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CDC2L5, a Cdk-Like Kinase With RS Domain, Interacts With the ASF/SF2-Associated Protein p32 and Affects Splicing In Vivo

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Abstract The human *CDC2L5* gene encodes a protein of unknown physiological function. This protein is closely related to the cyclin-dependent kinase (Cdks) family and contains an arginine/serine-rich (RS) domain. The Cdks were first identified as crucial regulators of cell-cycle progression, more recently they were found to be involved in transcription and mRNA processing. RS domains are mainly present in proteins regulating pre-mRNA splicing, suggesting CDC2L5 having a possible role in this process. In this study, we demonstrate that CDC2L5 is located in the nucleoplasm, at a higher concentration in speckles, the storage sites for splicing factors. Furthermore, this localization is dependent on the presence of the N-terminal sequence including the RS domain. Then, we report that CDC2L5 directly interacts with the ASF/SF2-associated protein p32, a protein involved in splicing regulation. Overexpression of CDC2L5 constructs disturbs constitutive splicing and switches alternative splice site selection in vivo. These results argue in favor of a functional role of the CDC2L5 kinase in splicing regulation. J. Cell. Biochem. 99: 890–904, 2006.

Key words: phosphorylation; cyclin-dependent kinases; RS domain; mRNA processing

Cyclin-dependent kinases (Cdks) are protein kinases which associate with and are activated by a cyclin regulatory sub-unit. These proteins display high sequence homology in their kinase domain (KD), containing in particular a totally or partially conserved PSTAIRE (single-letter amino acid codes) motif located in the cyclin-binding domain. The 10 Cdks (Cdk1–9 and Cdk11), characterized so far, can be grouped according to their cellular function. Cdk1 (Cdc2), Cdk2, 3, 4, and 6 regulate cell-cycle progression, Cdk5 is involved in the development and maintenance of central nervous system, while Cdk7, 8, and 9 behave as regulators of mRNA transcription [reviewed in Johnson and Walker, 1999; Bregman et al., 2000; Palancade and Bensaude, 2003; Murray, 2004]. More recent studies indicate that certain Cdks could also play a role in mRNA processing [Loyer et al., 2005]. The Cdk9/cyclin T complex is localized in splicing factor-rich regions, known as the nuclear speckles [Herrmann and Mancini, 2001]. The larger Cdk11 isoform, p110, interacts with general splicing factors and has been shown to promote pre-mRNA splicing [Hu et al., 2003]. In addition to these characterized Cdks, there are several CDC2-related kinases that have not yet been classified as Cdks due to the absence of an identified cyclin partner. Among these proteins is a sub-family of high molecular mass kinases with a Cdc2-like catalytic domain containing a PITAI/VRE motif [Marques et al., 2000]. In human, this sub-family contains two proteins, CDC2L5 [Lapidot-Lifson et al., 1992; Marques et al., 2000] and CrkRS [Ko et al., 2001], which display high

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similarity in the KD but are poorly related in the flanking large N- and C-terminal sequences. These two genes express several protein isoforms. Homologues can be identified by sequence comparison in the genome (or EST) of various species including mouse, rat, sea urchin [Marques et al., 2000], nematode [Boxem et al., 1999], drosophila, mosquito [Chiou et al., 1998], and the plants *Arabidopsis thaliana* and *Medicago sativa.* The function of these proteins is unknown; however, all of them contain a characteristic arginine/serine-rich (RS) domain at the N-terminus.

RS domain containing proteins are commonly involved in regulation of pre-mRNA splicing events. They can be broadly sub-divided into two groups: (i) the SR proteins, characterized by one or two N-terminal RNA recognition motifs (RRM) and a C-terminal RS domain; and (ii) the SR-related proteins, which have one or several RS domains not necessarily associated with an RRM [reviewed in Boucher et al., 2001]. SR proteins comprise a large set of nuclear proteins which are required for both constitutive and alternative pre-mRNA splicing [reviewed in Graveley, 2000; reviewed in Bourgeois et al., 2004]. Among the SR proteins, the human ASF/SF2 and SC35 pre-mRNA splicing factors have been well characterized. The RS domains of splicing factors, through protein–protein interactions, mediate the formation of a protein network important for splice site selection, and spliceosome assembly. Phosphorylation of RS domains by specific kinases regulates these interactions and provides a way of controlling splicing activity.

The SR-related protein family is continuously growing, uncovering novel proteins functionally associated with different nuclear processes, such as chromatin structure, mRNA transcription, coordination between pre-mRNA splicing, and 3′-end processing as well as protein targeting to nuclear domains like speckles [Boucher et al., 2001 and references therein]. Several kinases harboring RS domain were shown to participate in the phosphorylation of serine or threonine residues located in their own RS domain or in RS domain of other proteins. In particular, members of the Clk/Sty family phosphorylate SR proteins, regulating their splicing activity and their localization in speckles [Colwill et al., 1996; Prasad et al., 1999].

Here, we show that CDC2L5 is expressed in human cells as a nuclear protein accumulated in speckles, and that its N-terminal RS containing domain directly interacts with the ASF/SF2-associated protein p32 (p32) [Krainer et al., 1991]. Furthermore, we demonstrate that the overexpression of different CDC2L5 constructs affects constitutive and alternative splicing in vivo. These results argue in favor of a functional role of the CDC2L5 kinase in splicing regulation, although its precise mechanism of action remains to be elucidated.

