DLP, a Novel Dim1 Family Protein Implicated in Pre-mRNA Splicing and Cell Cycle Progression*

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In eukaryotes, primary transcripts undergo a splicing process that removes intronic sequences by a macromolecular enzyme known as the spliceosome. Both genetic and biochemical studies have revealed that essential components of the spliceosome include five small RNAs, U1, U2, U4, U5, and U6, and as many as 300 distinct proteins. Here we report the molecular cloning and functional analysis of a novel cDNA encoding for a protein of 149 amino acids. This protein has 38% amino acid sequence identity with and is evolutionarily related to yeast Dim1 protein. Hence we named this protein DLP for Dim1-like protein. We showed that DLP is required for S/G2 transition. We also demonstrated that DLP functions in cell nucleus and interacts with the U5–102-kDa protein subunit of the spliceosome, and blocking DLP protein activity led to an insufficient pre-mRNA splicing, suggesting that DLP is yet another protein component involved in pre-mRNA splicing. Collectively, our experiments indicated that DLP is implicated in not only cell cycle progression but also in a more specific molecular process such as pre-mRNA splicing.

During evolution, the size of the proteome of an organism (i.e. the complete set of proteins expressed by the genome during the lifespan of an organism) is expanded by increasing the number of genes via elaborating pre-existing mechanisms that generate protein diversity as well as via inventing new mechanisms. Alternative pre-mRNA splicing is considered to be the principal source of protein diversity in vertebrates (1). Pre-mRNA splicing operates via assembly of a series of highly dynamic spliceosomal complexes followed by two successive transesterification reactions. The high fidelity of the splicing is achieved through networks of interactions involving four small ribonucleoprotein (snRNP)1 particles (U1, U2, U5, and U4/U6) (2–5), each of which contains the corresponding small nuclear RNA and a set of specific and common proteins (6). The complex also contains multiple non-snRNP-associated proteins that are essential for spliceosome assembly and catalysis (6).

Pre-mRNA splicing is an essential step in the expression of most metazoan protein-coding genes, often regulated in a cell-type specific or development specific manner. Members of the highly conserved SR (arginine/serine) protein family of splicing regulators, including SRp20/X16/Rbp1, SF2/ASF (Srp30a), SC35 (Srp30b), and B52/Srp55, possess modular structures featuring one or two RNA recognition motifs and a carboxy-terminal arginine/serine-rich domain. In addition to the SR proteins, there are other non-SR proteins that are also critical players in pre-mRNA splicing. Interestingly, these proteins appear also to be required for cell cycle progression. For example, Prp4, by virtue of its kinase activity, is required for pre-mRNA splicing by promoting the formation of active spliceosomes through activating non-SR components (7). However, the phenotypic manifestations of loss of the kinase activity of Prp4 included impairments of G1-S and G2-M progression of the cell cycle (7). Loss of the function of another non-SR protein, Prp6, led not only to the accumulation of pre-mRNA, but also to a cell cycle arrest phenotype (8).

An essential step of pre-mRNA spliceosome assembly is the interaction between the snRNPs U4/U6 and U5 to form the U4/U6/U5 tri-snRNP. The tri-snRNP protein, Prp6, plays a key role in this process, at least in yeast (9). Prp6, containing multiple tetratrico peptide repeats that are typically involved in multiple protein-protein interaction, is a 102-kDa protein that interacts within the tri-snRNP with both the U5 and U4/U6 snRNPs and bridges the two particles. The U5 small ribonucleoprotein particle contains various proteins with catalytic activities involved in conformational rearrangements of the spliceosome. Among these proteins, the human U5–15-kDa protein (also known as hDim1) is an evolutionarily conserved U5 snRNP-specific protein, and its ortholog in Saccharomyces cerevisiae named Dim1p was identified as a factor essential for cell cycle progression and pre-mRNA splicing in yeast (10). Dim1 proteins associate with multiple proteins known or strongly predicted to be associated with pre-mRNA processing, with interactions of hDim1 with hnRNPF, hnRNPH, and NP/PQ (11, 12).

