

A Direct Interaction between the Carboxyl-terminal Region of CDC5L and the WD40 Domain of PLRG1 Is Essential for Pre-mRNA Splicing*

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The human proteins CDC5L (hCDC5) and PLRG1 are both highly conserved components of a multiprotein complex that is a subunit of the spliceosome. The respective homologues in yeast of both proteins are also associated with a sub-spliceosomal multiprotein complex that has been shown to be important for pre-mRNA splicing. We show that these two human proteins are associated *in vivo* and will interact directly *in vitro*. The regions containing the interacting domains in both proteins have been identified. Our results indicate that the carboxyl-terminal region of CDC5L and the WD40 domain of PLRG1 are essential for direct interaction between both proteins. By using a bacterially expressed mutant protein, containing the PLRG1 interacting domain in CDC5L, we show that the CDC5L-PLRG1 interaction in HeLa nuclear extract can be disrupted causing pre-mRNA splicing to be inhibited. Thus, a direct interaction between the CDC5L protein and PLRG1 in the CDC5L complex is essential for pre-mRNA splicing progression.

Pre-mRNA splicing occurs in the eukaryotic cell nucleus through a process that involves the removal of non-coding sequences (introns) in the pre-mRNA and the joining of adjacent or alternate coding sequences (exons) to produce mature mRNA. The splicing reaction takes place via a two-step transesterification mechanism involving nucleophilic attacks of phosphodiester bonds and creation of new bonds resulting in the formation of mature mRNA that is then exported to the cytoplasm for use in protein synthesis (for review see Ref. 1). Splicing is catalyzed by a large ribonucleoprotein complex made up of over a hundred proteins called the spliceosome. The spliceosome complex contains four ribonucleoprotein (snRNP)¹ particles (U1, U2, U5, and U4/U6), each of which contains the corresponding small nuclear RNA and a set of specific and common proteins (2–4). The complex also contains multiple non-snRNP associated proteins that are essential for spliceosome assembly and catalysis (1, 4). Spliceosome assembly involves the sequential association of the snRNP particles and

other proteins onto the pre-mRNA substrate prior to catalysis (reviewed in Ref. 5).

Several recent studies have suggested that both the human proteins CDC5L and PLRG1 and their respective homologues in yeast are in a large complex containing other pre-mRNA splicing factors (6–10). The possible involvement of the human proteins in pre-mRNA splicing was first suggested when both proteins were identified by mass spectrometry in purified spliceosomes assembled on adeno-pre-mRNA *in vitro* (6). However, other cellular roles for CDC5L have also been proposed; for example, it has been suggested that CDC5L may also be involved in transcription because of sequence similarities in the amino-terminal domain of the protein with the proto-oncogenic transcription factor, c-Myb (11, 12). Also, the *Arabidopsis thaliana* homologue of this protein (AtCDC5), when overexpressed in *Schizosaccharomyces pombe*, can complement a growth-defective phenotype of an *S. pombe cdc5+* temperature-sensitive mutant. A sequence-specific DNA binding activity has been reported for AtCDC5 (13). A possible role for this protein in the regulation of the cell division cycle was observed in a genetic screen of *S. pombe* for cell division cycle mutants. The study indicated that the *cdc5+* gene encodes an essential protein and that the function of the gene may be required in the G₂ phase of the cell cycle (11). More recently, it has been shown that overexpression of CDC5L in mammalian cells shortened the G₂ phase of the cell cycle. Also, a dominant negative mutant of the protein lacking the activation domain slowed G₂ progression and delayed entry into mitosis (12).

The human protein PLRG1, recently identified as a component of the CDC5L complex, is highly homologous to the *A. thaliana* PRL1 gene product. The PRL1 protein has been shown to be essential for the regulation of glucose and hormone responses in *A. thaliana* (14). Mutations in PRL1 have pleiotropic phenotypes in *A. thaliana*. For example, a *prl1* mutation can cause transcriptional de-repression of glucose-responsive genes; augment the sensitivity of the plants to growth hormones such as cytokinin, abscisic acid, ethylene, and auxin; stimulate the accumulation of sugars and starch in the leaves of the plants; and inhibit root elongation (14). In both *A. thaliana* and COS-1 cells, PRL1 shows nuclear localization and interacts with ATHKAP2, an α -importin nuclear import receptor (14). PLRG1 and the PRL1 protein both contain seven copies each of the phylogenetically conserved WD repeat domains that were first characterized in β -transducin (15). Proteins with WD domains are thought to have regulatory functions in the cell as well as being involved in protein-protein interactions. These WD domain proteins have been identified in a variety of species, from human to the facultatively thermophilic actinomycete *Thermospora curvata* (16, 17). In a phenotypic screen for cell cycle mutants of an *A. thaliana* cDNA

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¹ The abbreviations used are: snRNP, small nuclear ribonucleoprotein; GST, glutathione *S*-transferase; PIPES, 1,4-piperazinediethanesulfonic acid; PCR, polymerase chain reaction; PBS, phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis; GFP, green fluorescent protein; YFP, yellow fluorescent protein.

library in fission yeast, *PRL1* was identified as one of 11 genes that can cause severe morphological changes in the yeast. This was interpreted to indicate that *PRL1* may be involved in cell shape maintenance and/or regulation of the cell cycle (18). More recently, it has been shown that mutations in the *PLRG1* homologue in *S. pombe*; *prp5*⁺ results in defects in pre-mRNA splicing and also blocks progression of the cell division cycle at the G₂/M phase (19).

In yeast it has been demonstrated that cells lacking *cdc5*+ function are defective in pre-mRNA splicing (8). The *CDC5L* gene product is highly conserved across species, and homologues have been identified in several eukaryotic species including *S. pombe*, *Saccharomyces cerevisiae*, *Caenorhabditis elegans*, *Drosophila melanogaster*, and *Xenopus laevis* (7, 13, 20, 21). Like *CDC5L*, *PLRG1* is also very highly conserved across species (14), suggesting essential cellular functions for these proteins. However, a direct role for *PLRG1* in pre-mRNA splicing has not yet been shown despite its presence in splicing factor complexes.

We have recently purified a protein complex associated with *CDC5L* from HeLa nuclear extracts and shown that this complex contains at least six "core" proteins (10). Two of the core proteins, which also are the most highly conserved across species, are *CDC5L* and *PLRG1*. We show here that these two proteins will interact directly *in vitro* and provide evidence that a direct interaction between *CDC5L* and *PLRG1* is essential for pre-mRNA splicing.

EXPERIMENTAL PROCEDURES

cDNA Cloning and Sequencing—The *PLRG1* and *CDC5L* cDNAs were cloned from a HeLa cDNA library (CLONTECH) by PCR. Primers were designed for the amino and carboxyl termini of the proteins using previously deposited sequences for these cDNAs in the GenBank™ data base (*PLRG1* accession number AF044333 and *CDC5L* accession number U86753). The *PLRG1* primers contained *Bam*HI and *Xho*I sites added to the 5' ends of the 5' and 3' end primers, respectively. The *CDC5L* PCR primers contained *Sal*I sites added to their 5' ends. The PCR products were purified on a PCR purification column (Qiagen) according to the manufacturer's instructions. Purified PCR products from *CDC5L* and *PLRG1* cDNAs were digested with the appropriate enzymes and cloned using standard methods into the compatible sites of the vectors pGEX-4T1 (Amersham Pharmacia Biotech), pET-30a (Novagen), pEGFP-C1, pEYFP-C2 (CLONTECH) and pSG-9M, a modified form of the plasmid pSG5 (22) containing an amino-terminal Myc tag. The plasmid DNA was sequenced on the Applied Biosystems 377 automated DNA sequencer using the *Taq* dye terminator cycle sequencing method according to the manufacturer's instructions. pGEX-4T1 and pET-30a were used for expression of recombinant protein in *Escherichia coli*, whereas the pEGFP-C1 and pSG-9M vectors were used for expression in mammalian cells (HeLa). pET-30a clones were also used for *in vitro* transcription/translation experiments.

Expression of Recombinant Proteins in *E. coli*—cDNAs cloned into the pGEX-4T1 vector were used to transform *E. coli* BL21(DE3). Overnight cultures were grown from single colonies and then diluted 1:10 in fresh LB medium with ampicillin (100 µg/ml) and grown at 30 °C to an A₆₀₀ of 0.7–1.0 before induction with 0.1 mM isopropyl-1-thio-β-D-galactopyranoside. Three hours post-induction, cells were pelleted and resuspended in 10 ml of PBS, 0.5% Triton X-100 containing protease inhibitor mixture (Roche Molecular Biochemicals). Cell lysis was achieved by sonication. The cell debris was removed by centrifugation at 10,000 × *g* for 10 min. Pre-swollen glutathione-Sepharose beads pre-equilibrated in PBS were added to the supernatant (1 ml per liter of culture). The beads were incubated with the crude protein extract for 2 h at 4 °C with rocking. Beads were collected and washed 3 times in PBS, 0.5% Triton X-100 followed by 3 washes in PBS. Proteins were eluted from the beads by incubating in 25 mM glutathione in 50 mM Tris-Cl, pH 8.0. The proteins were dialyzed into a buffer containing 20 mM HEPES, pH 8.0, 20% glycerol, 100 mM KCl, 0.2 mM EDTA, 1 mM dithiothreitol, or PBS and stored at –80 °C.

pET-30a cDNA clones were treated as above for expression except that 1 mM isopropyl-1-thio-β-D-galactopyranoside was used for induction and the cells were grown at 37 °C prior to induction at 30 °C. The expressed protein was bound to nickel-nitrilotriacetic acid-agarose

beads (Qiagen) and the beads washed with a buffer containing 20 mM imidazole, 1 mM phenylmethylsulfonyl fluoride, 50 mM NaH₂PO₄, and 300 mM NaCl (pH 8.0). Recombinant protein was eluted from beads using the same buffer as above except that the concentration of imidazole was increased to 250 mM. Eluted proteins were dialyzed in the same buffer as above.

