in vivo systems.

**EXPERIMENTAL PROCEDURES**

**Cell Culture, Transfection, and Retroviral Infection**—HuH7, HEK 293T, HeLa, NB9, NB13, and human foreskin fibroblasts (HFFs) were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum and 2% l-glutamine. Histopaque-1077 (Sigma)-purified lymphocytes were cultured for 4 days from peripheral blood obtained from healthy donors in RPMI 1640 medium supplemented with 20% fetal bovine serum, 2% l-glutamine, and 2% phytohemagglutinin (PHA, M-form; catalog number 10576-015 Invitrogen).

**Recombinant Protein Production**—His<sub>6</sub>-tagged cDNAs encoding full-length cyclins L1<sub>α</sub>, L1<sub>β</sub>, L2<sub>α</sub>, and L2<sub>β</sub> and His<sub>6</sub>-tagged cyclin L1<sub>α</sub> peptide (amino acids 314–369) were cloned into the pQE expression vector (Qiagen), expressed in M15[pREP4] bacteria, and purified using nickel-nitrioltriacetic acid (Ni<sup>2+</sup>-NTA) affinity under denaturing conditions according to the manufacturer’s protocol (The QIAexpressionist<sup>TM</sup>, Qiagen). Purified proteins were renatured by gradual elimination of denaturing agent, and beads were resuspended in in vitro splicing assay compatible buffer containing 20 mM Heps-KOH (pH 8), 100 mM KCl, 0.2 mM EDTA, 0.5 mM dithiothreitol, 0.5 mM phenylmethanesulfonyl fluoride, and 20% glycerol. 9G8 was produced as described previously (6), and SF2/ASF was a gift from Dr. A. Mayeda (University of Miami, FL). GST-cyclin L2α (amino acids 307–379) was expressed in BL21 bacteria and purified using GSH-Sepharose<sup>TM</sup> 4B (Amersham Biosciences) using the manufacturer’s protocol.

**Antibodies**—The CDK11 antibodies P2N100 and P1C have been described previously (12, 24). The cyclin L antibodies were produced by immunization of rabbits with purified His<sub>6</sub>-tagged full-length cyclin L1<sub>β</sub> (pan-L antibody), His<sub>6</sub>-tagged cyclin L1<sub>α</sub> peptide (cyclin L1<sub>α</sub>-specific antibody, amino acids 314–369), or GST-cyclin L2α peptide (cyclin L2α-specific antibody, amino acids 307–379). The antibodies were affinity-purified by column chromatography using the recombinant proteins described above. Antibodies recognizing HA tag (Roche Applied Science), FLAG tag (M2, Sigma), CK2α (C-18), SF2/ASF (P-15), 9G8 (H-120) (Santa Cruz Biotechnology), PCNA (PC10, Dako), and SR proteins (1H4, Zymed Laboratories Inc.) were used as described by the manufacturer. The anti-SR mAb 1H4 recognizes five proteins (SRp75, SRp55, SRp40, SRp30a/b, and SRp20) and several other bands that have not been fully characterized (27).

**Chromatography/Size Fractionation**—Size fractionation analysis was carried out using a Superose 6 column (Amersham Biosciences). Protein standards of 737, 460, 170, 67, and 25 kDa were used to calibrate the column. Mouse liver was sonicated in 50 mM phosphate (pH 7), 150 mM NaCl, 0.2 mM EDTA, 0.1% Nonidet P-40, and 1× protease inhibitors (Complete EDTA-free, Roche Applied Science). Lysates were centrifuged twice at 14,000 rpm for 20 min. One mg of liver lysate or HeLaScribe<sup>®</sup>
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nuclear extract (Promega) was loaded onto the column, and 1-ml fractions were collected.

Immunobots, Immunoprecipitations, and Immunofluorescence—For immunoblot analysis, human cell lines and mouse tissues were washed in PBS and lysed by brief sonication as described previously (22). Lysates were centrifuged at 14,000 rpm for 20 min, and the protein concentration was determined. Immunoprecipitation (IP) of endogenous CDK11 protein kinases was performed using 293T and PBC cell lysates. IP of FLAG-tagged CDK11 and HA-tagged cyclin L proteins was performed using cell lysates of 293T cells harvested 48 h after transfection using JetPEI (Qiagen). In all IP experiments, cell lysates were incubated overnight with antibodies (500 µg of lystate, 1 µg of antibody) and then incubated for 2 h with GammaBind Plus-Sepharose (GE Healthcare) rotating at 4 °C. The beads were then washed four times with lysis buffer, and immunoblot analysis was performed as described previously (22). All immunofluorescence assays were performed using HFF cells grown on coverslips, fixed with cold methanol at −20 °C for 10 min, and rinsed with PBS. Expression of HA-tagged cyclin L proteins was accomplished using retrovirus encoding cyclin L proteins produced by transfection of the 293T Phoenix amphotropic cell line (ATCC) with pMSCV-HA-cyclin L-IRE-GFP vectors. The incubations with primary antibodies were performed for 1 h at 37 °C in PBS containing 0.1% casein. Anti-rabbit, anti-mouse, and anti-rat fluorescent secondary antibodies labeled with Alexa Fluor 488 (Molecular Probes) and Cy3 (Jackson ImmunoResearch) were used as described above.

Pulldown Assays, Nuclear Extract Depletion, and in Vitro Splicing Assays—Pulldown assays for SR proteins and depletion experiments were performed by incubating 36 µg (3 µl) of HeLaScribe® nuclear extract adjusted to 15 µl with in vitro splicing assay compatible buffer with His6-cyclin L N12^−-NTA beads (1 µg of protein/20 µl of beads) or control beads (20 µl) for 1 h on ice. The beads were extensively washed with the binding buffer prior to use for immunoblot analysis or splicing assays (22). For the splicing assays, 32P-labeled β-globin pre-mRNA substrate was prepared by in vitro transcription using SP6 RNA polymerase and linearized pSP64-Hβ3E6 plasmid as the template (28). In vitro splicing reactions were carried out in a final volume of 25 µl containing HeLaScribe® nuclear extract (36 µg/15 µl) and splicing assay mix (10 µl) containing 0.5 mM ATP, 20 mM creatine phosphate, 8 mM MgCl2, 200 mM Hepes-KOH (pH 7.3), 6.5% polyvinyl alcohol, and 2 fmol of 32P-labeled β-globin pre-mRNA substrate. Reactions were incubated at 30 °C for 4 h. The spliced RNA products were analyzed by autoradiography following electrophoresis on a 5.5% polyacrylamide, 7% urea gel.