Over the years, more protein components implicated in pre-mRNA splicing have been cloned and identified. Recently, Zhou et al. (13) identified ~145 distinct spliceosomal proteins. Of these, 88 were known splicing factors/snRNPs proteins/-spliceosomal proteins, including U5–102-kDa/Prp6 protein and RNAi, RNA interference; FACs, fluorescence-activated cell sorting; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.
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U5–15-kDa/Dib1 protein. They also found 58 proteins that had not been previously identified as splicingomalous proteins.

In this report, we show that a novel Dim1-like protein, DLP, is involved in cell cycle progression. We demonstrate that DLP interacts with splicing factor Prp6 and affects pre-mRNA splicing when its activity was blocked. These results indicate that DLP is implicated in both general cellular functions as well as more specific molecular processes.

EXPERIMENTAL PROCEDURES

Bioinformatics—The full-length of DLP gene was aligned out from 96 sequences from 89 cDNA clonal homologies in silico. The open reading frame, conserved domains, and chromosome location of DLP were predicted from the data base at NCBI (available at www.ncbi.nlm.nih.gov). The theoretical molecular weight and isoelectric point of DLP were predicted from the data base at www.expasy.ch/tools, and membrane-spanning motifs and signal peptide were analyzed at www.cbs.dtu.dk. The homologous alignment was analyzed by The ClustalW program using AdvantageTM eDNA PCR kit (Clontech) from the human cDNA panel followed by inserting these PCR products in pcDNA3.1 vector.

Plasmid Construction—The coding region of DLP was amplified in multiple tissue cDNA panels (Clontech, Palo Alto, CA) by RT-PCR with primers 5'-GAAAATGTCGAGAAGATCCTGTCTGT-3' and 5'-GCACTTCTGGCCTCTTCCTCTCATC-3' (forward) and cloned into the pCDNA3.1 vector (Invitrogen, Carlsbad, CA) to generate plasmid named pcDNA3.1-DLP. The sequence of human DLP is available in the GenBankTM database under accession AY566808. Plasmid pcDNA3.1-DLP was transformed into yeast strain AH109 as a bait. Yeast expression vector for DLP fusion protein, named pGADT7-Prp6, was made as follows: the entire coding region of the Prp6 was amplified by PCR using primers 5'-GGGAATTGTCATCTTGTGACAGTGAT-3' (forward) and 5'-CAGCTGATGCTGCAAAACAT-3' (reverse) from pcDNA3.1-Prp6 plasmid. The PCR product were digested with EcoRI and Clal and then ligated into the vector pGKT7 (Clontech). Yeast expression vector for Prp6 fusion protein, named pGADT7-Prp6, was made as follows: the entire coding region of the Prp6 was amplified by PCR using primers 5'-GGGAATTGTCATCTTGTGACAGTGAT-3' (forward) and 5'-CAGCTGATGCTGCAAAACAT-3' (reverse) from pcDNA3.1-Prp6 plasmid. The PCR product were digested with EcoRI and Clal and then ligated into the vector pGADT7 (Clontech).

RT-PCR and Northern Blotting—Total cellular RNA was isolated using the TRIzol reagent (Invitrogen), and first strand cDNA was prepared using the ProtoScriptTM first strand cDNA synthesis kit with a dT23 VN Primer (New England Biolabs, Beverly, MA). cDNA synthesis was performed using glyceraldehyde-3-phosphate dehydrogenase primers as a positive control. Human adult multiple tissue Northern blots (Clontech) were used to detect human DLP mRNA expression profile. A 447-bp fragment spanning 287–736 bp of human DLP cDNA was prepared as a probe and labeled by random priming with [α-32P]dCTP (Amersham Biosciences). Hybridization was performed in ExpressHyb hybridization solution (Clontech) according to the manufacturer’s instruction. After stringent washing the filters were exposed for autoradiography at ~80 °C.