Antibody Production and Affinity Purification—Peptide antibodies to *CDC5L* and *PLRG1* were prepared as described previously (10). Antibodies to bacterially expressed *PLRG1* and *CDC5L* were also produced in sheep and rabbits (SAPU, Lanarkshire, Scotland, UK).

Splicing Assays—Nuclear extracts used in the splicing assays were obtained commercially from Computer Cell Culture Center (Mons, Belgium). Splicing assays were done using uniformly labeled, capped pre-mRNAs incubated with nuclear extract as described previously (23). In experiments where recombinant proteins were added to the splicing reactions, proteins that gave low yields during expression in *E. coli* and purification by affinity chromatography were concentrated using centrifugal filter devices (molecular weight cut-off 3500) (Microcon) according to the manufacturer's instructions before addition to splicing reactions. The adeno-pre-mRNA was transcribed from *Sau*3AI-digested plasmid pBSAd1 (24). The splicing reactions were loaded on a 10% polyacrylamide, 8 M urea denaturing gel and run in 1× TBE to separate the splicing products. When samples were to be used for the analysis of splicing complexes, the reactions were loaded onto a polyacrylamide-agarose composite gel (25) and run for about 5 h at 25 mA.

Immunoprecipitation of Proteins from HeLa Nuclear Extract—Immunoprecipitations of the spliceosomal proteins from nuclear extract were done using affinity-purified peptide antibodies. 50 µl of nuclear extract (4–5 mg/ml) were pre-cleared for 1 h at 4 °C on 25 µl of settled protein G- or A-Sepharose (Amersham Pharmacia Biotech) or protein G/A-agarose beads (Roche Molecular Biochemicals) that had been preincubated with 10 µg of sheep preimmune IgG. The pre-cleared nuclear extract was diluted 10 times (except in immunodepletion experiments) with PBS buffer containing 0.5% Triton X-100 before adding to protein G-Sepharose or -agarose beads (25 µl) that had been preincubated with 30 pools of antibody for 1 h at 4 °C. Immunoprecipitations were carried out at 4 °C for 2–16 h. The immunoprecipitates were washed three times at 4 °C with 1 ml of PBS containing 0.5% Triton X-100. Protein G beads carrying the immune complexes were collected after each wash by centrifugation at 1500 × *g* for 1 min. For immunodepletion experiments a higher amount of antibody (0.33 nmol/250 µg) of HeLa nuclear extract was used during the immunoprecipitation step.

SDS-PAGE and Western Blotting—SDS-PAGE gel analysis was done as described previously (26). For immunoblotting, the washed immunoprecipitates were resuspended in 50 µl of 2× SDS-PAGE loading buffer and heated at 95 °C for 5 min. Approximately 10–15 µl of the supernatant were loaded on a 10% SDS-PAGE gel or 4–12% pre-cast gradient gel (NOVEX). The separated proteins were transferred onto Hybond-C extra membrane (Amersham Pharmacia Biotech) by electroblotting. The membranes carrying the transferred proteins were blocked with 5% non-fat milk powder in PBS, 0.3% Tween 20. The membranes were incubated with primary antibody for 1–16 h at room temperature, washed with blocking buffer, and incubated with the appropriate secondary antibody. The primary antibodies were used at the following dilutions: anti-*CDC5L* (1:1000), anti-*PLRG1* (1:1000), and anti-SPF30 (dilution 1:2000). After washing the blots in blocking buffer 3–4 times (5 min per wash at room temperature), the membranes were then incubated with a secondary antibody to which has been covalently coupled horseradish peroxidase or alkaline phosphatase. Protein bands were detected by developing blots using the ECL kit (Amersham Pharmacia Biotech) according to the manufacturer's instructions or using nitro blue tetrazolium (60 µl of a 30 mg/ml solution)/5-bromo-4-chloro-3-indolyl phosphate (60 µl of a 25 mg/ml solution) in 10 ml of alkaline phosphatase buffer (0.1 M NaHCO₃, 1 mM MgCl₂, pH 9.8) for colorimetric detection on the membrane.

In Vitro Transcription and Translation—The *in vitro* transcription and translation experiments were done with the T7 or SP6 RNA polymerase transcription/translation systems that used rabbit reticulocyte lysate (TNT Systems, Promega) and L-[³⁵S]methionine (Amersham Pharmacia Biotech, AG1094) to produce ³⁵S-labeled proteins according to the manufacturer's recommendations. Aliquots of these reactions were used for protein-protein interaction assays as described below.

Protein Binding Assays—About 0.2 nmol of the appropriate GST fusion recombinant protein was mixed with an equimolar amount of hexahistidine-tagged bacterially expressed protein or 8–10 µl of the *in vitro* translation reaction above and incubated in binding buffer (250 mM NaCl, 50 mM HEPES, pH 7.9, 2% bovine serum albumin) at room temperature for 15 min. The binding reaction was then added to 25 µl

of glutathione-Sepharose beads or antibody-bound protein G-Sepharose and mixed at 4 °C for 1–2 h. The beads were washed 3–4 times in 1 ml of PBS, 0.1% Triton X-100 and resuspended in 25 μ l of 2 \times SDS-PAGE loading dye before heating at 70 °C for 10 min or 95 °C for 5 min. Bound proteins were separated on a 10 or 12% SDS-PAGE gel after which the gel was fixed in 50% methanol, 10% acetic acid for 30 min. The fixed gel was then soaked in a fluorographic reagent (Amplify, Amersham Pharmacia Biotech) for 30 min before drying. Protein bands were detected by autoradiography at –80 °C after 4–16 h. For antibody probing, the gels containing proteins from the antibody beads were treated as described above under “SDS-PAGE and Western Blotting.”

In Vitro Mutagenesis of the CDCL and PLRG1 cDNAs—Point mutations generating stop codons were inserted into the cDNAs at ~100-amino acid intervals by PCR using the QuikChange site-directed mutagenesis kit (Stratagene) according to the manufacturer’s instructions. The cDNAs were sequenced to confirm the presence of the appropriate mutations. Regions of the cDNAs involved in binding and overlapping regions were subcloned into the vectors pGEX-4T1 (Amersham Pharmacia Biotech) or pET-30a (Novagen) for use in *E. coli* expression and protein-protein interaction assays.

Cell Culture and Transfection—HeLa cells were grown in Dulbecco’s modified Eagles’ medium supplemented with 10% fetal calf serum and 100 units/ml penicillin and streptomycin (Life Technologies, Inc.). For immunofluorescence assays, cells were grown on coverslips and transfected using Effectene transfection reagent (Qiagen) according to the manufacturer’s instructions.

Cell Staining and Immunofluorescence Analyses—Cells were washed in PBS and fixed for 5 min in 3.7% (w/v) paraformaldehyde in CSK buffer (10 mM PIPES, pH 6.8; 10 mM NaCl; 300 mM sucrose; 3 mM MgCl₂; 2 mM EDTA) at room temperature. Permeabilization was performed with 1% Triton X-100 in PBS for 15 min at room temperature. Cells were incubated with primary antibodies diluted in PBS with 1% goat serum for 35 min to 1 h, washed 3 times for 10 min with PBS, incubated for 35 min to 1 h with the appropriate secondary antibodies diluted in PBS with 1% goat serum, and washed 3 times for 10 min with PBS. Antibodies used were Y12 monoclonal antibody (anti-Sm) (27) (dilution 1:500), rabbit anti-CDC5L (1:500), and rabbit anti-PLRG1. Tetramethylrhodamine B isothiocyanate-conjugated goat anti-mouse and Cy5-conjugated goat anti-rabbit secondary antibodies were also used (Jackson ImmunoResearch). Microscopy and image analysis was carried out using a Zeiss DeltaVision Restoration microscope as described previously (28).

RESULTS

CDC5L and PLRG1 Are Co-immunodepleted from HeLa Nuclear Extract—The human CDC5L and PLRG1 proteins can be co-purified as part of a multiprotein complex as can their homologues in yeast (8, 10). We also observed that CDC5L and PLRG1 are highly phylogenetically conserved proteins in the core CDC5L associated complex in HeLa nuclear extracts. These observations prompted us to investigate whether all of the PLRG1 protein in HeLa nuclear extract was associated with CDC5L. A GST-PLRG1 fusion protein was expressed in *E. coli*, purified, and used to raise antibodies in sheep and rabbits for use in the analysis of the CDC5L-PLRG1 interaction. The immune serum obtained recognizes the protein in HeLa nuclear extract as well as the bacterially expressed protein (Fig. 1B lane 1, Fig. 3A, lane 3, and data not shown).

Anti-CDC5L antibodies (10) were used to immunodeplete CDC5L from HeLa nuclear extract, and both the proteins bound on the beads and the proteins remaining in the supernatants were separated by SDS-PAGE and transferred onto a nitrocellulose membrane by Western blotting as described under “Experimental Procedures.” Transferred proteins were probed using anti-CDC5L antibodies (Fig. 1A), anti-PLRG1 antibodies (Fig. 1B), and as a negative control, an antibody to the spliceosomal protein SPF30 (6), which was not identified in the human CDC5L complex (Fig. 1C). The results indicate that most of the PLRG1 is co-depleted upon immunodepletion of CDC5L from HeLa nuclear extract (Fig. 1, A and B, lanes 4 and 5). In contrast, the spliceosomal protein SPF30 remains in the supernatant, *i.e.* not associated with the CDC5L-PLRG1 complex. These data show that most of the PLRG1 protein in HeLa