MATERIALS AND METHODS

Plasmid Constructs

The human CDC2L5 isoform 2 coding sequence (GenBank accession No: AJ297710, nt 133–4688) was cloned into the pCDNA3 vector (Invitrogen), in fusion with a HA-tag in N-terminus, generating the plasmid pCDNA3-HA-CDC2L5. For this purpose, the DNA sequence containing the full-length ORF (nt 133–4688) was assembled from CDC2L5 restriction fragments obtained from previously sub-cloned CDC2L5 partial sequences [Marques et al., 2000] and the HA-tag was introduced by PCR. A *Sal*I fragment digested from pCDNA3-HA-CDC2L5, containing the full-length coding sequence, was sub-cloned into the pEGFP-C2 vector (Clontech) to express the GFP-CDC2L5 fusion protein (tagged in N-terminus). Fragments encoding different domains of CDC2L5 were generated by cleavage with restriction enzymes or by PCR amplification. The construct containing the N-terminal domain, pCDNA3-HA-N-ter (aa 1–706), was obtained from pCDNA3-HA-CDC2L5 by digestion with *Eco*RV and *Xho*I, filling of recessed termini and ligation of blunt ends. An *Eco*RV–*Bam*HI fragment (aa 706–982), containing the CDC2L5 KD, was sub-cloned into an HA-tag containing pCDNA3 (pCDNA3-HA) to generate pCDNA3-HA-DK (tagged in N-terminus). The pCDNA3-HA-C-ter, containing the CDC2L5 C-terminal domain (aa 1006–1452), and the pCDNA3-HA-ΔNLS-C-ter (aa 1044–1452), deleted of a putative nuclear localization signal (NLS), were generated by sub-cloning of PCR products into pCDNA3-HA. A recombinant GST-CDC2L5-C-ter fusion protein (aa 1194–1452) was expressed from a pGEX-4T2 construct containing an *Eco*RI–*Xho*I CDC2L5 fragment (pGEX-C-ter). For the two-hybrid screen, the bait containing the full-length CDC2L5 coding sequence, pGBKT7-CDC2L5,

was obtained by sub-cloning a *Sal*I fragment from pCDNA3-HA-CDC2L5 into the pGBKT7 vector (Clontech), in frame with the GAL4 DNA-binding domain. The same *Sal*I fragment, blunted, was inserted into pGAD-GE in frame with the GAL4 DNA-activating domain. The CDC2L5 N-terminal (aa 1–708) and C-terminal (aa 1006–1452) domains were inserted into pGBKT7 as PCR products. An *Eco*RV-*Bam*HI fragment (aa 706–982), containing the CDC2L5 KD, was sub-cloned into pGBKT7. The cyclinL/Ania6 in pGBKT7 and pCDNA3 vectors were gifts from V. Sgambato [Berke et al., 2001], pAS1-Clk2 [Nikolakaki et al., 2002] was a gift from L. Rabinow.

Antibodies

Two polyclonal antibodies were raised against portions of CDC2L5. The first one, anti-peptide, was raised against the C-terminal peptide (CP) sequence with an additional terminal cysteine for coupling purpose: CHIS TSTGRGRGRGLPY, linked to Keyhole Limpet Hemocyanin (KLH). The serum was affinity purified on beads cross-linked with the peptide. Antibodies collected by successive low and high pH solutions were then dialyzed against a phosphate-buffered saline solution (PBS) and stored at −80°C, at a final concentration of 7 mg/ml with 0.02% NaN_3. A second antibody, anti-C-ter, was raised in rabbit against the purified GST-CDC2L5-C-ter protein (aa 1254–1312) produced in bacteria from a pGEX-construct. The serum was pre-treated with GST-Affigel 10 before affinity purification by FPLC on GST-CDC2L5-C-ter bound to Affigel 10 column (Biorad). Antibodies were eluted in conditions described above and stored at 3 mg/ml. The polyclonal p32 antibody was a gift from W. Russel [Matthews and Russell, 1998]. Mouse anti-HA and anti-SC35 and goat anti-Cdk8 antibodies were purchased from Santa Cruz Biotechnology.

Yeast Two-Hybrid Screening

The CDC2L5 bait cDNA, cloned into pGBKT7, in frame with the GAL4 DNA-binding domain was transformed into the yeast strain Y190. The bait strain was mixed, for yeast mating, with a pre-transformed human liver MATCMAKER cDNA library (Clontech) that contained cDNAs fused to GAL4 AD in pACT2. Screening was performed according to the manufacturer instructions. The yeast cells were grown on selective minimal synthetic medium lacking leucine, tryptophan, and histidine, in the presence of 40 mM α-amino-1, 2, 4, triazole. The diploid yeast clones grown on these triple-nutriment-deficient plates were finally subjected to a β-galactosidase assay and the resulting positive blue clones selected for further DNA sequence analysis (Genomexpress).

Recombinant Protein Expression and GST Pull Down

The GST-CDC2L5-C-ter protein was expressed in *E. coli* BL21 transformed with the pGEX-C-ter construct. Bacteria were frozen in liquid nitrogen and cells were sonicated in TBS buffer, (Tris-HCl 50 mM pH 8, NaCl 150 mM) containing EDTA 0.5 mM, EGTA 0.5 mM, DTT 1 mM, protease inhibitors (PMSF 0.5 mM, benzamide 1 mM, leupeptin 4 μM, antipain 1 mM, aprotinin 1 mM, and soybean trypsin inhibitor 1 mg/ml), and Triton X-100 0.1%. Cell debris was removed by centrifugation at 30,000*g* for 15 min. The supernatant was incubated in batch for 30 min at 4°C with glutathione-Sepharose beads, which were previously blocked with milk and rinsed. Beads were then washed with TBS buffer supplemented with Tween 0.1%, Triton 1%. The GST-CDC2L5-C-ter protein was eluted with 10 mM reduced glutathione in 50 mM Tris pH 8. The pGEX-GTH-p32 plasmid (gift from J. Kjems) was transformed in *E. coli* BL21 and the encoded GST-p32 fusion protein was purified on glutathione-Sepharose beads. The GST-CTD of RNA polymerase II was expressed in bacteria and purified according to Peterson et al. [1992].