GST Pull-down Assays and Generation of Polyclonal Antibodies against DLP—GST fusion constructs were expressed in BL21 Escherichia coli cells, and crude bacterial lysates were prepared by sonication in TEDGN (50 mM Tris-Cl, pH 7.4, 1.5 mM EDTA, 10% (v/v) glycerol, 0.4 mM NaCl) in the presence of the Complete® protease inhibitor mixture (Roche Applied Science). The in vitro transcription and translation experiments were done with rabbit reticulocyte lysate (TNT systems, Promega, Madison, WI) and r-Methionine (Amersham Biosciences) according to the manufacturer’s recommendations. In GST pull-down assays, about 10 μg of the appropriate GST fusion proteins was mixed with 5–8 μl of the in vitro transcribed/translated products and incubated in binding buffer (75 mM NaCl, 50 mM HEPES, pH 7.9) at room temperature for 30 min in the presence of the Complete® protease inhibitor mixture (Roche Applied Science). The binding reaction was then added to 30 μl of glutathione-Sepharose 4B beads for 12 h at 4 °C. The beads were washed with binding buffer, resuspended in 30 μl of 2× SDS-PAGE loading buffer, and resolved on 10–12% gels. The gels were then fixed in 50% methanol, 10% acetic acid for 30 min and dried. Protein bands were detected by autoradiography at ~80 °C for 4–16 h. Antibodies to recombinant DLP (anti-DLP) was raised in rabbits against purified GST-DLP fusion protein.

Co-immunoprecipitation Assay—MCF-7 cells were grown in dishes of 100-mm diameter seeded with 105 cells and transfected with expression plasmid pCDNA3.1-Flag-Prp6. Forty hours after transfections, cells were washed twice with ice-cold phosphate-buffered saline (PBS) containing protease inhibitor mixture (Roche Applied Science) and resuspended in saline. The beads were washed with binding buffer, resuspended in 30 μl of 2× SDS-PAGE loading buffer, and resolved on 10–12% gels. The gels were then fixed in 50% methanol, 10% acetic acid for 30 min and dried. Protein bands were detected by autoradiography at ~80 °C for 4–16 h. Antibodies to recombinant DLP (anti-DLP) was raised in rabbits against purified GST-DLP fusion protein.

Yeast Two-hybrid Screening—The Matchmaker GAL4 Two-hybrid System 3 (Clontech protocol PT3247-1) was used to screen for proteins interacted with DLP from a human mammary gland library (Clontech). pGBK7-DLP was transformed into yeast strain AH109 as a bait. Approximately 5 × 106 transformants were screened. Plasmids were recovered from yeast and retransformed into native AH109 yeast with pGADT7-Prp6. The transformants were assayed for MEL1 activation by selecting on high stringency plates: SD/-Ade/-His/-Leu/-Tryp/-X-Gal. The protein expression construct used was C terminally tagged with a 3×Flag epitope (Clontech). Yeast colonies were assayed for β-galactosidase activity using a colony-lift filter as follows: colonies were transferred to 3 MM filter paper, permeabilized by brief immer-
sion in liquid nitrogen, and incubated on a filter paper saturated with Z-buffer containing 1 mg/ml X-gal at 30 °C for 0.5–8 h. Positive clones were then subjected to sequencing.

**Yeast Two-hybrid Interaction Assay**—The Matchmaker GAL4 Two-hybrid system 3 (Clontech protocol PT3247-1) was used to conduct the interactions between with DLP and Prp6. pGBK7T7-DLP and pGADT7-Prp6 were co-transformants into strain Y187 or AH109. We then assayed the transformants for MEL1 activation by selecting for transformants on high stringency plates: SD-Ade/His-Leu/TrpX-a-galactosidase. Positive and negative controls in parallel were performed. Yeast colonies were assayed for β-galactosidase activity using colony-lift filter as following: colonies were transferred to 3 M filter paper, permeabilized by brief immersion in liquid nitrogen, and incubated on a filter paper saturated with Z-buffer containing 1 mg/ml X-gal at 30 °C for 0.5–8 h.