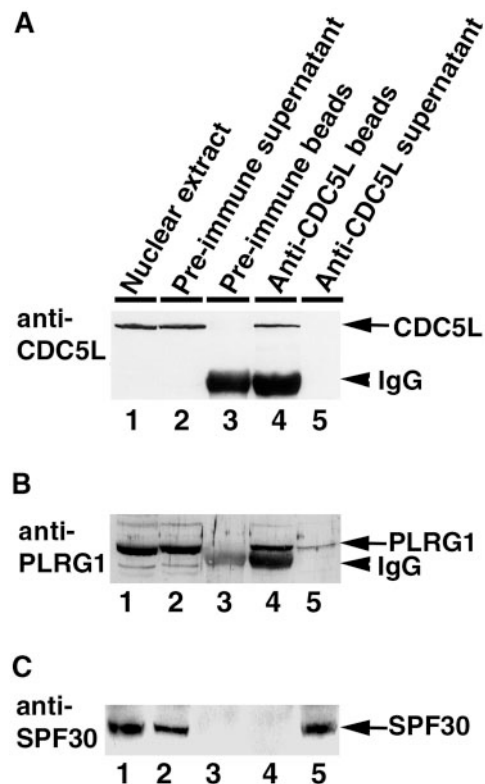


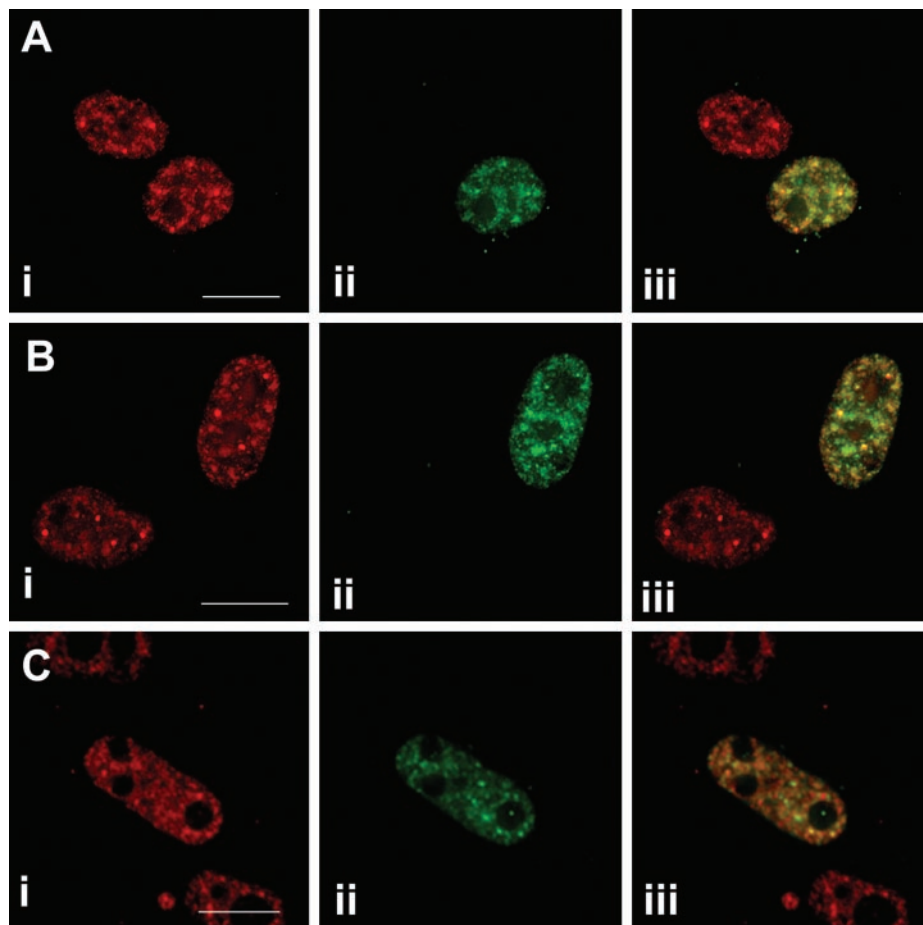
FIG. 1. Anti-CDC5L co-immunoprecipitates most of the PLRG1 from HeLa nuclear extract. Anti-CDC5L was used to immunodeplete CDC5L protein from nuclear extract. The beads containing immunoprecipitated proteins and supernatants from which CDC5L has been removed were loaded onto a 10% SDS-PAGE gel and probed by Western blotting using anti-CDC5L antibodies. Lanes 1 are the control lanes containing about 50 μ g of HeLa nuclear extract, and lanes 2 have the same amount of nuclear extract as lanes 1 except that the extract in lanes 2 was collected from an immunodepletion experiment using pre-immune IgG. Lanes 3 and 4 contained proteins eluted from protein G-agarose beads used in the immunodepletion of CDC5L from nuclear extract by pre-immune IgG and anti-CDC5L antibodies, respectively. The supernatant from the immunodepletion experiment using anti-CDC5L antibodies was loaded into the lanes 5 of the figure. Immunoprecipitation reactions in A were probed with anti-CDC5L antibodies. B and C contained identical samples as in A except that B and C were probed with anti-PLRG1 and anti-SPF30 antibodies, respectively.

nuclear extract is associated with CDC5L.

CDC5L Co-localizes with PLRG1 in Vivo in HeLa Cell Nuclei—Because of the tight association between CDC5L and PLRG1 found in immunoprecipitation experiments, we next decided to investigate whether these proteins are present in the same nuclear structures *in vivo*. HeLa cells were either transfected with expression vectors encoding GFP-tagged CDC5L and PLRG1 proteins or else stained with specific antibodies. PLRG1 and CDC5L both showed a speckled nuclear staining pattern, as judged by antibody staining and by expression of GFP fusion proteins, which co-localized with the pattern obtained by co-staining the cells with the monoclonal antibody Y12 (Fig. 2, A and B). The antibody Y12 recognizes the “Sm” proteins common to each of the major splicing snRNPs (27). When HeLa cells transiently expressing GFP-CDC5L were stained with anti-PLRG1, both proteins were found to co-localize in the same speckled structures (Fig. 2C). Similar results were obtained by staining cells transiently expressing GFP-PLRG1 with anti-CDC5L antibodies (data not shown). These results are consistent with the presence of CDC5L and PLRG1 in a common complex *in vivo* but do not address whether the proteins directly interact.

FIG. 2. CDC5L associates with PLRG1 *in vivo* in human HeLa cells.

HeLa cells were grown, transfected, and incubated with antibodies as described under "Experimental Procedures." The images shown in the panels are representative optical sections from the respective deconvolved data sets. All the panels *i* show antibody staining, and the panels *ii* indicate the nuclear structures obtained using GFP-CDC5L or YFP-PLRG1. All the panels *iii* show overlays of *i* and *ii* in each experiment. **A**, HeLa cells were transiently transfected with a plasmid expression vector encoding the GFP-PLRG1 fusion protein, fixed, and stained with the anti-Sm protein monoclonal antibody Y12 (27). Red represents Y12 staining, and green indicates GFP-PLRG1 expression. **B**, a plasmid expression vector encoding GFP-CDC5L was used to transiently transfect HeLa cells that were subsequently fixed and stained with the same anti-Sm antibody as above. Red indicates Y12 staining as above, and green represents GFP-CDC5L expression. **C**, HeLa cells were transfected with a YGFP-PLRG1 plasmid expression vector. The cells were then fixed and stained with anti-CDC5L antibodies. Red represents anti-CDC5L staining, and green shows YFP-PLRG1 expression. In all the panels, yellow indicates co-localization of the two proteins CDC5L and PLRG1. Similar localization patterns were obtained using the protein-specific antibodies instead of the GFP or YFP fusions (data not shown). Bar, 10 μm . Please note that the use of a fluorescent protein tag results in a stronger signal from the fusion protein. This shows up as a small fraction of the label that does not co-localize.



CDC5L and PLRG1 Interact Directly *In Vitro*—We next decided to investigate a possible direct interaction between the two proteins using *in vitro* methods. HeLa nuclear extracts were incubated with GST-PLRG1, and an immunoprecipitation was performed on the reaction mixture using anti-CDC5L antibodies. Immunoprecipitated proteins were then separated by SDS-PAGE and blotted onto nitrocellulose filters before probing with anti-PLRG1 antibodies (Fig. 3A, lane 3). The results show that bacterially expressed GST-PLRG1 as well as endogenous PLRG1 are co-immunoprecipitated by anti-CDC5L antibodies, and thus GST-PLRG1 will associate with CDC5L in HeLa nuclear extract. To determine whether bacterially expressed CDC5L will interact with PLRG1 in the nuclear extract, the bacterially expressed protein GST-CDC5L was incubated with HeLa nuclear extract and bound protein selected using glutathione-Sepharose beads. After separation by SDS-PAGE and transfer of proteins to nitrocellulose membranes as described above, the blots were probed with an antibody raised against GST-PLRG1 (Fig. 3A, lane 4). These results indicate that GST-CDC5L expressed in *E. coli* will interact with PLRG1 in HeLa nuclear extract, whereas GST alone does not (Fig. 3A, compare lanes 4 and 5; the PLRG1 arrow on the right of the panel shows endogenous PLRG1 pulled down by GST-CDC5L).

It is possible that the CDC5L-PLRG1 interaction may be indirect because other protein factors in the nuclear extract may be involved in mediating the interaction, *e.g.* acting as "bridging" factors. Therefore, *in vitro* translated CDC5L was used in a pull-down assay with *E. coli* expressed GST-PLRG1 fusion protein and glutathione-agarose beads (see "Experimental Procedures"; Fig. 3B). The results indicate that the GST-PLRG1 fusion protein will pull down L-[³⁵S]methionine-labeled

and *in vitro* translated CDC5L (Fig. 3B, lanes 1 and 2), whereas neither glutathione-Sepharose beads nor GST alone will (Fig. 3B, lanes 3 and 4, respectively). To rule out any involvement of some component in the reticulocyte lysate used for *in vitro* translation mediating the binding, a GST pull-down was performed using only purified, bacterially expressed proteins, *i.e.* GST-PLRG1 and His-tagged CDC5L (Fig. 3C). The results show that GST-PLRG1 will bind CDC5L directly *in vitro*, whereas GST-SPF30 (6), another spliceosomal protein not found in the CDC5L complex, does not interact directly with CDC5L (Fig. 3C, compare lanes 1 and 2 with lane 3). Taken together, the above data indicate that PLRG1 and CDC5L directly interact with each other.