For GST pull-down experiments, 400 μl of HeLa cell lysate (5 mg) or lysis buffer (Tris-HCl 20 mM pH 8, NaCl 150 mM, 10% glycerol, 1% NP-40, EDTA 5 mM, EGTA 0.5 mM, β-glycerophosphate 20 mM, NaF 50 mM, PMSF 0.5 mM, benzamidine 2 mM, Na_3VO_4 1 mM, and inhibitor cocktail 10% (Sigma), were added to 25 μl of beads coupled with GST-p32 or GST. After incubation at 4°C for 2 h, beads were pelleted and washed four times with 1 ml lysis buffer without protease inhibitors and then with 1 ml Tris 200 mM. Bound proteins were eluted in Laemmli buffer at 95°C for 3 min and subjected to SDS-polyacrylamide gel electrophoresis (PAGE) and immunoblotting.

Cell Culture, Transfection, and Preparation of Lysate

HeLa, HEK 293, U2OS, and NIH3T3 cells were cultured in Dulbecco's MEM (Gibco-Invitrogen) supplemented with 10% fetal bovine serum, 1 mM sodium pyruvate, 100 units/ml penicillin, and 100 μg/ml streptomycin, at 37°C with 5% CO_2. The day before transfection, 6×10^5 cells were seeded per 85-mm dish. Cell transfections were carried out with 10 μg DNA using standard calcium phosphate precipitation procedure. Transfected (48 h post-transfection) or untransfected (80% confluence) cells were washed, harvested with cold PBS, and lysed in 150 μl lysis buffer per plate for 30 min at 4°C. After 20 min of centrifugation at 13,000 rpm (4°C), the supernatants were collected, frozen in liquid nitrogen, and stored at −80°C. Proteins were quantified by Bradford assay.

Immunoprecipitation and Immunoblotting

For immunoprecipitation (IP), HEK or HeLa cell lysate (1 mg proteins) and 3 μg of anti-pept, anti-C-ter, or anti-p32 antibodies were added to 20 μl of protein A-Sepharose beads in a final volume of 200 μl lysis buffer. After incubation at 4°C for 2 h, beads were pelleted and treated as described above for pull-down experiments. Samples in Laemmli buffer were analyzed on 7% SDS–PAGE except otherwise stated. The proteins were transferred to PVDF membrane and the blots were probed with one of the following antibodies, anti-peptide (1:1,000), anti-C-ter (1:1,000), anti-HA (1:1,000), or anti-p32 (1:500) in buffer A: Tris 25 mM pH 7.5, NaCl 200 mM, $CaCl_2$ 1 mM, $MgCl_2$ 5 mM. Bound secondary anti-rabbit or anti-mouse antibodies (1:10,000) coupled with horseradish peroxidase (Sigma) were detected by enhanced chemioluminescence using ECL + reagents (Amersham).

Immunofluorescence

HeLa or U2OS cells were seeded onto coverslips in 30-mm plates and transiently transfected with 1.6 μg plasmid using the protocol described above. Cells were rinsed in PBS 48 h after transfection and fixed for 20 min in 4% paraformaldehyde in PBS at room temperature. Cells were permeabilized in PBS with 0.2% Triton X-100 for 5 min and incubated in PBS-5% goat serum overnight at 4°C. The following primary antibodies, diluted in PBS-BSA 1%, were added for 2 h at RT: anti-HA (1:50), anti-peptide (1:50), or anti-SC35 (1:5,000). Cells were rinsed in PBS and incubated in secondary anti-species antibodies (Sigma) in PBS-BSA for 1 h at RT: Texas red-conjugated anti-mouse antibody 1:500, FITC- or TRITC-conjugated anti-rabbit antibody 1:250. The cells were washed three times and mounted with Mowiol medium (40–88 Aldrich) containing 2% Dabco antifading (Sigma), and observed with a confocal laser-scanning microscope (Olympus).

In Vivo Splicing Assays

For tumor necrosis factor β (TNF β) and E1A splicing assays, NIH 3T3 and HeLa cells, respectively, were transfected in the same conditions as described above. The pCM plasmid containing the murine TNF β gene is described in Neel et al. [1995] and the pXJ41 containing the *E1A* gene (gift of C. Bourgeois) in Bourgeois et al. [1999]. In these experiments, the constructs containing the full-length CDC2L5, its N-terminal or kinase domains or the cyclinL/Ania6 were cotransfected with the TNF β or E1A constructs (5 μg DNA each). After 48 h, RNA were extracted: transfected cells were washed with PBS and scraped in guanidium thiocyanate and RNA was purified by centrifugation over a cesium chloride cushion.

For RNase mapping analysis of TNF β splicing, 4 μg RNA were hybridized overnight at 50°C with 300 pg RNA probe (labeled with [α-^{32}P] UTP to a specific activity of 6×10^7 cpm/μg). This probe overlaps exon 2 to exon 4, including introns 2 and 3 (Fig. 5A). The hydrids were digested with RNases A and T1 and analyzed by electrophoresis on a 5% polyacrylamide, 7 M urea gel. Bands were analyzed and quantified by Phosphorimager (Molecular Dynamics).

Alternative E1A splicing was performed by RT-PCR on 1.5 μg of total RNA as described in Bourgeois et al. [1999], with primers a, 5′-TTTG GACCAGCTGATCGAAG-3′ and d, 5′-AAGCTT GGGCTGCAGGTCGA-3′. Products were resolved on 6% polyacrylamide gels and visualized by ethidium bromide staining. Quantification of digital images was performed using Bioprofil-1D (Vilbert Lourmat).

RESULTS

The Human CDC2L5 Is a 200-kDa Nuclear Protein Accumulated in Speckles

We initially identified the *CDC2L5* gene in sea urchin and further showed that the echinoderm protein expressed in eukaryotic-transfected cells

resides in the nucleus [Marques et al., 2000]. Then we cloned the human orthologue. To characterize the human protein, an anti-peptide antibody directed against the human C-terminal sequence (aa 1436–1452) was produced. An immunoblot analysis on immunoprecipitated HEK 293 cell lysates showed an apparent molecular mass of 200 kDa for endogenous as well as ectopically expressed CDC2L5 (Fig. 1A). CDC2L5 IP was prevented by pre-incubation of the anti-peptide antibodies with competing CP. The CDC2L5 apparent molecular weight differs from the theoretical molecular weight (160 kDa), suggesting potential post-translational modifications of the protein by glycosylation or phosphorylation, or a slower electrophoretic migration due to the proline-rich domain.