**RNA Interference and Western Blotting**—Vector-based RNAi was utilized. Plasmids were constructed by inserting a synthesised 64-mer oligonucleotide containing a specific sequence for a forward 19- to 215-bp region of DLP open reading frame into pSUPER vector (15). The sequences synthesized were: oligonucleotide 1, 5′-GATCCCCGTCGACATATACGATTTACAGTGTTATTAACTGGTATATTTTGGAAA-3′ and oligonucleotide 2, 5′-AGTTTTTTCTAAAAACTGCTGAATTTTGTTATATCTTGTTTTTGCTACAGTGTTATTAACTGGTATATTTTGGAGGGG-3′. The oligonucleotides were resuspended in an annealing buffer (100 mM potassium acetate, 30 mM HEPEs-KOH, pH 7.4, 2 mM magnesium acetate) and heated to 65 °C for 5 min, 70 °C for 10 min, and then cooled to room temperature to generate double-stranded DNA. The double-stranded DNA was then phosphorylated and cloned into the BglII/Hind III site of the pSUPER vector. The vector was then transfected into cells with the LipofectAMINE 2000 Reagent (Invitrogen). Total proteins were extracted after 48 h of transfection. Transfection efficiency was monitored by co-transfection with an E. coli lacZ construct (pDNA4/His/Max1lacZ, Invitrogen). For Western blotting, 48 h after the transfection, cell culture media were collected and total cellular protein was extracted. Extracts were fractionated on 12% SDS-PAGE gels, transferred to Immobilon P polycrystalline membrane (Millipore), and extracted after 48 h of transfection. Transfection efficiency was monitored by co-transfection with an E. coli lacZ construct (pDNA4/His/Max1lacZ, Invitrogen). The plasmids used to transfect into 293T cells. Forty-eight hours after the transfection, cell culture media were collected and total cellular protein was extracted. Extracts were fractionated on 12% SDS-PAGE gels, transferred to Immobilon P polycrystalline membrane (Millipore), and probed with anti-c-Myc monoclonal antibodies (Invitrogen) or anti-GST-DLP polyclonal antibodies.

**Fluorescence Confocal Microscopy**—MCF-7 cells were plated into six-well chamber slides and transfected with pEGFP or pEGFP-DLP by LipofectAMINE 2000 reagent (Invitrogen). Twenty-four hours after the transfection, cells were washed with PBS, fixed in 4% paraformaldehyde, and permeabilized with 1% Triton X-100. Cells were washed for four times and a final concentration of 0.1 μg/ml 4,6-diamidino-2-phenylindole dihydrochloride (Sigma) was included in the final washing to stain nuclei. Images were visualized on an Olympus IX70 inverted microscope equipped with a charge-coupled device camera. The resulting images were deconvolved with Deltavision software. For co-localization analysis, pEGFP-Red-Prp6 and pEGFP-DLP were co-transfected into MCF-7 cells. Twenty-four hours after transfection, cells were washed in PBS, fixed with 4% paraformaldehyde, and visualized under Nikon confocal microscope (Nikon, Bio-Rad).

**Cell Cycle Analysis**—To prepare cells for fluorescence-activated cell sorter (FACS) analysis, 10^5 to 10^6 MCF-7 cells were fixed in 70% ethanol for overnight at 4 °C. After washing with PBS, cells were incubated with 1 μl (10 mg/ml) of RNase A (Sigma) in PBS/0.25% Triton X-100 for 30 min at room temperature and then stained with 50 μg/ml propidium iodide in 50 mM sodium citrate for 20 min at room temperature in the dark. Cell cycle data were collected with a FACSscan (BD Biosciences immunochemistry system) and analyzed with CELLQuest software.

**Cell Proliferation Analysis**—Cell proliferation of transiently transfected 293T, HeLa, C2C12, and MCF-7 cells was measured by MTT dye reduction assay (16). Briefly, cells were seeded into 96-well plates, and on the day of harvest, 100 μl of medium was replaced with an equal volume of fresh medium containing 10% FBS 5 mg/ml stock. Plates were incubated at 37 °C for 4 h before addition of 100 μl of dimethyl sulfoxide, and the plates were shaken at room temperature for 10 min. Cellularity was determined by measuring the absorbance of converted dye at a wavelength of 570 nm.