In Vivo β-Galactosidase/Luciferase Splicing Assays—293T cells were transfected using FuGENE 6 (Roche Applied Science) as described by the manufacturer with the splicing reporter vector pTN24 (1 µg) (29) and constructs expressing cyclin L (3 µg) and CDK11 (3 µg). The cyclin L constructs were subcloned into the pFlex vector with an N-terminal FLAG tag, and the CDK11 p110, p110ΔRE (deletion of amino acids 127–220), and p58 constructs were subcloned into the pUHD 10-3 vector with a C-terminal FLAG tag. The CDK11 p46 construct, containing a nuclear localization signal at the N terminus and a

FLAG tag at the C terminus, was subcloned into the pTet-1 vector. The pTet-1 vector has a pcDNA 3.1 backbone with the tetracycline-responsive promoter replacing the cytomegalovirus promoter. Cells were harvested 24 h after transfection for the enzymatic assays. β-Galactosidase and luciferase activities were measured using the Dual-Light® Assay System (Applied Biosystems) (24). 3–9 transfected samples were measured in quadruplicate per data point. Immunoblot analyses of equal volume cell lysates were performed. Statistical significance analyses for various pairwise comparisons were performed using a nonparametric rank-based test (see supplemental “Experimental Procedures” for an R subroutine used in the analyses).

EIA in Vivo Splicing Assays—HuH7 hepatoma cells in 60-mm dishes were co-transfected using transfectin (Bio-Rad) via the manufacturer’s instructions with pCEP4-EIA (1.2 µg) (30) (gift from Dr. Tarn W-Y, Institute of Biomedical Sciences, Taipei, Taiwan) and expression vectors (5 µg) for CDK11 p110 WT or DN (pMSCV-IRE-GFP) and HA-cyclin L isoforms (pcDNA 3.0). Total plasmid amount was equalized for each transfection using the appropriate empty vector. Cells were harvested 48 h after transfection, and total RNA was extracted using the RNeasy kit (Qiagen). The RNAs were further treated using the RNase-free DNase I kit (Qiagen). RT was performed using 5 µg of total RNAs, the SuperScriptTM II RNase H− reverse transcriptase kit, and the EIA-specific primer CGGATATCCACATTGGGACACT (P2). For detection of E1A splice variants, 2 µl of the RT reaction was used as template, and PCR amplification (Takara ExTaq, 50 µl, 35 cycles) was performed using primers CAAGCTTGAGTGCTCAGCGAGTAG (P1) and CTCAGGTTTCAGACAGG (P3). PCR products were Visualizations via 1% agarose gels stained with ethidium bromide using Bio-Vision fluorescence image acquisition system (Vilber-Lourmat, Fisher-Bioblock, France) and quantitated using Bio-1D software (Vilber-Lourmat, Fisher-Bioblock). The percentage of each transcript signal relative to the total amount of the five splice forms was calculated for each sample and expressed relative to the appropriate control splice form percentages, which were set equal to 1.

RESULTS

Cyclin L1 and L2 Genomic Organization, mRNA Splice Variants, and Translation Products—We identified nine distinct mRNA splice variants from the human cyclin L1 gene by RT-PCR in human tissues (supplemental Data 1) and HFF cells (data not shown). This gene, located on chromosome 3q23.2–3 (7), contains 14 exons and spans ~12.3 kb. From these nine mRNAs, three distinct open frames were found encoding the three cyclin L1 protein isoforms, L1α, L1β, and L1γ. All of the cyclin L1 isoforms share a similar N-terminal sequence, encoded by exons 1–3, which contains the cyclin box domain and diverge in their C termini (2). The cyclin L1α protein exhibits an extended C terminus with an Arg/Ser (RS) di-peptide region characteristic of splicing factors, which is not present in the β or γ isoforms. Importantly, because of the location of the translational stop sequences, cyclin L1β and L1γ proteins contain 7 and 9 amino acid C-terminal peptides, respectively, which are specific for these two isoforms (Table 1). The human
cyclin L2 gene spans ~11.8 kb on chromosome 1 (1p36.33) (8, 26). This gene is comprised of 14 exons and encodes five distinct mRNA variants identified by RT-PCR in human tissues (supplemental Data 2) and HFF cells (data not shown). The products contain three distinct open reading frames. The largest isof orm, cyclin L2, is encoded by the shortest mRNA transcript, and the protein domain composition of L2 is identical to that of L1. The cyclin L2A and L2B isoforms differ by only 2 and 12 amino acids, respectively, in their C-terminal ends (Table 1). The exon and intron lengths for the cyclin L1 and L2 genes are summarized in supplemental Table 1.

The detection of both cyclin L1 and L2 transcripts by RT-PCR in all human tissues (supplemental Data 1 and 2) and HFF cells tested suggests ubiquitous expression of these two genes. Similar data were obtained by Northern blot analyses of cyclin L1 and L2 gene expression in various human tissues and cell lines (supplemental Data 3). Furthermore, these analyses confirmed the expression of 2.3- and 4.5-kb cyclin L1 and L2 mRNA species in human cells as reported by others (7, 26). Although the RT-PCR experiments are not quantitative, the relative abundance of the L1 and L2 splice variants detected differs slightly between tissues suggesting there are different levels of cyclin L isoform expression in the cell types tested.