To investigate the sub-cellular localization of the human CDC2L5 protein, we performed immunofluorescence analysis with the anti-peptide antibody in HEK 293 and HeLa cells. As its echinoderm counterpart, the human CDC2L5 localized in the nucleus, mainly in the nucleoplasm (Fig. 1B). No staining was observed with antibodies pre-incubated with the antigenic peptide.

CDC2L5 contains nine putative NLS distributed along the full-length sequence, three being bipartite NLS located in the N-terminal domain (Fig. 2A). To determine which region of CDC2L5 is required to target the protein in the nucleus, we generated several different HA-tagged deletion constructs of CDC2L5 (Fig. 2A). The full-length HA-CDC2L5 expressed in HeLa cells exhibited a nucleoplasmic pattern similar to the endogenous protein with some accumulation in discrete loci (Fig. 2B). The HA-tagged N-terminal domain is localized in the nucleus in patches brighter than for the full-length

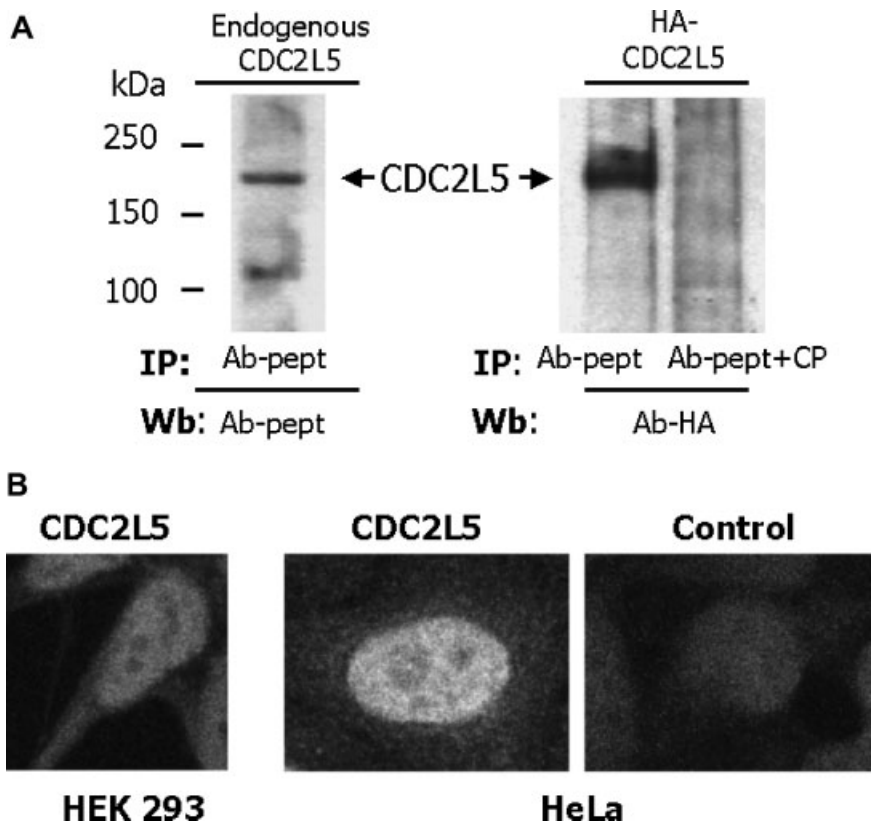

Fig. 1. The human CDC2L5 is a 200 kDa nuclear protein. **A**: HEK 293 cells either untransfected or transfected with pCDNA3-HA-CDC2L5 were harvested and lysed. One milligram of cell lysate was immunoprecipitated with the anti-peptide antibodies (Ab-pept) pre-incubated or not with a competing C-terminal peptide (CP). Immunoprecipitates were immunoblotted with anti-peptide antibodies to label endogenous CDC2L5 or with anti-HA antibodies to stain the HA-tagged overexpressed protein. **B**: Indirect immunofluorescence analysis of endogenous CDC2L5 localization in HEK 293 and HeLa cells. Cells were fixed, permeabilized, and labeled with anti-peptide antibodies previously incubated (control) or not (CDC2L5) with the CP. Labeling was detected using fluoresceine isothiocyanate (FITC)-conjugated secondary antibodies and visualized by confocal laser-scanning microscopy.