**In Vitro Pre-mRNA Splicing Assays**—The plasmids used to transcribe the adenoviral major late-M3 pre-mRNA (pAdML-M3) were kindly provided by Dr. Robin Reed (Harvard Medical School, Boston, MA). Plasmids were linearized and transcribed in vitro in the presence of 20 units of T7 RNA polymerase (Promega), 1 μg of the linearized plasmids, and 5 μl [γ-32P]dCTP (Amersham Biosciences) in a 1 μM monomethyl cap (New England Biolabs) in 25 μl of reaction buffer. The radiolabeled RNAs were resolved on 6% denaturing polyacrylamide gels. The gels were briefly exposed to films, and the band corresponding to the full-length RNA was excised. RNAs were eluted from the gel slice in elution buffer (0.75 x ammonium acetate, 10 mM magnesium acetate, 0.1% (v/v) SDS, 0.1 mM EDTA) overnight at 37 °C. The eluted RNAs were separated from the gel pieces by a Millipore Ultrafree-MC filter unit (0.45 μm), ethanol-precipitated, dissolved in nucleic-acid free-water, and stored at −80 °C. Splicing reaction mixture, in a total volume of 25 μl, contained 3 M E. coli, 2.5 mM MgCl2, 1.5 mM ATP, 20 μM creatine phosphate, 0.5 mM dithiothreitol, and 20 μl of RNAse (Promega). Splicing reactions were incubated at 32 °C for 1 h. The assay was stopped by addition of proteinase K and SDS to final concentrations of 4 μg/ml and 0.1%, respectively. The samples were incubated at 37 °C for 20 min and diluted to 100 μl with 125 mM Tris, pH 8.0, 1 mM EDTA, 0.3% NP-40. RNA was extracted with 200 μl of phenol/ chloroform (50:50, v/v) followed by 200 μl of chloroform and precipitated with 300 μl of ethanol at −80 °C. The extracted RNAs were subjected to electrophoresis on 15% polyacrylamide/7 M urea gels. The radioactive RNA bands were revealed by autoradiography.

**RESULTS**

**Cloning and Characterization of DLP**—The full-length sequence of DLP gene was assembled in silico first through searching the GenBankTM data base, and clones were obtained from human MCF-7 cells, HepG2 cells, and Ishikawa cells by RT-PCR. These clones were 2540 bp in length (GenBankTM AY566808) and contained an open reading frame encoding for a protein of 149 amino acids. The predicted molecular mass of this protein is 17 kDa with a theoretical isoelectric point 5.63. The corresponding gene was mapped to chromosome 16q22.3 and consists of four exons and six introns (Fig. 1A). Sequence analysis of the deduced protein indicated that it has 38% amino acid sequence identity with and is evolutionally related to yeast Dim1 protein. Thus this protein was given the name of DLP for Dim1-like protein. Bioinformatics analysis indicated that DLP has three putative functional domains, three putative motifs (residues 104–109 and 132–135), and two N-myristoylation sites (residues 104–109 and 122–127) (Fig. 1B). Structural analysis revealed that DLP contains only one putative functional domain, i.e. a Dim1 domain, from 4 to 126 amino acids. Amino acid sequence alignment indicated that DLP shares 97% identity with its mouse homolog, NP 079575. The similarity of the amino acid sequence sequence of DLP with a homolog in other organisms was 55% in Arabidopsis thaliana, 40% in Orzya, 38% in Schizosaccharomyces pombe and Drosophila melanogaster, and 35% in S. cerevisiae (Fig. 1C). Phylogenetic analysis also indicated that DLP is a well conserved protein, and there is an evolutionary relationship between DLP and Dim1 protein (Fig. 1D).