Cyclins L1α and L2α Are Nuclear Proteins That Co-localize with CDK11p110 Protein Kinase—To study expression of cyclin L proteins and investigate formation of complexes with CDK11 kinases in mammalian cells, we raised rabbit polyclonal anti-cyclin L antibodies recognizing various cyclin L isoforms. Independent immunizations were carried out using bacterial recombinant full-length cyclin L1β and two peptides corresponding to amino acids 314–369 for cyclin L1α and 307–379 for cyclin L2α. These two peptides, which are located in a short region connecting the cyclin box and RS domain, demonstrate very low homology between cyclins L1α and L2α and are absent in cyclins L1β/γ and L2β. To assess the specificity of these antibodies, immunoblot analyses were performed using cell extracts from 293T cells transfected with expression vectors encoding HA-tagged cyclin L proteins (Fig. 1A). An anti-HA immunoblot confirmed expression of the HA-tagged cyclin L proteins. Immunoblot analysis using the rabbit polyclonal antibody raised against full-length cyclin L1β (pan-L) demonstrated this reagent was able to detect all cyclin Lβ isoforms and cross-reacted with all of the cyclin Lα isoforms. However, the bands obtained for cyclin L1 proteins were more intense than those for cyclin L2 and those obtained with anti-HA antibody, indicating pan-L recognized the cyclin L1 proteins with a higher affinity than the cyclin L2 proteins. The pan-L antibody also detected endogenous cyclins L1α and L2α but did not detect endogenous cyclin L1β, L1γ, or L2β in 293T cells. This

### TABLE 1

| 14 alternatively spliced mRNAs encode six cyclin L1 and L2 protein isoforms |
|-----------------------------|---------------------|-----------------------|
| 14 mRNAs                   | 6 proteins          | Last coding exon      | Length |
| L1α                        | L1α                 | Exon 14, –SRSGHGHRRR* | 526    |
| L1β                        | L1β                 | Exon 7, VVHDGKS*      | 323    |
| L1γ                        | L1γ                 | Exon 4, SDQULLLHPKPG* | 307    |
| L2α                        | L2α                 | Exon 14, –DHGPISHRRR* | 520    |
| L2βA                       | L2βA                | Exon 6, –GK*          | 226    |
| L2βB                       | L2βB                | Exon 7, DPLLKWDSWQR*  | 236    |

* indicates the presence of a stop codon at that position.

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**TABLE 1**

14 alternatively spliced mRNAs encode six cyclin L1 and L2 protein isoforms.

Alternative splicing of cyclin L1 and L2 genes generates 14 mRNAs that encode six proteins. The final coding exon and corresponding terminal amino acid sequence are indicated in the table with the length in amino acids of each protein isoform.
suggests that the abundance of these proteins is very low and/or that the affinity for the shorter cyclin L isoform is relatively low. Antibodies against cyclin L1α and L2α peptides specifically recognized these two proteins and not the shorter cyclin L isoforms. As expected, these three antibodies also detected endogenous cyclins L1α and L2α co-migrating with HA-tagged cyclins L1α and L2α in extracts of nontransfected 293T cells. Both cyclins L1α and L2α are detected in the apparent molecular mass range of ~64 to 68 kDa, which is larger than the predicted molecular masses of 59.6 and 58.4 kDa, respectively, thus strongly suggesting the presence of post-translational modifications.

We also examined the intracellular distribution of endogenous cyclin L proteins by indirect immunofluorescence. Immunodetection with pan-L, cyclin L1α, and L2α antibodies showed nuclear staining with both large speckles and diffuse nucleoplasmic localization (Fig. 1B). As expected, CDK11p110 exhibited a very similar nuclear staining and co-localized with cyclin L1α and L2α proteins. However, the cyclin L staining pattern differed from that obtained with antibodies recognizing PCNA, a nuclear protein involved in DNA replication, which served as a control for specificity of co-localization. Given that our cyclin L antibodies cannot differentiate between α and β/γ isoforms by this method of detection, we also determined the intracellular localization of epitope-tagged isoforms of cyclin L. Expression of HA-tagged cyclin L proteins and endogenous CDK11 was detected using anti-HA monoclonal and P1C anti-CDK11 polyclonal antibodies. Cyclins L1/2α, L1/2β, and L1/γ each localized to both the nucleoplasm and large nuclear speckles and co-localized with endogenous CDK11 (supplemental Data 4). Importantly, the immunofluorescence localization patterns of the tagged proteins were indistinguishable from that of the endogenous protein. Given that the HFF cells are in interphase and nonapoptotic, the CDK11 proteins detected by the P1C antibody are likely CDK11p110.

Cyclin L1α and L2α Proteins Are Ubiquitously Expressed in Human Cell Lines and Mouse Tissues and Co-immunoprecipitate with CDK11p110, CDK11p58, and CDK11p46 Protein Kinases—Cyclins L1α and L2α were detected in 293T, HeLa, NB13, and NB19 human cell lines as well as in cultured activated human peripheral blood cells (PBCs) and nontransformed HFF cells using the isoform-specific antibodies (Fig. 2A). In addition to the cyclin Lα proteins, the pan-L antibody also detected low levels of 25–30 kDa proteins co-migrating with cyclin Lβ. As reported previously, CDK11p110 protein was ubiquitously expressed at various levels in human cells, whereas CDK11p58 and CDK11p46, which are expressed in mitosis and during apoptosis, respectively, are generally undetectable in log phase growing cells (14, 19), although both CDK11p58 and CDK11p46 were highly expressed in PBCs stimulated with PHA. Because of the high conservation between human and mouse cyclin L proteins, the antibody generated against human full-length cyclin L1β should cross-react with mouse cyclin L proteins. In mouse tissues, pan-L antiserum detected several bands in the ranges of 50–65 and 25–35 kDa (Fig. 2B) co-migrating with HA-tagged human cyclin L1/2α and L1/2β proteins, respectively. These data indicate that cyclins L1/2α and L1/2β are expressed at high levels in mouse tissues.