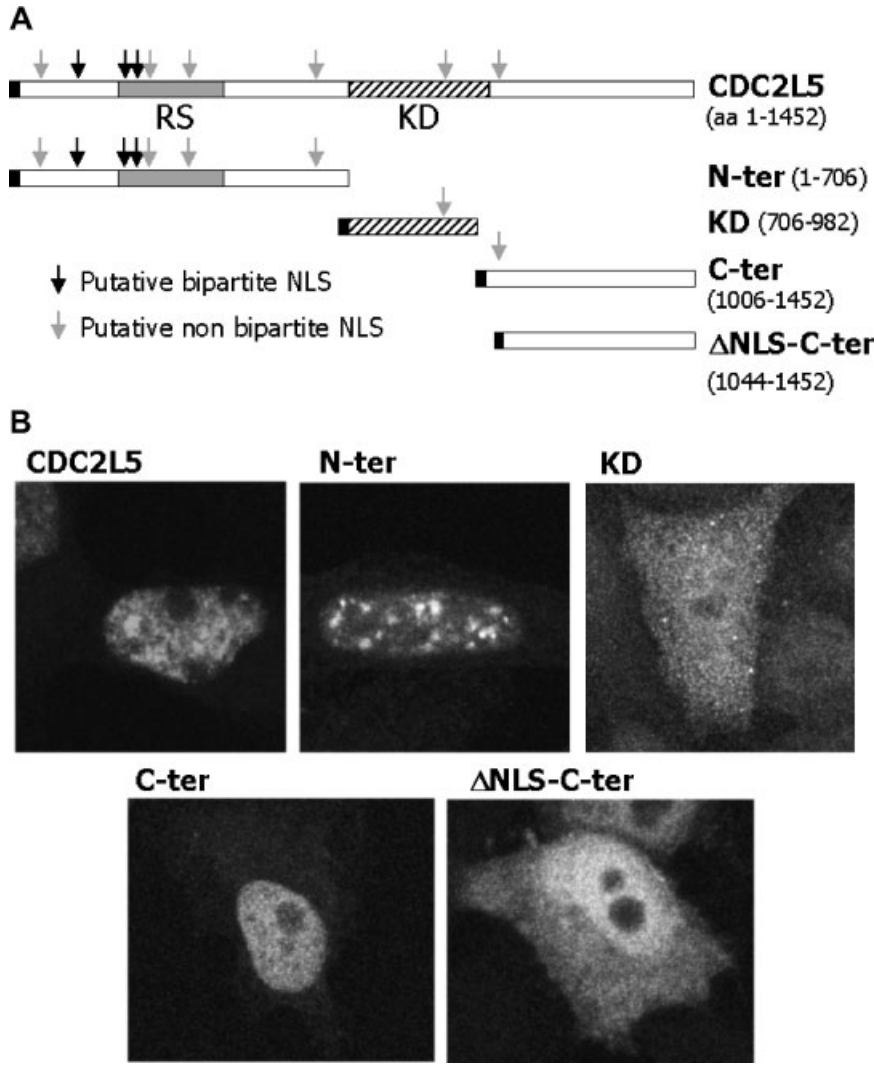

Fig. 2. NLS-dependent nuclear localization of CDC2L5. **A**: Domain structure and potential nuclear localization signals (NLS) of full length and truncated CDC2L5 proteins: RS containing N-terminal domain (N-ter) (aa 1–706), kinase domain (KD) (aa 706–982), C-terminal domain with one putative NLS (C-ter) (aa 1006–1452), and C-terminal without any NLS (ΔNLS-C-ter) (aa 1044–1452). RS, RS domain; KD, kinase domain. Black rectangles correspond to HA tag. **B**: Immunolocalization of full-length and truncated CDC2L5. HeLa cells, transfected with the indicated HA-fusion proteins, were stained with anti-HA antibodies and with FITC-coupled secondary antibody. Images were obtained by confocal microscopy.

protein, whereas the HA-C-terminal domain is homogeneously distributed within the nucleoplasm. In contrast, the KD and the truncated C-terminal domain of CDC2L5 lacking any NLS was found throughout the cell except in the nucleoli. These data suggest that the N-terminal domain and the first 15 amino acids of the C-terminal domain are involved in the nuclear targeting of CDC2L5. Furthermore, CDC2L5 and the N-terminal construct, both containing the RS domain, are assembled in patches in the nucleus. Because RS domains have been shown to drive proteins to nuclear speckles, we speculated that CDC2L5 could be localized in these compartments. Immunofluorescence was performed on CDC2L5-transfected U2OS cells, using the CDC2L5 anti-peptide antibody and an antibody directed against the splicing factor SC35, which specifically stains nuclear speckles. The fluorescence pattern of CDC2L5 was found to partially overlap with SC35 staining (Fig. 3A, upper panel). A similar colocalization of CDC2L5 and SC35 was obtained in GFP-tagged CDC2L5-transfected HeLa cells (Fig. 3A, lower panel). When the GFP-tagged CDC2L5 N-terminal domain was