Identification of the PLRG1 Binding Domain in CDC5L—By having shown that CDC5L and PLRG1 will interact directly *in vitro*, we next decided to identify the protein domain in CDC5L needed for binding to PLRG1. Protein truncation mutants were prepared by inserting stop codons at ~100-amino acid intervals in the CDC5L sequence progressively from the amino terminus (Fig. 4A). The mutated CDC5L expression plasmids were then used for *in vitro* transcription/translation to produce L-[³⁵S]methionine-labeled truncated proteins lacking overlapping regions of the carboxyl terminus of the protein (Fig. 4B). The PLRG1 interaction domain in CDC5L was determined by using full-length GST-PLRG1 in pull-down experiments with each of the respective CDC5L truncation mutants. The full-length and the $\Delta\text{CDC5L}_{\text{lf}}$ mutant proteins were efficiently pulled down (Fig. 4C, lanes 6 and 7), whereas little or none of the mutants *a–e* were pulled down (Fig. 4C, lanes 1–5). These results indicate that truncated proteins lacking the carboxyl terminus of the protein, as well as GST, will not bind efficiently to PLRG1. This indicates that the major PLRG1 binding region in CDC5L

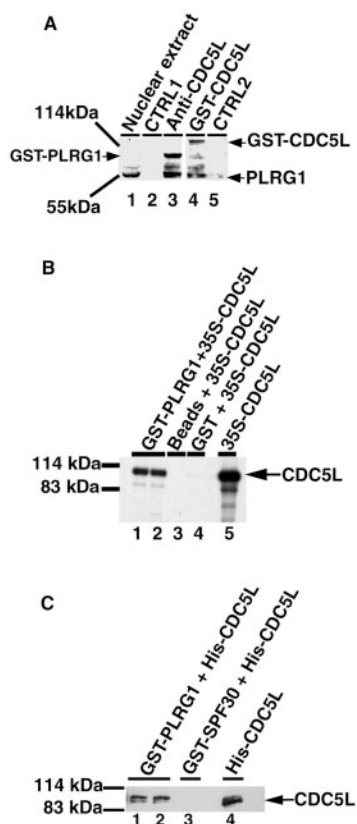


FIG. 3. CDC5L interacts directly with PLRG1 *in vitro*. A, GST-PLRG1 (lanes 2 and 3) was incubated for 20 min at room temperature with 0.5 mg of HeLa nuclear extract. An immunoprecipitation was performed on the reaction either using control preimmune serum, CTRL1 (lane 2) or anti-CDC5L antibodies (lane 3). The blotted proteins from the immunoprecipitates were probed with anti-PLRG1 antibodies. Lane 4 shows a pull-down experiment in which GST-CDC5L was incubated with nuclear extracts as above and the interacting proteins pulled down using 25–30 μ l of glutathione-Sepharose beads. The blot was probed with anti-GST-PLRG1 antibodies. Lane 5 is a control reaction using GST alone in the pull-down experiment instead of GST-CDC5L. Lane 1 contained \sim 40 μ g of HeLa nuclear extract. Note that the anti-GST-PLRG1 antiserum (which also contains GST antibodies) recognizes full-length GST-CDC5L and a minor band in lane 4 corresponding to a partial cleavage product of the GST-CDC5L protein used in the pull-down experiment as well as endogenous PLRG1. B, GST-PLRG1 (0.2 nmol) was used to pull down L -³⁵S]methionine-labeled *in vitro* translated CDC5L (5–8 μ l). Lanes 1 and 2 are duplicate samples of the pull-down experiment, and lanes 3 and 4 are controls using glutathione-Sepharose beads alone and GST, respectively, for pull-downs. Lane 5 contained about 30–40% of the input labeled CDC5L. C, GST-PLRG1 was used to pull down bacterially expressed His-tagged CDC5L. Lanes 1 and 2 contained duplicate samples of the pull-down of His-CDC5L by GST-PLRG1. Lane 3 had a control pull-down using the spliceosomal protein SPF30 that does not interact with CDC5L. Lane 4 is the positive control and contained His-CDC5L alone used in the binding assays. All the arrows on the right of the panels indicate proteins identified in the pull-down experiments.

is likely located toward the carboxyl-terminal end of the protein from about amino acid residues 600–800.

To determine the region in the CDC5L sequence containing the binding domain for PLRG1, overlapping regions toward the 3' end of the CDC5L cDNA were subcloned (Fig. 4D). The protein fragments translated *in vitro* from these cDNA regions (Fig. 4E) were used with GST-PLRG1 in pull-down experiments (Fig. 4F). The results obtained show that full-length CDC5L and the mutant proteins translated *in vitro* from regions g (Δ CDC5Lg) and h (Δ CDC5Lh) were pulled down using full-length GST-PLRG1 (Fig. 4F, lanes 1–3, respectively). The mutant proteins obtained from the regions i (Δ CDC5Li) and j (Δ CDC5Lj) did not bind to GST-PLRG1 (Fig. 4F, lanes 4 and 5).

These experiments show that the carboxyl terminus sequence (amino acid residues 706–800) is essential for binding to PLRG1, whereas sequences upstream from amino acid 706 will not bind PLRG1 in this assay. However, we consistently observed an enhancement in binding (compare Fig. 4F, lanes 2 and 3) when the carboxyl-terminal domain of CDC5L also contained the sequences upstream from amino acid position 706 (*i.e.* Δ CDC5Lh). Taken together, these data indicate that although the carboxyl-terminal amino acids 706–800 (Δ CDC5Lg) are essential and sufficient for PLRG1 binding, upstream amino acids can either enhance or stabilize this interaction.

Identification of the Region Containing the CDC5L Binding Domain in PLRG1—By having identified the PLRG1 binding region in CDC5L, we next decided to determine the region in PLRG1 containing the CDC5L-binding site. Protein truncation mutants were engineered by inserting stop codons at regular intervals of about 130 amino acids in a 5'–3' direction in the PLRG1 cDNA sequence (Fig. 5A). The mutations were created in the GST-PLRG1 construct and the mutant proteins expressed in *E. coli* and purified. The expressed proteins were then used in GST pull-downs with L -³⁵S]methionine-labeled full-length CDC5L (Fig. 5B). The pull-down experiments showed that full-length GST-PLRG1 (Fig. 5B, lane 5) and the PLRG1 truncated protein that stops at amino acid position 390 (Fig. 5B, lane 4) will bind to CDC5L, whereas GST alone will not (Fig. 5B, lane 1). The proteins terminating at positions 130 or 262 showed little or no binding (Fig. 5B, lanes 2 and 3). To determine the region in PLRG1 containing the minimum binding sequence for CDC5L, we next prepared sub-clones of the PLRG1 cDNA containing overlapping sequences upstream and downstream from the amino acid positions 262–390 (Fig. 5C). The cDNA sub-clones were expressed and L -³⁵S]methionine labeled *in vitro* by translation in reticulocyte lysate (Fig. 5D). The labeled proteins were then used in pull-down experiments with full-length GST-CDC5L. These experiments show that the overlapping fragments containing the amino acid sequence positions 257–396 will bind to CDC5L, whereas GST alone will not interact with L -³⁵S]methionine-labeled PLRG1 (Fig. 5E, compare lane 1 with lanes 2–4).

In Vitro Analysis of the Interaction between the Carboxyl-terminal Region of CDC5L and the WD Domain of PLRG1—Because all the pull-down experiments described so far involved using either full-length CDC5L to pull down PLRG1 mutants, or full-length PLRG1 to pull down CDC5L mutants, it is possible that other regions in the full-length proteins, apart from those determined using our assays, might also play a role in the binding. To confirm that the interacting domains in CDC5L and PLRG1 that we have identified are alone sufficient for binding, the respective mutant proteins with regions containing the minimal binding sequences were used in pull-down experiments (Fig. 6). In the first experiment GST- Δ PLRG1f (*i.e.* comprising amino acid positions 257–396) was used to pull-down *in vitro* translated, L -³⁵S]methionine-labeled Δ CDC5Lg and Δ CDC5Lh. The results indicate that the *in vitro* translated carboxyl-terminal sequence of CDC5L consisting of amino acids 602–800 and 706–800 (Fig. 6A, lanes 3 and 4) will interact with the bacterially expressed PLRG1 mutant protein containing the amino acids from positions 257–396 (GST- Δ PLRG1f).

To provide a quantitative estimate of the relative binding efficiencies of the wild type and mutant proteins, the GST pull-downs described above (Fig. 6A) were repeated in quadruplicate, and the amount of L -³⁵S]methionine-labeled protein in the pull-downs was measured as a percentage of input using a PhosphorImager (Fuji-FLA2000) (Fig. 6B). The results ob-