To detect endogenous cyclin L-CDK11 complexes, anti-cyclin L and CDK11 antibodies were used to immunoprecipitate these complexes from 293T cell lysates (Fig. 3A). Immunoprecipitations (IPs) were performed using the anti-CDK11 P1C antibody, which recognizes CDK11p110, CDK11p58, and CDK11p46, the anti-CDK11 P2N100 antibody which recognizes only CDK11p110, the pan-L antibody which recognizes all of the cyclin L1 and L2 isoforms, the cyclin L1α and cyclin L2α-specific polyclonal antibodies, and purified rabbit immunoglobulins as negative controls. IP complexes analyzed by immunoblotting for the co-IP of CDK11 with cyclins L1α or L2α demonstrated that CDK11p110 co-immunoprecipitates with both cyclins L1α and L2α. The reciprocal experiment confirmed that cyclins L1α and L2α were present in the CDK11p110 IP. These experiments further substantiated the specificity of our anti-cyclin L antibodies because cyclin L1α was not found in cyclin L2α IP and vice versa, whereas cyclins L1α and L2α were both found in the pan-L antiserum IP. CK2, shown previously to interact with CDK11p110, was also found in CDK11 and cyclin L IPs but not in the control IP, suggesting these three proteins form a ternary complex in vivo.

Because CDK11p58 is highly expressed in PHA-activated PBCs, we used activated PBCs to determine whether endogenous CDK11p58 interacts with endogenous forms of cyclin L1.
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Interactions detected between the different CDK11 and cyclin L1 and L2 isoforms by co-IP from activated PBC lysates and untransfected 293T cell lysates, and from 293T cells co-transfected with expression constructs for CDK11-FLAG and HA-cyclin L. + + represents interactions with signal strength >40% of input signal; + represents interactions with signal strength <30% of input signal; + /- indicates signal strength <2% of input signal; - represents no interaction detected.

or L2. PBC lysate was immunoprecipitated using P2N100, P1C, cyclin L1α, cyclin L2α, and ERK1 (negative control) polyclonal antibodies and analyzed by immunoblot for the co-IP of CDK11 with cyclin L1α or L2α. As is shown in Fig. 3B, the P1C antibody

effectively immunoprecipitated CDK11p58 and CDK11p46 from the PBC lysate and also co-immunoprecipitated both cyclins L1α and L2α. The reciprocal co-IPs using the cyclin L antibodies were also positive, and the strength of the p58 versus p46 CDK11 bands suggests that the p58 isoform interacts with cyclin Lα more robustly than p46.

Endogenous human cyclin Lβ is barely detectable in cultured cells (Fig. 2A). Therefore, to identify possible interactions between CDK11 protein kinases and cyclin L1/2 and L1γ isoforms, FLAG-tagged CDK11p110, CDK11p58, and CDK11p46 expression vectors were co-transfected with expression vectors encoding the HA-tagged cyclin L isoforms, and co-IP experiments were performed. CDK11 proteins were immunoprecipitated using anti-FLAG antibody, and IPs were analyzed by immunoblot using anti-HA antibody (Fig. 3C). Cyclin L1α was found in the CDK11p110 IP under these conditions but was barely detectable in CDK11p58 or CDK11p46 IPs. This was surprising because we were able to show interaction between endogenous cyclin L1α and CDK11p58 and CDK11p46 proteins in activated PBCs. In contrast, cyclin L1β was found in CDK11p58 and CDK11p46 IPs and was not easily detected in the CDK11p110 IP (Fig. 3C), and cyclin L1γ did not co-immunoprecipitate with any CDK11 proteins. The reason for the difference between the binding of the endogenous cyclin L1 protein and the overexpressed proteins is not clear, but the data suggest that these proteins might undergo post-translational modifications that are either rate-limiting or tissue-specific and/or that the complexes are stabilized via interaction with other, possibly tissue-specific, proteins. In contrast to specific binding between cyclin L1 and CDK11 protein isoforms, both cyclins L2α and L2β were found in CDK11p110, CDK11p58, and CDK11p46 IPs (Fig. 3C). Together, these data confirm that cyclins L1α and L2α interact with CDK11p110 and demonstrate that several additional complexes can form between cyclin L and CDK11 proteins. The CDK11/cyclin L protein interactions are summarized in Table 2.

Both Cyclin L1 and Lβ Proteins Are Present in Macromolecular Complexes—Our group has demonstrated previously that CDK11p110 protein is present in macromolecular complexes of two size ranges that contain transcription and splicing factors (5, 6, 22). Co-IP and nuclear co-localization of cyclin L proteins with CDK11p110 strongly suggest that cyclin L proteins should also be present in these nuclear complexes. To test this hypothesis, HeLa nuclear extract (NE, high mitotic index) and mouse liver tissue extract (low mitotic index, active metabolism) were
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Both Cyclin La and Lβ Proteins Interact with Components of the Splicing Machinery—To further examine whether cyclin La and Lβ proteins interact with SR proteins, we performed pulldown assays using bacterially expressed His6-tagged cyclin L1α, L1β, L2α, and L2βA proteins bound to Ni2+-NTA beads and incubated with HeLa NE. SR proteins were detected by immunoblot analysis using anti-SF2/ASF, 9G8, and 1H4 antibodies. Multiple SR proteins bound to all four cyclin L isoforms, including SF2/ASF and 9G8 and the five SR proteins recognized by 1H4, SRp75, SRp55, SRp40, SRp30a/b, and SRp20 (27). No SR proteins were detected in the negative control pulldown (Fig. 5A). Additionally, CDK11p110 also bound all four cyclin L isoforms (Fig. 5A).

To determine whether this interaction has functional significance, we incubated HeLa NE with cyclin L1α, L1β, L2α, and L2βA-Ni2+-NTA beads, removed the beads, and used the remaining supernatant to perform in vitro splicing assays with human β-globin pre-mRNA. Depletion of cyclin L interacting factors resulted in a marked 70–90% decrease in the ability of the NE to catalyze the excision of the pre-mRNA intron (Fig. 5B). As expected, adding the cyclin L beads back to the depleted NE resulted in a marked recovery of the splicing activity. Importantly, addition of recombinant SF2/ASF or 9G8 to depleted NE fully restored splicing activity (Fig. 5B and supplemental data 5). Together, these data demonstrate that cyclin L proteins bind multiple splicing factors and CDK11p110 and that complexes containing SR proteins and cyclin L-CDK11 play essential role(s) in splicing.