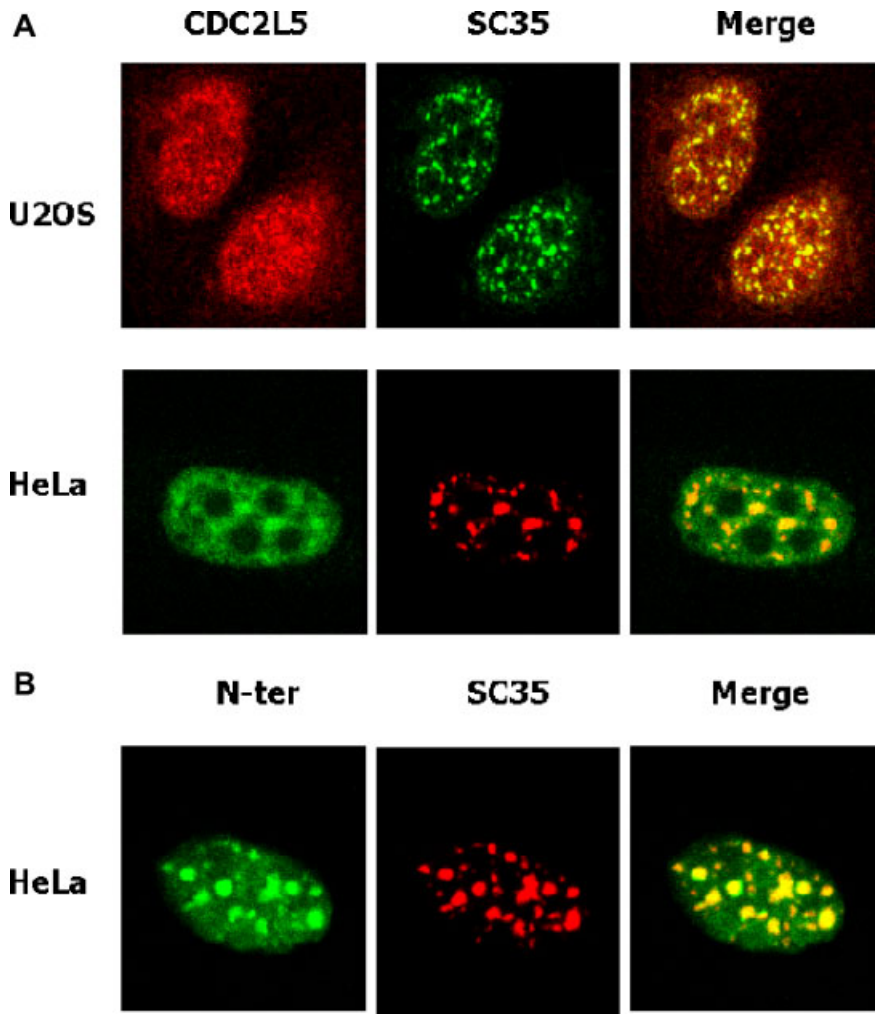

Fig. 3. Ectopically expressed CDC2L5 accumulates in nuclear speckles. **A**: U2OS cells were transfected with pCDNA3-HA-CDC2L5, and localization of the expressed protein (red) and the speckle accumulating protein SC35 (green) were analyzed using rabbit anti-peptide and mouse anti-SC35 antibodies, respectively. HeLa cells transfected with pEGFP-CDC2L5 (green) were labeled with mouse anti-SC35 antibodies (red). **B**: HeLa cells transfected with the GFP-tagged CDC2L5 N-terminal domain (green) were labeled with anti-SC35 antibodies (red). Colocalization resulted in the appearance of yellow in the merged images (A and B). Images were obtained by confocal microscopy.

expressed in HeLa cells, it accumulates in foci coincident with speckles and sometimes extending to their edge (Fig. 3B). The truncated protein containing the N-terminal and kinase domains displays an analogous pattern (data not shown). These results show that CDC2L5 is mainly concentrated in speckles and that the N-terminal domain of CDC2L5 is responsible for this accumulation.

CDC2L5 Interacts with the ASF/SF2-Associated Protein p32

To identify cellular proteins interacting with CDC2L5, we performed a two-hybrid screening by yeast mating of a pre-transformed human liver cDNA library fused to the GAL4 activation domain (GAL4-AD) and the full-length CDC2L5 fused to the GAL4 DNA-binding domain (GAL4-BD) as bait. From a total of 2×10^6 transformants screened, four independent clones fulfilled the criteria for interaction of gene products. Checking the corresponding sequences against the EMBL database revealed that the four clones contain full-length or partial sequences of the gene encoding the splicing factor ASF/SF2-associated protein p32, originally described by Krainer et al. [1990]. The p32 protein is synthesized as a 282 amino acid pre-cursor which is processed by removal of the N-terminal 73 residues to form

the mature p32 protein [Honore et al., 1993]. Every isolated clone contained the full-length sequence of the mature protein and all or only a part of the signal peptide sequence (Fig. 4A). This demonstrates that CDC2L5 can interact with the pre-cursor or mature p32 protein. To delineate the domain(s) of CDC2L5 required for interaction with p32, CDC2L5 truncation constructs containing either the central KD (aa 706–982) or the N-terminal domain (aa 1–706)