**Tissue Expression Profile of DLP**—The expression of DLP was investigated by Northern blots and by RT-PCR experiments. The results indicated that DLP gene was expressed as a message of about 2.5 kb in eight normal tissues assayed (Fig. 2A). Highest expression was seen in skeletal muscle, liver, heart, and pancreas, whereas the expression of DLP was modest in kidney, brain, and placenta, and low in lung tissue (Fig. 2A). RT-PCR experiments indicated that DLP was also expressed in cancer cell lines such as MCF-7, HepG2, and Ishikawa (Fig. 2B). To confirm the predicted molecular weight of the DLP protein, a c-Myc-tagged DLP expression construct was transfected into 293T cells. Forty-eight hours after the transfection, cells were lysed and total proteins were subjected to Western blotting analysis with a monoclonal antibody against c-Myc. As shown in Fig. 2C, Western blotting indicated that the DLP was expressed as a protein of 17 kDa in DLP-transfected 293T cells, confirming its predicted molecular weight.

**Subcellular Localization of DLP**—To gain insight into the biological function of DLP protein, we first analyzed the subcellular localization of this protein. For this purpose, MCF-7
cells were transfected with either an enhanced green fluorescent protein (EGFP) expression construct pEGFP or with a DLP-EGFP fusion construct pEGFP-DLP. Twenty-four hours after transfection, the fluorescence was visualized under fluorescence microscopy. As shown in Fig. 3A, while cells transfected with pEGFP displayed diffuse fluorescence throughout subcellular compartments of the cells, in pEGFP-DLP transfected cells, intensive fluorescence were observed mainly in cell nucleus (Fig. 3B), suggesting that DLP may mainly function in cell nucleus.

Functional Analysis of DLP Protein in Cell Cycle Progression—As stated before, previous studies indicated that Dim1 protein was implicated in the cell cycle (17). To investigate whether or not DLP is also involved in cell cycle progression, the expression of the DLP protein was silenced by RNAi or enhanced by transfection of a DLP expression vector, and the cell cycle profile was analyzed under these conditions. In these experiments, MCF-7 cells were transfected with pSUPER vector carrying a specific sequence of DLP for its mRNA targeting or with pCDNA3.1-DLP for DLP expression. Twenty-four hours after the transfection, the MCF-7 cells were arrested at G0/G1 phase by growing in culture media deprived of estrogen for another 48 h before estrogen treatment for 16 h (18). Cells were then collected and analyzed for cell cycle profile by flow cytometry.
etry. As shown in Fig. 4A, silencing DLP expression resulted in a decreased percentage of cells in G2/M phase and an increased percentage of cells in S phase. On the other hand, overexpression of DLP led to an increased percentage of cells in G2/M phase (Fig. 4B), whereas treatment of cells overexpressing DLP with ICI 182,780, which causes cells arrested in G0/G1 phase resulted in little changes in cell cycle profile (Fig. 4C). The corresponding protein level was detected by Western blots. These results strongly suggested that DLP function in S-G2/M transition of the cell cycle.

DLP Stimulates Cell Proliferation—Given the observation that DLP may play roles in cell cycle progression, we next investigated whether DLP could stimulate cell growth. In these experiments, DLP was overexpressed in HeLa, C2C12, 293T, and MCF-7 cells via transfection, and the cell proliferation was assessed by MTT assays. As shown in Fig. 4D, overexpression of DLP stimulated the proliferation of HeLa, C2C12, 293T, and MCF-7 cells. Together with the observation that DLP promoted cell cycle progression, these results indicated that DLP could stimulate the growth of cells through accelerating S-G2/M transition of the cell cycle.

Physical Interaction of DLP with Proteins Implicated in Pre-mRNA Splicing—As mentioned earlier, Dim1 protein is mainly implicated in pre-mRNA splicing. Thus, it is reasonable to assume that DLP is also functionally involved in this process. To test the hypothesis that DLP is also implicated in pre-mRNA splicing, we first investigated the potential interaction of DLP with pre-mRNA splicing-related factor U5–102 kDa, PQBP, hnRNPF, or APC4 by GST pull-down experiments. In these experiments, DLP was fused to GST, and U5–102 kDa, PQBP, hnRNPF, and APC4 were in vitro transcribed/translated in the presence of [35S]methionine from pcDNA3.1-Prp6, pcDNA3.1-PQBP, pcDNA3.1-hnRNPF, and pcDNA3.1-APC4 constructs, respectively. GST pull-downs were performed, and the results indicated that DLP indeed interacted with Prp6 (U5–102 kDa) but not PQBP, hnRNPF, or APC4 (Fig. 5A). In addition, GST pull-down experiments with Prp6 and a series...
Deletion of DLP mapped the interaction domain in DLP was within 1–33 amino acids (Fig. 5B).