Expression of Both Cyclin L1/2α-β Isotypes Increases Intron Splicing Activity in Vivo—A double reporter system designed to measure intron splicing activity in vivo was employed to ascertain whether α and β isoforms of cyclins L1 and L2 affect intron splicing activity. In these experiments, unspliced transcripts produced by the pTN24 construct yield a protein with only β-galactosidase activity, whereas spliced intronless transcripts yield a fusion protein with both β-galactosidase and luciferase activities. By calculating the ratio of luciferase to β-galactosidase activity in the cell lysate, we can measure the proportion of spliced mRNA. 293T cells were co-transfected with pFlex-β-galactosidase and luciferase plasmids, pTN24 reporter vector. Co-transfection of pTN24 with empty pFlex expression vector was used for the base-line activity, and expression of the cyclin L isoforms was verified by immunoblot (Fig. 6A, upper panels). Expression of each cyclin L isoform resulted in increased splicing activity (Fig. 6A, lower panel). Although the cyclin L2 isoforms were expressed slightly better than the cyclin L1 isoforms in this cell type, titration experiments suggest that the higher expression is not entirely responsible for the enhanced splicing activity seen with the cyclin L2 constructs. Indeed, when experimental conditions were adjusted so that the expression levels of the cyclin Lα isoforms were similar, cyclin L2α still enhanced splicing activity more than cyclin L1α (data not shown). Thus, expression of both the α and β isoforms of cyclins L1 and L2 in cultured cells augments the endogenous intron splicing activity by 1.5-1.9-fold.

FIGURE 4. Cyclin L proteins are present in large macromolecular complexes. Extracts from HeLa nuclei and mouse liver (1 mg) were fractionated using a Superose 6 column and analyzed by immunoblot using the cyclin L1α, L2α, pan-L, and CDK11 p110 mAbs and the anti-SR proteins mAb. The anti-SR 1H4 mAb recognizes SRp75, SRp55, SRp40, SRp30a/b, and SRp20 proteins. Input lanes contain 10–20 μg of HeLa nuclear extract or 40 μg of mouse liver lysate, whereas other lanes contain 40 μl from each 1-ml fraction. Shorter exposure films were used for the input lanes for the HeLa nuclear extracts blots. The bands for the HNE SR proteins in fractions 9–11 between the 26- and 37-kDa markers were substituted from a replicate blot. * indicates a faster migrating signal of cyclins L1α and L2α.

fractionated using Superose 6 size exclusion chromatography (Fig. 4). CDK11p110 was easily detected in HeLa NE and eluted in fractions 8–15 corresponding to >1-MDa to ~170-kDa size complexes. CDK11p110 was not as easily detected in mouse liver extracts and was detected in 1-MDa (fractions 8 and 9) and in ~170–400 kDa (fractions 14–15) size ranges. Similar to CDK11p110, both cyclins L1α and L2α were found in fractions 8–15 of HeLa NE, as well as in fraction 17 corresponding to monomer size (67 kDa). Interestingly, the strongest elution signal in HeLa NE for all three proteins CDK11p110, cyclin L1α, and cyclin L2α corresponded to fraction 13 (460 kDa). Faster migrating signals, which possibly represent proteolytic fragments of cyclins L1α and L2α, were also detected in various fractions of HeLa NE (8–11, 15–17); however, we have not further characterized these bands. The two cyclin Lα isoforms were also detected in mouse liver although in slightly smaller sized protein complex fractions (12–16). In addition, immunoblot analysis using the pan-L antibody allowed detection of cyclin Lβ proteins in mouse liver fractions 16–20, corresponding to molecular masses of approximately <25–120 kDa. In both extracts, immunoblot analysis using an anti-SR protein antibody that detects SRp75, SRp55, SRp40, SRp30a/b, and SRp20 revealed that the SR proteins co-eluted with CDK11p110 and cyclin L in fractions of multiple molecular masses.
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FIGURE 5. Cyclin L proteins bind essential splicing factors from nuclear extract. A, bacterially produced full-length cyclins L1α, L1β, L2α, and L2β/βα bound to Ni²⁺-NTA beads were incubated with HeLa NE. Endogenous splicing factors and CDK11p110 were detected by immunoblot using anti-SF2/ASF and 9G8-specific pAbs, anti-SR protein 1H4 mAb (SRp75, SRp55, SRp40, SRp30a/b, and SRp20), and anti-CDK11 P1C pAb. Unbound nonspecific splicing factors and CDK11p110 were detected by immunoblot using anti-SF2/ASF and 9G8-specific pAbs, then used to perform splicing assays (right panels, upper labels). Splicing activity was recovered following re-addition of the cyclin L incubation beads or addition of recombinant splicing factors SF2/ASF and 9G8 (20 or 200 ng) (lower labels).

Kinase Activity and the RE Domain of CDK11p110 Are Required to Enhance Splicing Activity in Vivo—The β-galactosidase/luciferase double reporter system was also used to determine whether the different CDK11 isoforms individually enhance in vivo splicing activity. Both wild-type and kinase-dead forms of CDK11p110, CDK11p58, and CDK11p46 and control expression vector were expressed by transient co-transfection with pTN24 in 293T cells. RNA markers of 955, 623, and 281 nucleotides (nt) were loaded in the far-left lane and stained with ethidium bromide. The pre-RNA, splicing intermediates, and final product are labeled. Cyclin L1α, L1β, L2α, and L2β/βα-Ni²⁺-NTA beads and unbound Ni²⁺-NTA beads were incubated with NE for 1 h. The supernatants were then used to perform in vitro splicing assays (right panels, upper labels). Splicing activity was recovered following re-addition of the cyclin L incubation beads or addition of recombinant splicing factors SF2/ASF and 9G8 (20 or 200 ng) (lower labels).

we were curious whether the N-terminal splicing factor interacting domain of p110 (arginine/glutamate (RE)-rich domain, amino acids 127–220) was necessary to stimulate splicing. We previously determined that deletion of the RE domain from CDK11p110 causes relocation of this protein from SFCs to the nucleoplasm (supplemental Data 6). The splicing assays demonstrated that expression of p110ΔRE suppressed splicing activity to 82% (Fig. 6B, lower panel), and further reduced splicing activity in a titratable manner (data not shown). We also compared the expression of the unspliced and spliced forms of the pTN24 reporter transcripts following expression of each cyclin L and CDK11 isoform by RT-PCR. Side-by-side comparison of enzyme activities versus RT-PCR transcript levels gave a correlation coefficient of 0.9473. This value is slightly better than that obtained in the original paper describing this system (29) and validates the use of enzyme activity to measure the effect on RNA splicing following expression of these proteins. Consequently, we conclude that CDK11p110 requires both kinase activity and the RE splicing factor interaction domain to enhance splicing activity in vivo.