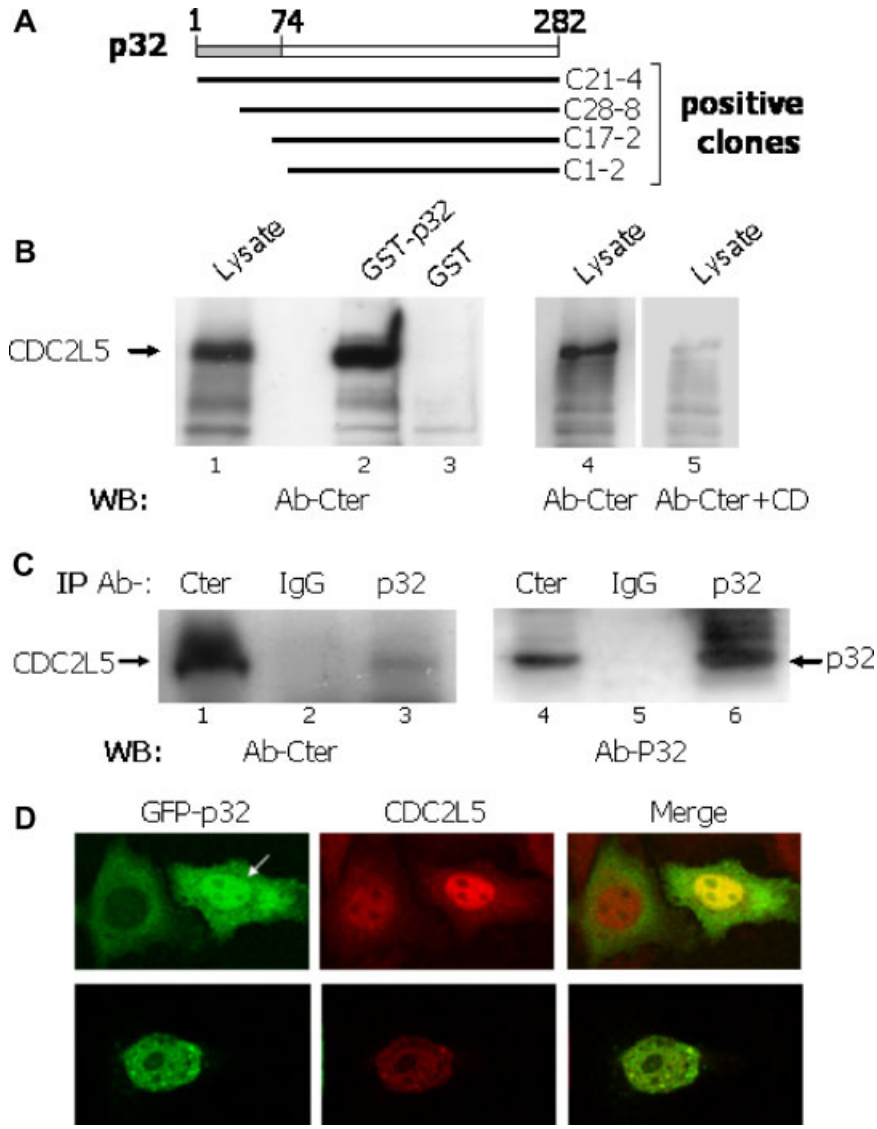

Fig. 4. CDC2L5 directly interacts with the splicing regulator p32. **A**: Schematic representation of the p32 protein sequence and the corresponding positive clones isolated by two-hybrid screening. The grey rectangle represents the p32 signal peptide. **B**: GSH-beads, coated with either GST-p32 or GST, were incubated with lysate of HEK 293 cells transfected with HA-CDC2L5. Total proteins of cell lysate (10 μg, lanes **1**, **4**, and **5**) or lysate proteins retained on GST-p32-beads (lane **2**) and on GST-beads as control (lane **3**) were immunoblotted with anti-C-ter antibodies (1:1,000). The specificity of anti-C-ter antibodies was confirmed by disappearance of the signal obtained with lysate proteins after incubation of the antibodies with GST-CDC2L5-C-ter protein (CD) fixed on GSH-agarose beads (lane 5). **C**: Protein complexes were immunoprecipitated (IP) either with anti-C-ter (lanes 1 and 4) or anti-p32 antibodies (lanes 3 and **6**) from 1 mg of total lysate of HA-CDC2L5-transfected HEK 293 cells. A non-specific antibody (IgG) was used as control for immunoprecipitation (IP) (lanes 2 and 5). Immunoprecipitates were immunoblotted with anti-C-ter antibodies (1:1,000) (**left panel**) or with the anti-p32 antibodies (1:500) (**right panel**). **D**: HeLa cells cotransfected with pEGFP-p32 and pCDNA3-HA-CDC2L5 (**upper panel**) were labeled with anti-HA antibodies, detected by Texas-Red-coupled secondary antibodies. GFP-p32 and HA-CDC2L5 were visualized by confocal microscopy. White arrow indicates a cell expressing a high level of HA-CDC2L5 and displaying a nucleoplasmic GFP-p32 localization. As a control, HeLa cells were transfected with pEGFP-p32 alone (**lower panel**) and labeled with anti-CD2L5 antibodies detected by Texas-Red-coupled secondary antibodies. The p32 protein can be visualized in the nucleus without overexpression of the CDC2L5 protein.

in fusion with GAL4-BD were expressed in yeast together with the GAL4-AD-p32 construct. The C-terminal domain fused to GAL4-BD autonomously transactivated the reporter genes and consequently could not be used as bait. No interaction was found with the KD while the N-terminal domain alone was able to interact with p32 (Table I). This result suggests that CDC2L5 interacts with p32 via its N-terminal domain. Because the CDC2L5 N-terminus contains an RS domain, two RS domain-containing proteins were used as control to determine the specificity of interaction. Neither the rat cyclin L/Ania 6 [Berke et al., 2001], nor the kinase Clk2 [Nikolakaki et al., 2002] associated with p32 (Table I) suggesting that p32 interacts with CDC2L5 via its N-terminal domain in a specific manner. Interaction between the endogenous p32 protein and CDC2L5 was confirmed by GST-pull-down assays. The recombinant GST-p32 protein produced in *E. coli* and bound to GSH-Sepharose beads was incubated with HeLa cell lysates. An immunoblot showed that CDC2L5 is retained on GST-p32-Sepharose beads (Fig. 4B). In vivo interaction in mammalian cells was further confirmed by coIP on HeLa cell lysates. These experiments were conducted with antibodies raised against the entire CDC2L5 C-terminal domain which are more efficient in immunoblotting and IP than the anti-peptide antibody. The p32/CDC2L5 complexes were indeed immunoprecipitated either with anti-CDC2L5 or anti-p32 antibodies as revealed by immunoblot (Fig. 4C). The p32 protein is translated as preprotein from which a mitochondrial import signal is cleaved off to create the mature protein. The majority of p32 is consequently found in the mitochondria [Muta et al., 1997; Dedio et al., 1998]. However, in a small percentage of cells, p32 could also be demonstrated in a nucleoplasmic localization, indicative of a traffic of mature p32 from cytoplasm to nucleus [Matthews and Russell, 1998; Brokstad et al., 2001]. As CDC2L5 is a nuclear protein, it could thus interact with the mature protein. Indeed, an immunofluorescence assay in HeLa cells demonstrated that, when nuclear, the mature p32 protein, expressed in fusion with GFP, colocalized with CDC2L5 (Fig. 4D). These data are in agreement with a p32/CDC2L5 interaction in vivo.

TABLE I. CDC2L5 Interacts with p32 Through its N-Terminal Domain

	Interaction with p32
CDC2L5	+
N-ter	+
KD	−
C-ter	nd
CyclinL/ania6	−
Clk2	−

This table summarizes the protein interactions with p32 as observed in directed two-hybrid assay. +, protein interaction with p32; −, no interaction. Interaction with the CDC2L5 C-terminal domain could not be determined (nd) due to the transactivating property of the expressed protein.