We next investigated whether the interaction between DLP and Prp6 observed in vitro could also be detected in an in vivo environment. In these experiments, MCF-7 cells were transfected with pcDNA3.1-FLAG-Prp6, total proteins were extracted and immunoprecipitated with an antibody against the FLAG tag, and Western blots were performed using anti-DLP antibodies to detected DLP. As shown in Fig. 5C, DLP indeed was co-immunoprecipitated with FLAG-tagged Prp6, supporting the data described in the GST pull-down assays.

The interaction between DLP and Prp6 was further solidified by yeast two-hybrid assays. In an attempt for screening for DLP-interacting proteins, a plasmid expressing a GAL-DLP fusion protein was used to screen a human breast library in yeast strain AH109. Seven positive clones were obtained from screening 1 × 10⁶ clones. Sequencing analysis indicated that these clones included Prp6 along with Homo sapiens nuclear receptor coactivator 7, α-2-glycoprotein 1, calmodulin 1, and several other proteins with unknown function. Meanwhile we confirmed the interaction between DLP and Prp6 using the yeast two-hybrid system (Fig. 6A).

Cellular Co-localization of DLP with Prp6—To further support the physical interaction between DLP and Prp6 and to support the role of DLP in pre-mRNA splicing, we next investigated the subcellular co-localization of DLP and Prp6. For this purpose, pGFP-DLP fusion and pDsRed-Prp6 fusion construct were co-transfected into MCF-7 cells. Twenty-four hours after the transfection, the localization of fluorescent proteins were recorded by fluorescent microscopy. As shown in Fig. 6B, both green fluorescence and red fluorescence were observed mainly in the cell nucleus and are superimposed, suggesting that DLP and Prp6 were co-localized in cell nucleus. These experiments supported the interaction between DLP and Prp6 and suggested that DLP may be also involved in pre-mRNA splicing.

Functional Analysis of DLP in Pre-mRNA Splicing—To establish the role of DLP in pre-mRNA splicing, in vitro splicing experiments were performed. In these experiments, ³²P-labeled Adml-M3 pre-mRNA was synthesized and incubated with HeLa cell nuclear extracts in standard splicing condition (4). Also included in these experiments was addition of anti-DLP polyclonal sera as a convenient means of ablating DLP function in HeLa cell nuclear extracts. As shown in Fig. 7, whereas addition of nonspecific sera did not affect the Adml-M3 pre-mRNA splicing, addition of anti-DLP sera severely inhibited the splicing activity of HeLa cell nuclear extract, and...
addition of GST-DLP again could restore the splicing activity, suggesting that DLP is required for ADML-M3 pre-mRNA splicing.