Cyclin L-mediated Stimulation of Intron Splicing Activity in Vivo Is Diminished by Co-expression of Kinase-dead CDK11p110—We also performed experiments to determine whether co-expression of CDK11p110 with cyclin L isoforms would further enhance splicing activity. Using the same double reporter in vivo splicing assay, intron splicing activity was measured following co-expression of wild-type and kinase-dead CDK11p110 with cyclins L1α, L2α, or L2β (Fig. 7), but not cyclin L1β because this isoform demonstrated no appreciable interaction with CDK11p110 (Fig. 3C). Each protein was also expressed individually under conditions where the total amount of DNA in the transfection was equalized to that of the “paired” transfections by addition of the appropriate empty vector. Protein expression levels for the paired transfections are “paired” transfections by addition of the appropriate empty vector. Protein expression was verified by immunoblot (Fig. 6B, upper panels). Similar to cyclin L, expression of wild-type CDK11p110 increased splicing activity by an average of 1.6-fold (Fig. 6B, lower panel). Not surprisingly, flooding the cells with kinase-dead CDK11p110 decreased splicing activity (0.65-fold). In strong contrast to active CDK11p110, neither CDK11p58 nor CDK11p46 protein expression was capable of enhancing splicing. In fact, both wild-type and kinase-dead forms of p58 and p46 dramatically decreased splicing activity by more than 60%. Given the inability of the kinase domain isoforms p58 or p46 to positively affect splicing activity,
Expression of both CDK11p110 and cyclin L2 resulted in greater splicing activity than either of these proteins alone. In contrast, co-expression of CDK11p110 with cyclin L1 or L2 resulted in a suppression of the splicing enhancement by cyclin L.

**Fig. 6.** Cyclins L1α and L2α and CDK11p110WT enhance in vivo intron splicing activity. A: FLAG-cyclin L1α, L1β, L2α, and L2β and empty FLAG expression vector (pFlex) were transiently co-expressed in 293T cells with the intron-splicing double reporter plasmid pTN24 for 24 h. Cell lysates were analyzed by immunoblot for expression of all cyclin L isoforms using M2 anti-FLAG mAb and for CDK11 isoforms using anti-CDK11 P2N100 pAb (upper panel). The vectors lane represents co-transfection of empty pFlex and pUHD 10.3 vectors with pTN24 and demonstrates the comparative expression of endogenous versus ectopic CDK11 isoforms. Relative splicing activities were calculated as described for Fig. 6 with the activity of the combined empty expression vectors set at 1.0 (histogram shown in lower panel). The results shown represent 3–5 independent transfection experiments in which one plate of cells was transfected, and each lysate was measured in quadruplicate. The error bars represent standard deviation. Statistically significant comparison data is indicated by the following symbols: *, p < 0.02 for comparisons to the control vector; +, p < 0.02 for p110WT/cyclin L or pFlex compared with p110DN/cyclin L or pFlex; #, p < 0.03 for p110WT/cyclin L compared with pUHD10-3/cyclin L; $, p < 0.04 for p110DN/cyclin L compared with pUHD10-3/cyclin L; %, p < 0.02 for p110WT/pFlex compared with p110DN/pFlex compared with p110DN/cyclin L. p values were calculated using a nonparametric rank-based test.

**Fig. 7.** Expression of kinase-dead CDK11p110 suppresses in vivo splicing enhancement by cyclin L. FLAG-cyclin L1α, L1β, L2α, and L2β and empty FLAG expression vector (pFlex) were transiently co-expressed in 293T cells with either CDK11p110WT or CDK11p110DN or empty CDK11 expression vector (pUHD 10.3) and the intron splicing reporter plasmid pTN24 for 24 h. Cell lysates were analyzed by immunoblot for expression of all cyclin L isoforms using M2 anti-FLAG mAb and for CDK11 isoforms using anti-CDK11 P2N100 pAb (upper panel). The vectors lane represents co-transfection of empty pFlex and pUHD 10.3 vectors with pTN24 and demonstrates the comparative expression of endogenous versus ectopic CDK11 isoforms. Relative splicing activities were calculated as described for Fig. 6 with the activity of the combined empty expression vectors set at 1.0 (histogram shown in lower panel). The results shown represent 3–5 independent transfection experiments in which one plate of cells was transfected, and each lysate was measured in quadruplicate. The error bars represent standard deviation. Statistically significant comparison data is indicated by the following symbols: *, p < 0.02 for comparisons to the control vector; +, p < 0.02 for p110WT/cyclin L or pFlex compared with p110DN/cyclin L or pFlex; #, p < 0.03 for p110WT/cyclin L compared with pUHD10-3/cyclin L; $, p < 0.04 for p110DN/cyclin L compared with pUHD10-3/cyclin L; %, p < 0.02 for p110WT/pFlex compared with p110DN/pFlex compared with p110DN/cyclin L. p values were calculated using a nonparametric rank-based test.
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did not increase splicing activity above that of wild-type CDK11p110 alone. Interestingly, expression of kinase-dead CDK11p110 significantly reduced splicing activity below baseline levels both for CDK11p110 alone and in combination with cyclins L1α and L2β. However, co-expression of cyclin L2α with kinase-dead CDK11p110 maintained splicing activity above baseline levels.