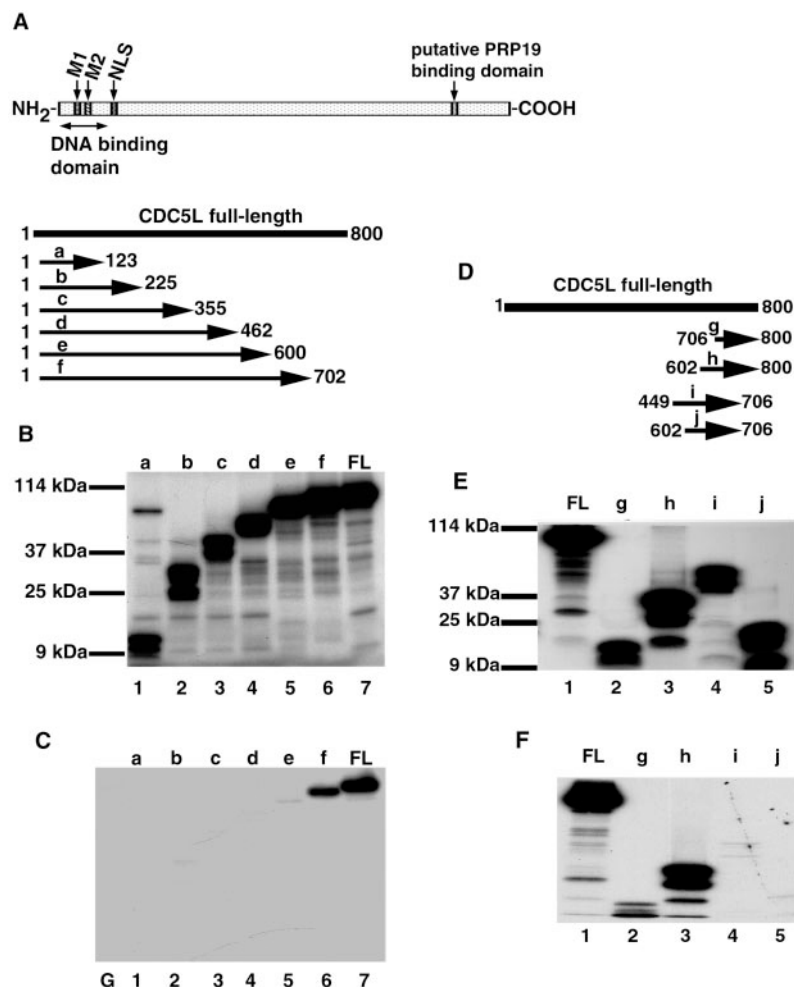


FIG. 4. Identification of the PLRG1 binding domain in CDC5L. *A*, linear diagram showing domain structure of CDC5L and mutagenesis strategy. *NLS* indicates a nuclear localization signal. Point mutations were inserted into the CDC5L cDNA creating stop codons that resulted in truncation mutants when the protein is expressed. The arrows indicated the approximate length of expressed protein, and the numbers to the right of the arrows show the position of the inserted stop codon. The letters *a–f* represent the different mutant proteins expressed. *B*, the CDC5L cDNAs with mutations were *in vitro* translated in the presence of L-[³⁵S]methionine and 3–4 μ l of the expressed protein loaded onto a 12% SDS-PAGE gel. Protein bands were revealed by autoradiography. The lanes marked 1–6 represent the respective deletion mutants marked *a–f*. Lane 7 contained full-length CDC5L. *C*, approximately 0.2 nmol of GST-PLRG1 was used in pull-down experiments with the deletion mutants *a–f* translated *in vitro* (8–10 μ l). The lane marked *G* contained the control sample where GST instead of GST-PLRG1 was used to pull-down CDC5L. Lanes 1–6 contained the mutant proteins *a–f*, and lane 7 had a pull-down of CDC5L with GST-PLRG1. *D*, overlapping carboxyl-terminal sequences of the CDC5L cDNA were sub-cloned into the expression vector pET-30a (Novagen). The arrows indicate the cloned fragments, and the numbers on the left- and right-hand side of each arrow represent the positions of the first and last amino acids, respectively, of the protein expressed from each cDNA fragment. The letters *g–j* represent the respective sub-clones of the CDC5L cDNA. *E*, the sub-clones above were *in vitro* translated, and 3–5 μ l of the translated proteins were run on a 12% SDS-PAGE gel. The protein bands were revealed by autoradiography. The lanes marked 2–5 contained the expressed proteins from the sub-clones *g–j*, respectively. Lane 1 contained the full-length CDC5L protein. *F*, GST-PLRG1 was used to pull down the proteins translated from the clones *g–j*. Lane 1 is a positive control, i.e. a pull-down of CDC5L using GST-PLRG1. Lanes 2–5 contained samples from pull-down experiments using GST-PLRG1 and the mutant proteins from *g* to *j*, respectively.

tained indicate that Δ CDC5Lh will bind much more efficiently to GST- Δ PLRG1f (Fig. 6B, bar 3) than to Δ CDC5Lg (Fig. 6B, bar 4). These results also show that the interaction between the mutant proteins appears to be more stable than the interaction between the full-length CDC5L and PLRG1 proteins (compare Fig. 6B, bar 2 and bars 3 and 4). This may be because the binding domain in the mutant proteins is more exposed and thus more accessible to the interacting partners.

We next decided to study the interaction between Δ PLRG1f and Δ CDC5Lg using only bacterially expressed proteins to exclude the possibility that some component in the reticulocyte lysate used above in the *in vitro* translation experiments may mediate the interaction between the two mutant proteins. GST- Δ PLRG1f and hexahistidine-tagged Δ CDC5Lg were expressed in *E. coli* and affinity-purified using glutathione-Sepharose (Amersham Pharmacia Biotech) and nickel-agarose beads (Qiagen), respectively. The purified proteins were used

in a GST pull-down assay (see “Experimental Procedures”). Bound proteins were separated by SDS-PAGE and blotted onto nitrocellulose filters. The filters were stained using Blot FastStain, (Chemicon International) according to the manufacturer’s instructions, to reveal all the proteins on the nitrocellulose membrane from the GST pull-downs (Fig. 6C). The results show that GST- Δ PLRG1f will pull-down His-tagged Δ CDC5Lg, whereas GST alone does not (compare Fig. 6C, lanes 1 and 2 with lanes 5 and 6). We also observed that GST- Δ PLRG1f does not bind to the truncated protein Δ CDC5Lj that lacks the carboxyl-terminal sequence of CDC5L (Fig. 6C, lanes 3 and 4). Because the reagent used above to stain the blot (i.e. Blot FastStain) will stain any protein transferred onto the membrane, it means that to rule out the possibility that the Δ CDC5Lg band may be a partial cleavage product of GST- Δ PLRG1f, the nitrocellulose membrane was stripped and then probed with protein-S alkaline phosphatase conjugate (Nova-

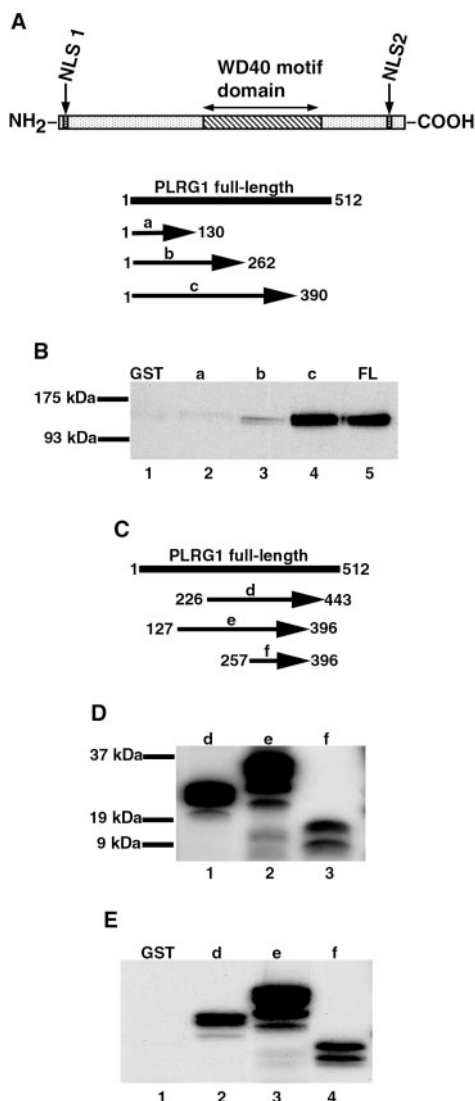


FIG. 5. Identification of the CDC5L binding region in PLRG1. A, linear diagram showing domain structure of PLRG1 and mutagenesis strategy. *NLS1* and *NLS2* indicate nuclear localization signals. GST-tagged PLRG1 cDNA was point-mutated such that stop codons were inserted at specific sites. The arrows indicate the length of the reading frames after stop codon insertion. The numbers on the right of the arrows indicate the positions of the stop codons in the PLRG1 cDNA sequence. The letters *a–c* represent the cDNAs generated by the mutations. B, the GST-PLRG1 mutants were expressed in *E. coli*, and the purified proteins were used in pull-down assays with L-[³⁵S]methionine-labeled CDC5L. Lane 1 is a negative control pull-down using GST, whereas lanes 2–4 are pull-downs using the proteins from *a* to *c*, respectively. Lane 5 represents a positive control experiment where full-length GST-PLRG1 was used to pull-down CDC5L. C, overlapping sequences containing the CDC5L binding region were sub-cloned into the pGEM-T vector (Promega) and pGEX4-T1 (Amersham Pharmacia Biotech). The arrows indicate the cloned fragments, and the numbers on the left- and right-hand side of each arrow represent the positions of the first and last amino acids, respectively, of the protein expressed from each cDNA fragment. The letters *d–f* have been used to label the sub-clones produced. D, the cDNAs in pGEM-T were used for *in vitro* transcription/translation and the expressed protein bands revealed by autoradiography. The lanes 1–3 represent proteins expressed from the clones *d–f*, respectively. E, the proteins expressed above were used in pull-down experiments with GST-CDC5L. Lane 1 is a control experiment in which GST alone was used to pull-down full-length L-[³⁵S]methionine-labeled PLRG1. The lanes 2–4 contained pull-downs using GST-CDC5L, and the proteins were expressed using the clones *d–f*.

gen) according to the manufacturer's instructions (Fig. 6D). Δ CDC5Lg used in these experiments was expressed in *E. coli* from the plasmid pET-30a (Novagen) that contains an S-tag

that is downstream from the hexahistidine sequence and upstream from the Δ CDC5Lg sequence. Protein-S specifically binds to the S-tag that is present in proteins expressed from the pET-30a plasmid vector. The results obtained after probing the membrane with protein-S alkaline phosphatase confirm the observation above that GST- Δ PLRG1f pulls down His₆- Δ CDC5Lg (Fig. 6D, lanes 1 and 2) and GST does not (Fig. 6D, lanes 5 and 6). Taken together, our results show that the truncated proteins GST- Δ PLRG1f and His- Δ CDC5Lg will interact directly *in vitro*. This means that the protein sequences that we have tested are both necessary and sufficient for binding, whereas other amino acid sequences outside these regions in both proteins are not strictly required for binding to occur.