DISCUSSION

The small evolutionarily conserved protein Dim1/hDim1 (U5–15KD)/Dib1p/DML-1 was initially identified as a factor essential for cell cycle progression and pre-mRNA splicing (10, 17, 19) and was shown to be required in the maintenance of the steady state of the anaphase/promoting complex/cyclosome in fission yeast (12). Dim1/hDim1 (U5–15KD)/Dib1p/DML-1 appears also to play significant roles in animal development as elimination of DML-1 expression in Caenorhabditis elegans by RNA interference led to embryonic lethality during gastrulation, which accompanied by a failure to express early zygotic transcripts (17). Yeast two-hybrid and biochemical assays have identified there proteins interacting with hDim1p. The proteins that were identified are known or likely pre-mRNA alternative splicing factor, including the pre-mRNA alternative splicing enhancer protein hnRNP F and the RNA-binding protein NPW38/PQBP-1 (20, 21); both are known to be associated with other molecules involved in pre-mRNA splicing and/or cell cycle. In this report, we cloning and characterized yet another member of the Dim1 protein, DLP. DLP had 38% identity with human Dim1 protein (hDim1p/U5–15KD), which is located in the nucleus. We showed that DLP was both necessary and sufficient for S-G2/M transition as silencing DLP expression by RNAi led to an S phase accumulation and G2/M phase decrease of MCF-7 cells, and overexpression of DLP resulted in an increased G2/M phase cell population. In addition, if MCF-7 cells were arrested in G0/G1 phase by treatment with ICI 182,780, overexpression of DLP had limited effects on the cell cycle profile of MCF-7 cells, an indication that DLP was not essential for cell cycle entry. These results were consistent with previous observations that Dim1 regulated G2 progression in both S. pombe and S. cerevisiae (12). Other pre-mRNA processing factors linked to cell division cycle progression have also been
reported, e.g. CDC5L/hCDC5 (22, 23); Prp17, syf1p, syf2p, and syf3 (24, 25); SAP130/SF3b130 (26); Prp4 p kinase (7); S. pombe Prp5 and Prp6 (27); Prp8 (28, 29); and U 2AF65 (30).

In addition to the general cellular role, Dim1/hDim1 (U5–15KD)/Dib1p/DML-1 protein has also been implicated in more specific molecular processes. For example, Dib1p was identified as a component of the U4/U6.U5 tri-snRNP required for pre-mRNA splicing (11, 12); genetic studies have shown that Dim1 is essential for pre-mRNA splicing in yeast (17); and hDim1 was identified as an essential component of the nuclear pre-mRNA splicing machinery (19). We showed that DLP was capable of interacting with Prp6, the U5–102-kDa protein, by yeast two-hybrid interaction assays, an in vitro binding assay, and co-immunoprecipitation of cellular DLP and Prp6. MCF-7 cells were transfected with pcDNA3.1-FLAG-prp6 for 40 h. Whole cell lysates were then immunoprecipitated with an antibody against FLAG. The immunoprecipitated material was subjected to Western blotting analysis with anti-DLP polyclonal antibodies.

In addition to the general cellular role, Dim1/hDim1 (U5–15KD)/Dib1p/DML-1 protein has also been implicated in more specific molecular processes. For example, Dib1p was identified as a component of the U4/U6.U5 tri-snRNP required for pre-mRNA splicing (11, 12); genetic studies have shown that Dim1 is essential for pre-mRNA splicing in yeast (17); and hDim1 was identified as an essential component of the nuclear pre-mRNA splicing machinery (19). We showed that DLP was capable of interacting with Prp6, the U5–102-kDa protein, by yeast two-hybrid interaction assays, an in vitro binding assay, and co-localization. This result was consistent with a previous observation that Dib1, the homolog of hDim1, interacted with Prp6 in yeast (31). Moreover, our experiments indicated that DLP interacted with Prp6 with its amino-terminal, which encompasses the amino short stretch of the non-Dim1 domain as well as the amino-terminal of the Dim1 domain. In vitro pre-mRNA splicing assay indicated that DLP indeed was able to affect the pre-mRNA splicing as addition DLP antibodies to cell nuclear extracts led to an insufficient
experiments indicated that DLP is required for S-G2/M transi-
tional analysis of a novel Dim1 family member, DLP. Our
activities of these proteins.

In summary, we described in this report the cloning and
functional analysis of a novel Dim1 family member, DLP. Our
experiments could provide clues for delineating the biological
activities of these proteins.

In our effort to screen for DLP-interacting proteins, positive
clones were identified to include, along with Prp6, H. sapiens
nuclear receptor coactivator T (32), α-2-glycoprotein 1, calmod-
ulin 1, and several other proteins with unknown function. Our
experiments could provide clues for delineating the biological
mechanisms.

Further experiments are required to elucidate the roles of the novel molecule, DLP, in
pre-mRNA splicing and cell cycle regulation, and the related mechanisms.

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