Expression of Cyclin L1/2 Isoforms Alone or in Combination with CDK11p110 Differentially Modulates Alternative Splicing of the E1A Gene in Vivo—An in vivo E1A minigene reporter system (Fig. 8A) that encodes unspliced and five alternatively spliced variant transcripts (13, 12, 11, 10, and 9S) was employed to investigate whether ectopic expression of wild-type (WT) and kinase-dead (DN) forms of CDK11p110 and cyclins L1α, L2α, and L2β (those isoforms that interact with CDK11p110) affects alternative splicing activity in HuH7 hepatoma cells. In these experiments, total RNA was extracted from HuH7 cells following transient transfection with the E1A minigene and CDK11p110 and cyclin L expression vectors. Protein expression was verified by immunoblot (Fig. 8B). The RNAs were used to perform RT-PCR, and the resulting E1A splice variant products were analyzed by gel electrophoresis (Fig. 8C). To quantitate the effect of the transfected proteins, the relative expression level of each splice form in control cells is set at 1, and the expression of each splice variant from the experimental transfections is reported relative to the corresponding form in control cells (Fig. 8D). In control cells, all five of the E1A variants were detected, with 13, 12, and 10S mRNA being most easily detected (Fig. 8C). Expression of cyclin L1α, L2α, or L2βA altered the E1A splice variant pattern (Fig. 8, C and D). Both cyclins L1α and L2βA substantially repressed formation of 13, 12, and 11S by 1.4- to 3-fold and induced up to a 1.5-fold increase in the levels of 10 and 9S mRNA. In direct contrast, cyclin L2α simultaneously increased 13, 12, and 11S and decreased 10 and 9S E1A mRNAs levels. Expression of CDK11p110WT resulted in small changes in E1A splice form expression with a 1.3-fold increase in 9S. Conversely, expression of CDK11p110DN strongly favored 13 and 11S formation (1.4–1.9-fold) and slightly decreased 10 and 9S formation.

Using the same in vivo E1A gene reporter construct, we measured alternative splicing following co-expression of wild-type and kinase-dead CDK11p110 with cyclins L1α, L2α, or L2βA (Fig. 8, C and D). Co-expression of CDK11p110WT with cyclin L1α amplified the effects of cyclin L1α alone, whereas expression of CDK11p110DN with cyclin L1α essentially reversed the effects of cyclin L1α. In opposition to cyclin L1α, co-expression of CDK11p110WT altered some effects of cyclin L2α alone with increased 12 and 11S and increased 10S mRNAs, whereas co-expression of CDK11p110DN with cyclin L2α did not change the mRNA pattern. Co-expression of CDK11p110WT with cyclin L2βA restored the splice form pattern to one near control levels and almost identical to CDK11p110WT alone. However, expression of CDK11p110DN with cyclin L2βA induced higher 9S expression than observed with L2βA alone.

**DISCUSSION**

Here we report that the cyclin L1 gene produces nine mRNAs in human cell lines and tissues, whereas the cyclin L2 gene produces five mRNAs. Detailed analyses of these sequences allowed us to identify a sixth cyclin L protein that diverges from L2β by 12 amino acids at the C-terminal end. Thus, the original cyclin L2β has been renamed L2βA and the new form named
L2βB. In humans, the two distinct genes Cdc2L1 and Cdc2L2 encode the three CDK11 protein isoforms. CDK11p110 is expressed ubiquitously throughout the cell cycle, CDK11p58 is expressed at the G2/M transition, and CDK11p46 is expressed during apoptosis. To further investigate formation of complexes between cyclin L and CDK11 isoforms, we have raised rabbit polyclonal anti-cyclin L antibodies specifically recognizing cyclins L1α and L2α and a third antisera capable of detecting all cyclin L proteins. Using these reagents we have identified endogenous complexes between cyclin L1/H9251 and L2/H9252, and CDK11p110, CDK11p58, and CDK11p46 for the first time. In encode the three CDK11 protein isoforms. CDK11p110 is

Cyclin L proteins can be divided into two groups as follows: the longer cyclin Lα isoforms containing a cyclin box in their N terminus and an arginine/serine-rich domain (RS domain) in the C terminus, and the shorter cyclin Lβ proteins that contain only the cyclin box domain. An RS-rich motif similar to that present in cyclins L1α and L2α is also found in the SR protein family members involved in regulation of splicing (31). Various reports have proposed that cyclin Lα proteins are involved in splicing regulation (7, 8, 13, 26). This hypothesis was reinforced by our data showing localization of endogenous cyclin L and the specific HA-cyclin L isoforms in nuclear SFCs. In contrast, previous transient expression experiments using a GFP-cyclin L1 isoforms in nuclear SFCs. In contrast, previous transient expression experiments using a GFP-cyclin L1 expression construct suggested a cytoplasmic subcellular location (13). This discrepancy may be due to the presence of the green fluorescent protein moiety and/or aberrant localization because of overexpression of the fusion protein. Previous studies using in vitro splicing assays reported that recombinant cyclins L1α and L2α can promote splicing of β-globin pre-mRNA (7, 8). It was also reported that transiently expressed cyclins L1α and L2α can each regulate alternative splicing of an E1A reporter gene in vivo whereas cyclin L1β does not (2, 3). These results support the conclusion that the cyclin Lα isoforms regulate splicing via their C-terminal RS domain, whereas cyclin Lβ isoforms have no direct effect on splicing. However, it was also recently demonstrated that both cyclins L1α and L1β co-transfected with CDK12 and cyclin L1β co-transfected with CDK13 potentiate CDK-dependent effects on alternative splicing (2, 3). No data were reported on whether co-expression of cyclin L2 isoforms, which also interact with CDK12 and 13, potentiate or antagonize the effects of these CDKs on E1A alternative splicing.