Disruption of the CDC5L-PLRG1 Interaction in HeLa Nuclear Extract Inhibits pre-mRNA Splicing—To characterize further the role of these highly conserved proteins, we next investigated the effect of adding the mutant proteins containing the interaction domains in both proteins on the CDC5L-PLRG1 complex in HeLa nuclear extract (Fig. 7). We decided to use Δ CDC5Lh for most of the subsequent experiments because this mutant protein consistently showed a higher binding efficiency to PLRG1 than Δ CDC5Lg (Fig. 6B and data not shown). Δ CDC5Lh was added to HeLa nuclear extract and wild type CDC5L immunoprecipitated from the extract using a peptide anti-CDC5L antibody (10), whose recognition sequence is outside the sequence contained in Δ CDC5Lh. The immunoprecipitates were then probed with anti-CDC5L antibodies (Fig. 7A, *i*) and anti-PLRG1 antibodies, respectively (Fig. 7A, *ii*). The data indicate that Δ CDC5Lh will disrupt the CDC5L/PLRG1 interaction in HeLa nuclear extract because it markedly reduces co-immunoprecipitation of PLRG1 by anti-CDC5L antibodies (Fig. 7A, *ii*, lane 3). However, when the CDC5L mutant is preincubated with Δ PLRG1f, the disruption of the CDC5L-PLRG1 complex in HeLa nuclear extract is blocked (Fig. 7A, *ii*, lane 7). This is presumably because of the titration of the Δ CDC5Lh interaction domain by Δ PLRG1f. We observed that although the PLRG1 mutant protein will bind to Δ CDC5Lh, it was unable to disrupt the CDC5L/PLRG1 interaction in HeLa nuclear extract (compare Fig. 7A, *ii*, lanes 3 and 6). The reason for this is not clear. It is possible that although Δ PLRG1f alone is sufficient for binding to CDC5L *in vitro*, another region of PLRG1 may be required to stabilize this interaction in HeLa nuclear extract. The absence of such a region in Δ PLRG1f may reduce the ability of the mutant protein to compete with wild type PLRG1 in nuclear extract for binding to CDC5L. The disruption of the CDC5L/PLRG1 interaction in nuclear extract by Δ CDC5Lh is consistent with the relatively high affinity of this mutant protein for Δ PLRG1f (Fig. 6B, bar 3) compared with CDC5L and Δ CDC5Lg (Fig. 6B, bars 2 and 3 respectively).

We next investigated the effect on pre-mRNA splicing of the disruption of the CDC5L-PLRG1 interaction in HeLa nuclear extract by the mutant proteins. The mutant proteins, as well as full-length proteins, were expressed in *E. coli* and purified as described under "Experimental Procedures." Under our expression conditions, some of the proteins gave high yields, e.g. Δ CDC5Lh and PLRG1 (Fig. 7B, lanes 2 and 4), whereas others, such as the Δ PLRG1f and CDC5L, gave relatively lower yields (Fig. 7B, lanes 3 and 6). When Δ CDC5Lh was added to splicing reactions, splicing of the pre-mRNA substrate was inhibited (Fig. 7C, lanes 4–6), whereas splicing was unaffected upon addition of GST, GST-tagged, or hexahistidine-tagged CDC5L or SPF30 (Fig. 7C, lanes 3, 9, and 10 and data not shown). Addition of Δ PLRG1f alone (Fig. 7C, lanes 12–14) inhibited splicing, although not as efficiently as Δ CDC5Lh, consistent with our previous observation of its inefficient disruption of the CDC5L/PLRG1 interaction in HeLa nuclear extract (Fig. 7A).

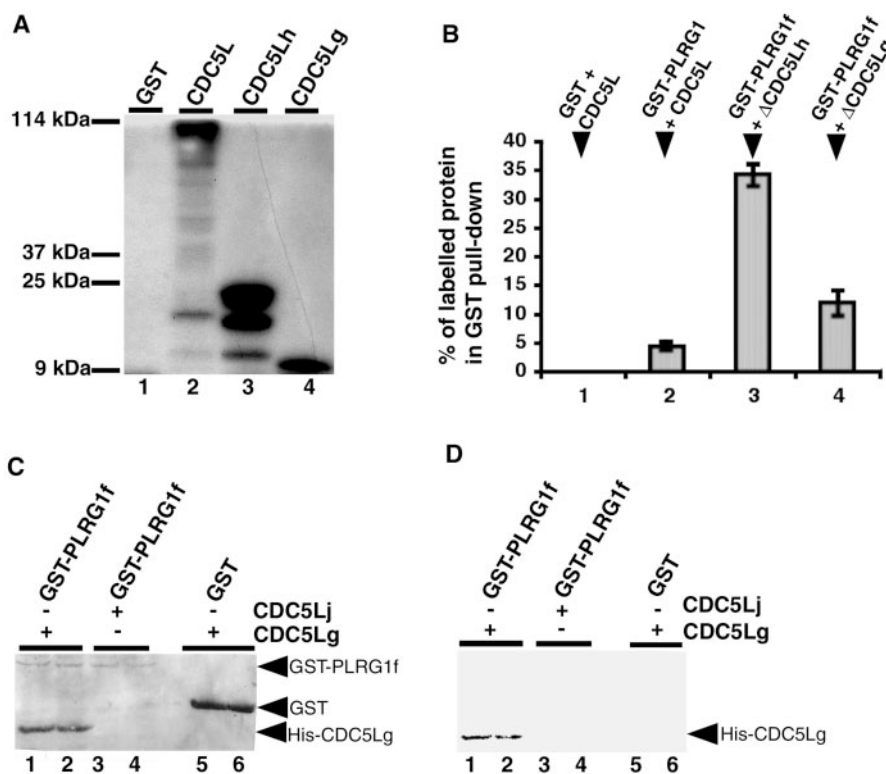


FIG. 6. The WD motif rich region of PLRG1 interacts with the carboxyl-terminal of CDC5L. *A*, GST- Δ PLRG1f (PLRG1 mutant from clone f) was used to pull-down the carboxyl-terminal truncated proteins of CDC5L, *i.e.* Δ CDC5Lg and Δ CDC5Lh. *Lane 1* is a control pull-down of CDC5L using GST alone. *Lane 2* is a pull-down of CDC5L using full-length GST-PLRG1. *Lanes 3 and 4* contained samples from experiments where GST- Δ PLRG1f was used to pull down L -[35 S]methionine-labeled Δ CDC5Lg and Δ CDC5Lh, respectively. *B*, quantitative analysis of the relative GST-pull-down efficiencies of binding assays in *A*. Equimolar amounts of the GST fusions above (*A*) were used in pull-down assays containing 10 μ l of L -[35 S]methionine-labeled proteins (CDC5L, Δ CDC5Lg, and Δ CDC5Lh, respectively). Each pull-down was performed in quadruplicate. The amount of activity in each pull-down was measured using a PhosphorImager (Fuji FLA-2000). Data analysis was performed using the two-dimensional densitometry procedure according to the manufacturer's instructions in the Advanced Image Data Analyzer software (AIDA version 2.11 from Raytest Isotopenmeßgeräte, GmbH, Germany). The total amount of activity (Integral/Area *i.e.* [PSL/mm 2]) in the pull-down was divided by the activity in the input band for each L -[35 S]methionine-labeled protein \times 100 to obtain the relative amounts of labeled protein pulled down by GST fusion protein. *C*, GST- Δ PLRG1f, Δ CDC5Lg, and Δ CDC5Lj were expressed in *E. coli* and purified as described under "Experimental Procedures." GST- Δ PLRG1f was used to pull down Δ CDC5Lg or Δ CDC5Lj onto glutathione-Sepharose beads. All the reactions were carried out in duplicate. *Lanes 1 and 2* contained reactions from a pull-down experiment using Δ CDC5Lg, and *lanes 3 and 4* contained samples from a pull-down of hexahistidine Δ CDC5Lj using GST- Δ PLRG1f. *Lanes 5 and 6* represent control pull-down experiments using GST and Δ CDC5Lg. Note that GST- Δ PLRG1f did not bind to the glutathione-Sepharose beads as efficiently as GST alone. *D* is identical to *C* except that the nitrocellulose filter was probed with a protein-S alkaline phosphatase conjugate (Novagen).

On the other hand, when equimolar amounts of Δ CDC5Lh and Δ PLRG1f were preincubated together before addition to the splicing reaction, the splicing inhibition was much reduced compared with when Δ CDC5Lh was added alone (Fig. 7C, compare lanes 5 and 6 with lanes 7 and 8). This means that the interaction between the two mutant proteins had reduced their ability to disrupt the CDC5L-PLRG1 complex in the HeLa nuclear extract, thus allowing splicing to progress. The addition of full-length PLRG1 to the splicing reactions did not inhibit pre-mRNA splicing (Fig. 7C, lane 11). Taken together, these results show that the interaction between CDC5L and PLRG1 in HeLa nuclear extract is essential for the ability of the extract to splice pre-mRNA.

Effect of the Addition of Δ CDC5Lh and Δ PLRG1f Proteins to HeLa Nuclear Extract on Spliceosome Assembly—We have found previously that removal of the CDC5L complex from HeLa nuclear extract by immunoprecipitation will inhibit formation of splicing products, whereas spliceosome assembly on the pre-mRNA was not prevented (10). Therefore, we decided to investigate whether spliceosome assembly was affected by the disruption of the CDC5L/PLRG1 interaction in HeLa nuclear extract (Fig. 8A). When Δ CDC5Lh was added to nuclear extract and the extract preincubated with the mutant proteins before addition to a splicing reaction, spliceosome assembly was inhibited and neither the pre-spliceosome nor spliceosome com-

plexes were formed (Fig. 8A, lane 3). The Δ CDC5Lh mutant was found to be more efficient in this inhibition than the Δ PLRG1f protein which showed little or no inhibition (Fig. 8A, compare lanes 3 and 4). However, when equimolar amounts of the Δ PLRG1f and Δ CDC5Lh mutants were preincubated before addition to the nuclear extract, the inhibitory effect of Δ CDC5Lh was blocked (Fig. 8A, lane 5). This implies that the interaction between Δ PLRG1f and Δ CDC5Lh has prevented Δ CDC5Lh from interfering with the CDC5L-PLRG1 complex in the nuclear extract and spliceosome assembly.

We next attempted to determine the step at which Δ CDC5Lh inhibits spliceosome assembly. Splicing reactions were set up as described under "Experimental Procedures," and Δ CDC5Lh was added to these reactions at different time points. After the addition of Δ CDC5Lh, the reactions were allowed to proceed up to 1 h. The reactions were then loaded onto native polyacrylamide-agarose composite gels and run for several hours. Bands corresponding to splicing complexes were revealed by autoradiography. The results obtained indicate that when Δ CDC5Lh was added to the nuclear extract and either preincubated for 20 min or added immediately prior to commencement of the reaction (time points -20 and 0 respectively, Fig. 8B, lanes 3 and 4), the formation of spliceosome complexes was blocked. At the other time points, *i.e.* adding Δ CDC5Lh between 20 and 60 min, progressively more spliceosomal complexes are detected

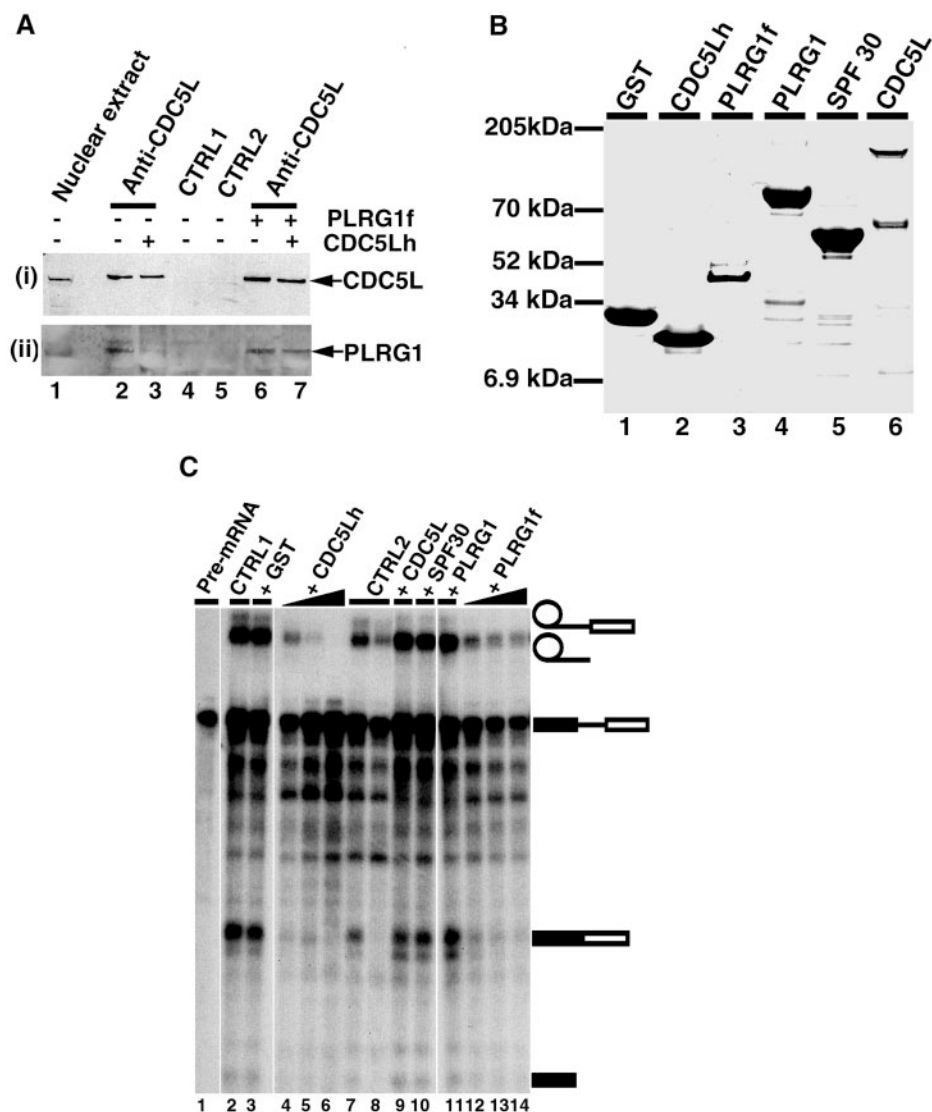


FIG. 7. The CDC5L-PLRG1 interaction in nuclear extract is essential for splicing. A, Δ CDC5Lh or Δ PLRG1f (~ 1 nmol) was added to ~ 100 μ g of HeLa nuclear extract followed by immunoprecipitation using about 15–20 μ g of rabbit anti-CDC5L antibodies. The immunoprecipitates were subsequently probed with sheep polyclonal anti-CDC5L and anti-PLRG1 antibodies. Lane 1 contained nuclear extract alone. Lanes 2, 3, 6, and 7 contained immunoprecipitates of anti-CDC5L except that Δ CDC5Lh alone was added to the nuclear extract in lane 3, and Δ PLRG1f was added to the extract in lane 6, and Δ CDC5Lh and GST- Δ PLRG1f were both added to the nuclear extract in lane 7. Lanes 4 and 5 contained negative control immunoprecipitation reactions using antibodies to HCF (a nuclear protein) and rabbit preimmune IgG. The panels *i* and *ii* were probed with anti-CDC5L and anti-PLRG1 antibodies, respectively. The arrows on the right of the figure indicate the protein bands. B, full-length and truncated proteins to be added to splicing reactions were expressed in *E. coli* and purified to the same extent by affinity chromatography. 2–10 μ g of the purified proteins were run on a 4–12% gradient gel (NOVEX) and stained using colloidal Coomassie stain according to the manufacturer's instructions. Lane 1 contained GST. Lanes 2 and 3 had Δ CDC5Lh and GST- Δ PLRG1f, respectively. Lane 4 contained GST-PLRG1. Lane 5 contained SPF30, and lane 6 contained His-CDC5L. Minor bands in the gel represent degradation products of the expressed proteins. C, 0.05–0.2 nmol of the bacterially expressed proteins were added to about 55 μ g of HeLa nuclear extract and preincubated at room temperature for about 20 min before addition to pre-mRNA splicing reactions. The splicing reactions were incubated at 30 °C for about 90 min. Lane 1 contained the pre-mRNA used in the splicing experiments. Lanes 2 and 3 contained control splicing reactions except that 0.2 nmol of GST were added to the reaction in lane 3. Lanes 4–6 represent splicing reactions to which increasing amounts of Δ CDC5Lh (0.05–0.2 nmol) were added, whereas the reactions in lanes 12–14 contained increasing amounts of GST- Δ PLRG1f. Lanes 7 and 8 contained splicing reactions to which were added increasing amounts (0.1 and 0.2 nmol, respectively) of both Δ CDC5Lh and GST- Δ PLRG1f. The reactions in lanes 9–11 contained 0.2 nmol of the *E. coli* expressed proteins CDC5L, SPF30, and PLRG1, respectively.

assembled on the pre-mRNA (Fig. 8B, lanes 5–7). These results indicate that the addition of Δ CDC5Lh inhibits any further assembly of spliceosomes after its addition to the reactions. It is also possible that the presence of the mutant protein disrupts some forms of assembled complexes.

To determine whether Δ CDC5Lh was disrupting assembled splicing complexes or inhibiting the formation of new complexes after its addition, a splicing reaction was set up under standard conditions and then allowed to run for about 50 min. The reaction was then split into 2 aliquots as follows: GST was added to 1 aliquot, *i.e.* the control; and to the other an equimo-

lar amount of Δ CDC5Lh was added. Both aliquots were then incubated for a further 10 min at 30 °C before being stopped. Splicing complexes formed were separated on a native gel, and bands corresponding to these complexes were revealed by autoradiography (Fig. 8C). The results obtained show that Δ CDC5Lh will not disrupt spliceosomal complexes after they have been formed (Fig. 8C, lane 4). Incubating the splicing reactions with Δ CDC5Lh for longer periods (up to 30 min) after spliceosome assembly did not cause disruption of the assembled spliceosomes (data not shown). Although Δ CDC5Lh was found to be unable to disrupt the pre-formed spliceosome com-