Here we used two different in vivo assays to investigate the effects of the various CDK11 isoforms and both α and β cyclin L1/2 proteins on constitutive and alternative splicing. The first assay uses a double reporter system to monitor constitutive splicing (29). Using this assay, we found that expression of α and β cyclin L1/2 isoforms caused an increase in constitutive splicing (Fig. 6A). Expression of active CDK11p110WT also significantly enhanced constitutive splicing activity, whereas kinase-dead CDK11p110DN markedly repressed constitutive splicing (Fig. 6B). Moreover, expression of CDK11p110 lacking the RE splicing factor interaction domain or either active or inactive CDK11p58 or CDK11p46 decreased overall splicing, suggesting that these proteins may act in a dominant negative fashion (Fig. 6B). These data demonstrate an in vivo role for CDK11p110 and cyclin Lβ in pre-mRNA splicing for the first time. Co-expression of all of the cyclin L isoforms with active CDK11p110WT further increased the level of splicing above that seen in cells expressing only the cyclin L construct (Fig. 7). However, only co-expression of cyclin L2α with CDK11p110WT increased the level of splicing above that of either the overexpressed kinase or cyclin alone. Strikingly, co-expression of inactive CDK11p110 with cyclins L1α and L2β completely ablated the effects of the cyclin L protein expression, whereas co-expression of the inactive kinase with cyclin L2α reduced splicing activity to a level lower than the activity seen with cyclin L2α alone, but still higher than control vector activity. These data support the conclusion that cyclin L proteins interact with CDK11p110 in vivo. They also suggest that interaction with CDK11p110 is important for the ability of cyclins L1α and L2β to affect splicing of this reporter, and that flooding the cells with kinase-dead CDK11 effectively displaces these two cyclins from interaction with a functional endogenous kinase partner(s). Given our data supporting in vivo interaction between CDK11p110 and the cyclin L proteins, it is somewhat surprising that co-expression of cyclins L1α and L2α with active CDK11p110 cannot further enhance the effect of the kinase alone. However, it is possible that the association of CDK11 with splicing complexes is already near its maximum or that the signaling pathway influenced by association of CDK11p110 with cyclin L1α/L2β has reached maximal amplification in 293T cells. The results further suggest that interaction with CDK11p110 is not essential for the ability of cyclin L2α to enhance splicing of this reporter and that cyclin L2α may preferentially use another CDK in 293T cells.

The second splicing assay we employed uses an E1A mini-gene splicing construct that generates a pre-mRNA, one constitutive splice product (13S), and four alternatively spliced transcripts (9S, 10S, 11S, and 12S) (32). Using this construct, we demonstrated that CDK11p110, cyclin L1α, L2α, and L2β complexes strongly affect alternative splice site selection (Fig. 8). Expression of active CDK11p110WT caused a slight change in E1A splice site usage resulting in the production of more 9S transcript, although this effect was less than the 2–3-fold increases in 9S seen following CDK12 and CDK13 expression (2–4). On the other hand, expression of inactive CDK11p110DN decreased production of the 9S splice form and considerably increased 13 and 115 splice form levels, an effect similarly observed when endogenous CDK12 expression was decreased using RNA interference (2). Co-expression of kinase-dead CDK11p110DN with cyclin L1α dramatically suppressed the effects of expressing cyclin L1α alone, strongly suggesting that cyclin L1α requires CDK11p110 to signal 10 and 9S splice site selection. In addition, co-expression of active CDK11p110WT, but not kinase-dead CDK11p110DN, changed the splicing pattern of L2α alone, suggesting that cyclin L2α also interacts with CDK11p110 to influence splicing of the E1A pre-mRNA substrate. However, the lack of effect following expression of CDK11p110DN on the cyclin L2α splicing pattern implies the additional use of another CDK partner in these cells. Surpris-
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ingly, co-expression of both active and kinase-dead CDK11p110 with cyclin L2β altered the effects of cyclin L2β alone. These results suggest that cyclin L2β is using at least one other CDK partner in addition to CDK11 for splicing of E1A transcripts, and that interaction of L2β with CDK11p110WT biases spliceosome activity in the cells against 10S splice site usage. These results demonstrate for the first time that CDK11p110 regulates alternative splicing and that this regulation requires kinase activity. Importantly, these results also demonstrate that the cyclin L isoform strongly influences splice site selection, most likely through the use of multiple CDK partners.

Together, our data demonstrate that CDK11p110, cyclin La, as well as cyclin Lβ proteins that lack the C-terminal RS domain regulate both constitutive and alternative splicing and influence splicing activity through protein abundance. Furthermore, our data indicate that it is the combination of the cyclin L isoform and the CDK partner protein, which varies in a cell type-specific manner, which affects splice site usage. During the course of our experiments, we tested E1A splicing in several cell lines and observed that expression of the E1A mRNAs was very different in 293T, HuH7, HFF, and HepaRG cells (data not shown), confirming that alternative splicing machinery differs between cell types and suggesting that enforced expression of splicing signaling components such as cyclin L and CDK proteins will result in different effects in various cell types. In addition to the in vivo splicing data, the demonstration that all of the cyclin L1/2 α and β isoforms bind SR proteins, including SF2/ASF and 9G8, and can deplete splicing activity from nuclear extract further supports the conclusion that both the cyclin Lα and Lβ isoforms function in splicing regulation.

The prolific interactions between cyclin L and CDK11 isoforms raises the questions of relevance and specificity of multiple CDK-cyclin L complexes in splicing regulation. Further experiments are required to identify substrates of these CDK-cyclin complexes and to determine whether they regulate different steps during pre-RNA maturation and splice site selection or respond to diverse signaling events in the cell. Very little is known about regulation of cyclin L, CDK11, CDK12, and CDK13 expression during development, cell cycle, and differentiation by extracellular signals. However, induction of cyclin L1α by cocaine and dopamine in the striatum of rat brain demonstrates that cyclin L expression can be regulated by specific extracellular signals via intracellular pathways that remain to be identified (13, 33). These data also clearly demonstrate that extracellular regulation of gene expression involves modulation of both transcriptional and splicing machineries. Thus, a major challenge is to identify genes regulated by CDK-cyclin L complexes to identify pathways connecting extracellular signals, regulation of splicing machinery, and gene expression.

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