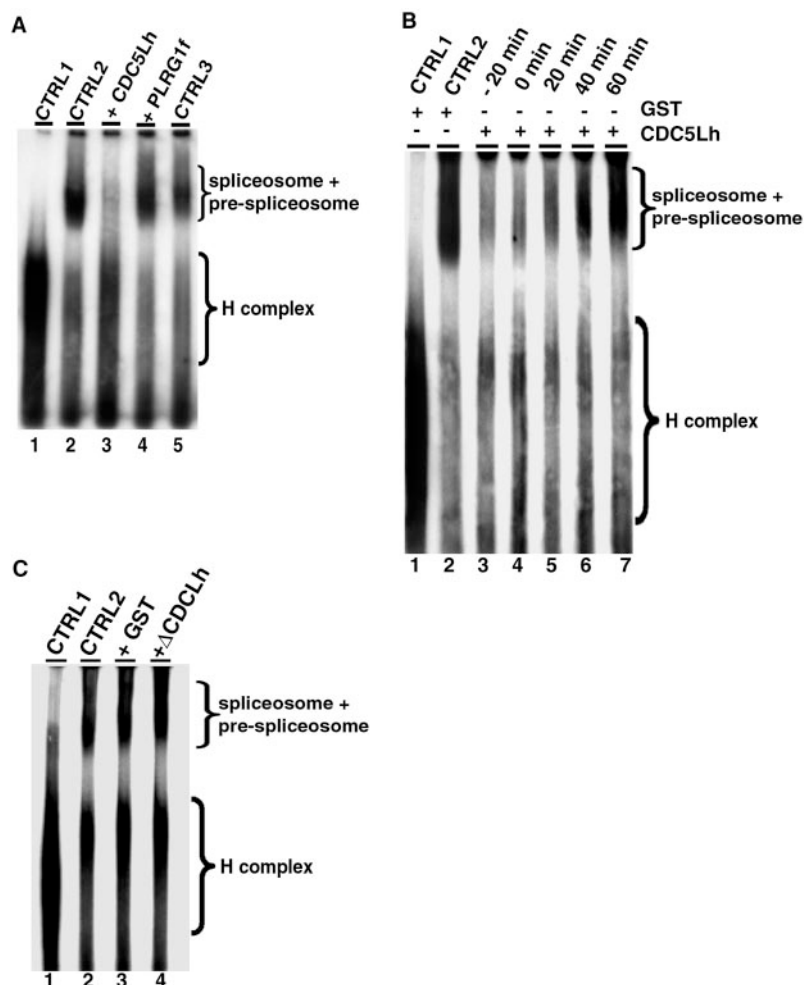


FIG. 8. Inhibition of spliceosome assembly by Δ CDC5Lh. A–C represent native polyacrylamide-agarose gels used to separate spliceosome complexes. The bands on the gels were revealed by autoradiography. Approximately 0.2 nmol of bacterially expressed proteins were added to about 55 μ g of HeLa nuclear extract and preincubated at room temperature for 20 min before addition to pre-mRNA splicing reactions as above except that the reactions here were incubated at 30 °C for 1 h before loading on to native gels. A, lane 1 contained a splicing reaction prepared and left on ice. CTRL2 (lane 2) is a control reaction to which was added GST. Lanes 3 and 4 contained reactions which had the Δ CDC5Lh and GST- Δ PLRG1 proteins added, respectively. Lane 5 (CTRL3) contained a splicing reaction to which was added both Δ CDC5Lh and GST- Δ PLRG1 proteins. The two proteins had been preincubated together for 10 min at room temperature before addition into the splicing reactions. B, Δ CDC5Lh was added to splicing reactions at different time points. The time point –20 indicates that Δ CDC5Lh was preincubated with nuclear extract at room temperature for 20 min before the splicing reaction was started. The time points 0, 20, 40, and 60 correspond to the respective times in minutes at which Δ CDC5Lh was added to the splicing reactions after they had been started. All the reactions contained ATP at the start of the experiment, i.e. time 0. Lane 1 (CTRL1) contained a splicing reaction prepared and incubated on ice. The lane labeled CTRL2 is a control reaction to which GST was added at time point –20. All the reactions were allowed to run at 30 °C for up to 60 min after addition of the Δ CDC5Lh protein. Lanes 3–7 contain samples representing the different time points. C, effect of Δ CDC5Lh on pre-assembled spliceosomes. A single splicing reaction was prepared and incubated for about 50 min after which the reaction was split into 2 aliquots. GST was added to the aliquot in lane 3, and Δ CDC5Lh was added to the aliquot in lane 4. The reactions were then allowed to continue for a further 10–15 min before stopping. Spliceosomal complexes were then separated on a native gel. CTRL1 (lane 1) contained a splicing reaction prepared and left on ice. CTRL2 (lane 2) is a positive control reaction containing no bacterially expressed protein.

plexes, it is possible that its presence in the reaction may make the assembled complexes less stable.

Heparin is routinely added to splicing reactions at low concentrations before separation on native gels because it enhances the migration of the complexes into the gel, leaving relatively less material stuck in the wells. However, heparin also has the property of disrupting aggregates and large protein complexes. We next decided to investigate whether the presence of Δ CDC5Lh caused pre-assembled spliceosome complexes on the pre-mRNA to be more sensitive to heparin treatment. Pre-mRNA splicing reactions were set up as described above and in duplicate tubes. After allowing the reactions to run for 50 min, GST or Δ CDC5Lh was added to separate duplicate tubes, and the reaction was allowed to proceed for a further 10–15 min. Heparin was then added to each of the reactions, and the reactions were incubated for 5 min at room

temperature before loading onto native polyacrylamide-agarose gels as mentioned above. The results obtained from this experiment showed that the presence of Δ CDC5Lh does not make the spliceosomal complexes formed more sensitive to heparin treatment (data not shown). Taken together, these results indicate that Δ CDC5Lh will inhibit the formation of any new complexes after its addition to a splicing reaction and that the inhibition of spliceosome assembly occurs at an early step, perhaps prior to or concomitant with the formation of the commitment complex.

DISCUSSION

In yeast and humans, the CDC5L protein has been found to be in a large multiprotein complex that also contains PLRG1 and other splicing factors (8, 10). However, a direct interaction between these two proteins has not been reported previously

nor has the functional significance of the association of these two phylogenetically conserved proteins in sub-spliceosomal complexes been studied. Their high degree of sequence conservation across species suggests that both proteins may perform essential biological roles in the cell.

In this study we have shown that CDC5L and PLRG1 are associated with each other *in vivo* and in HeLa nuclear extract. We have also shown that CDC5L interacts directly with PLRG1 *in vitro*. The regions containing sequences that mediate the direct interactions between the two proteins *in vitro* have been mapped to the carboxyl-terminal domain of CDC5L and the WD40 region of PLRG1. By using the CDC5L mutant protein containing the PLRG1 binding domain, we have also shown that the CDC5L-PLRG1 interaction in nuclear extract can be disrupted and that this disruption leads to the inhibition of pre-mRNA splicing. These observations indicate that a direct interaction between specific domains of the two proteins, CDC5L and PLRG1, is required for pre-mRNA splicing to proceed. Our results also indicate that the presence of a disrupted CDC5L complex in HeLa nuclear extract caused by the mutant protein Δ CDC5Lh interferes with the spliceosome assembly process. This is presumably because of a dominant negative effect of the disrupted CDC5L-PLRG1 complex on spliceosome assembly. It is thus possible that the disrupted components of the CDC5L complex will inhibit spliceosome formation, although the complex itself may not be strictly required for assembly to proceed. This possibility is supported by the observations that the mutant protein Δ PLRG1f, which is not efficient in disrupting the CDC5L-PLRG1 interaction in HeLa nuclear extracts, does not prevent spliceosome assembly. Next, the fact that Δ CDC5Lh blocks formation of splicing complexes once added to a reaction, but does not disrupt complexes that have already been formed, further supports the view that the presence of a disrupted CDC5L complex may interfere with the *de novo* assembly of spliceosomal complexes. Thus our previous observations that the removal of the CDC5L complex from HeLa nuclear extract by immunoprecipitation does not lead to inhibition of spliceosome assembly (10) may be because of the incomplete removal of all the proteins in the complex, with the low level of remaining proteins sufficient to promote spliceosome assembly and, to a lesser extent, the first catalytic step of splicing. The inhibition of spliceosome assembly by Δ CDC5Lh may also suggest a role for the CDC5L-PLRG1 interaction in spliceosome assembly. Here excess mutant protein in the nuclear extract interferes with the efficient assembly of the spliceosome by competing with the endogenous CDC5L protein for binding to PLRG1 during assembly of the spliceosomal complex.

In *S. cerevisiae*, the CDC5L homologue CEF1 has been shown to interact with the splicing factor Prp19p in yeast two-hybrid assays through a region in the carboxyl-terminal half of the protein, and this region has also been shown to be essential for CEF1 self-interaction (7). *S. cerevisiae* Prp19p is required for the first catalytic step of splicing and may associate with the spliceosome either after or, simultaneously, with U4 snRNP dissociation from the complex. It has therefore been suggested that Prp19p may play a role in mediating the structural rearrangements in the spliceosome during U4 dissociation (29, 30). The human homologue of yeast Prp19 has not yet been characterized, although we have identified a Prp19-like protein in our recent study of the CDC5L complex in HeLa nuclear extract (10). It is thus possible that both Prp19 and PLRG1 may interact, either simultaneously or alternatively, with the carboxyl-terminal domain of CDC5L. The length of the Prp19 binding domain identified in the yeast CEF1 protein is about 30 amino acids. The equivalent sequence to the above

region in the CDC5L protein, determined by comparative sequence analysis, is about 100 amino acids upstream from the 94 amino acid region we have identified as the PLRG1-binding sequence in CDC5L. The involvement of the WD40 domain of PLRG1 in binding to CDC5L is consistent with the suggestion that proteins containing these regions may have a role in multiple simultaneous or consecutive protein-protein interactions (17).

In yeast, the CDC5L homologue CEF1 has been implicated in the targeting of the CDC5L complex to the spliceosome as a single multiprotein complex (7). These findings are consistent with our observations that Δ PLRG1f on its own did not strongly inhibit spliceosome assembly, whereas Δ CDC5Lh was more efficient in inhibiting assembly. This may be because the CDC5L mutant protein containing the PLRG1 binding domain may not only disrupt the CDC5L-PLRG1 complex in nuclear extract but also interfere with interactions between other splicing factors that need to be targeted to the assembling spliceosome. Further studies on characterizing the direct protein-protein interactions between the core components (10) of the CDC5L complex should shed more light on how these interactions affect the pre-mRNA splicing mechanism.

CDC5L and its homologues in other species have already been implicated in several cellular roles in addition to pre-mRNA splicing, for example cell cycle progression (11, 31, 32), sequence-specific double-stranded DNA binding, and transcription activation in HeLa cells. The transcription activation ability of the protein was determined by using a vector containing a luciferase reporter fused downstream from a CDC5L-binding site. It was observed that luciferase activity is increased by about 28-fold when the HeLa cells were cotransfected with a plasmid expressing CDC5L (33). It is thus possible that CDC5L may be needed in the cell for pre-mRNA splicing as well as for gene transcription and cell division.

Although PLRG1 has not been shown previously to interact directly with specific pre-mRNA splicing factors, there is some evidence in *A. thaliana* that the homologue of this protein (PRL1) interacts with other cellular proteins not directly involved in splicing, such as protein kinase C- β II and α -importin ATHKAP2 (14). The PLRG1 homologue in *A. thaliana* has been shown to have a pleiotropic role in several regulatory pathways in this species. These include glucose metabolism, hormonal responses, transcription of genes regulated by sucrose and cytokinin, and regulation of SNF1-like kinases (14, 34). Thus, the CDC5L and PLRG1 proteins in eukaryotes may be directly involved in several cellular functions as mentioned above. Alternatively, it may be that some of the above observations are an indirect consequence of changes in the expression of specific genes in these organisms mediated by the role of CDC5L and PLRG1 in the mechanism of pre-mRNA splicing. Further studies are now required to characterize in more detail the interaction of the CDC5L-PLRG1 complex with other splicing factors to understand the contribution of such interactions to the regulation of pre-mRNA splicing.

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