Overexpression of CDC2L5 Constructs Affects Constitutive and Alternative Pre-mRNA Splicing In Vivo

Considering that CDC2L5 contains an RS domain characteristic of proteins involved in pre-mRNA splicing that this kinase partially colocalizes with speckles, the storage sites of splicing factors, and that the protein is able to form a complex with the ASF/SF2-associated protein p32, a splicing regulator, we investigated if CDC2L5 could be involved in pre-mRNA processing. To explore a potential CDC2L5 function in constitutive pre-mRNA splicing, in vivo splicing assays were performed using the lymphotoxin α/TNF β gene which contains three successively spliced introns [Weil et al., 1990]. The TNF β gene was transiently cotransfected in NIH3T3 cells with the pCDNA3 vector either empty or containing the full-length CDC2L5, or its N-terminal domain or its KD. Since the cyclinL/Ania6 protein, which associates with $Cdk11^{p110}$, was shown to stimulate splicing in vitro [Dickinson et al., 2002], we initially used it as a positive control. The resulting TNF β splicing products were analyzed by RNase mapping using a probe overlapping introns 2 and 3, as shown in Figure 5A [Neel et al., 1995]. The CDC2L5 N-terminal domain dramatically inhibited TNF β splicing while the KD had no effect (Fig. 5A and Table II). Furthermore, a truncated construct containing the N-terminal and KDs inhibited TNF β splicing to the same extent as the N-terminal domain alone (data not shown). Unexpectedly, the full-length CDC2L5 protein had no effect on TNF β splicing, which is probably due to the low expression of the exogenous CDC2L5 protein. On transfection, the number of cells expressing CDC2L5 detectable levels (<2%) is by far smaller than that of the N-terminal domain expressing cells (10%) as seen by immunofluorescence (data not

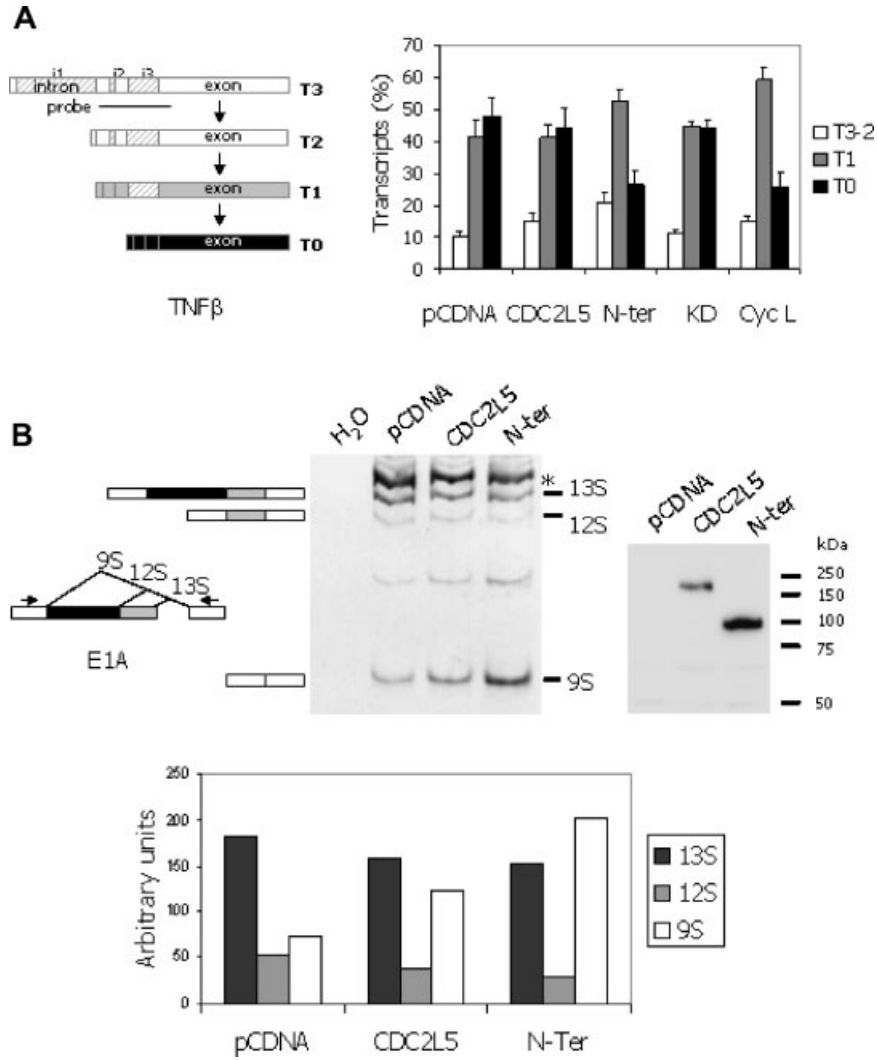

Fig. 5. CDC2L5 constructs inhibit constitutive splicing of TNF β and regulate alternative splicing of E1A pre-mRNA in vivo. Splicing analyses were performed with NIH3T3 (**A**) and HeLa (**B**) cells. A: For constitutive splicing, cells were transiently cotransfected with a plasmid encoding the entire TNF β gene and either the empty pCDNA3 vector or the plasmid containing the HA-tagged full-length CDC2L5, its N-terminal (N-ter) or kinase (KD) domain or cyclinL (Cyc L) as a positive control. As schematically shown, the three lymphotoxin introns are successively spliced: intron 1 (i1) is first removed, then intron 2 (i2), and finally intron 3 (i3). The different transcripts generated during splicing have been analyzed by the RNase mapping method, using a complementary probe shown in the diagram. This probe, as detailed in Materials and Methods, allows detection of transcripts containing 3 or 2 introns (T3–T2), those with only 1 intron (T1), and those completely spliced without any intron (T0). Bands were quantified by Phosphoimager and the percentage of the different splicing products was calculated for each electrophoresis lane. The results, expressed in percentage of transcripts, are the mean of at least three observations. The data obtained for the independent experiments are provided in Table II. B: The effect of overexpression of CDC2L5 or of its N-terminal domain was examined on alternative splicing of the *E1A* gene. As schematically shown, the E1A pre-mRNA contains three main alternative 5′ splice sites giving rise to three spliced products: 9, 12, and 13S. Spliced products were amplified by RT-PCR of mRNA from HeLa cells cotransfected with the adenovirus E1A plasmid and either the empty pCDNA3 vector or the plasmids containing the full-length CDC2L5 or its N-terminal domain. RT-PCR products were separated by polyacrylamide gel electrophoresis (PAGE) and visualized by ethydium bromide staining. The asterisk indicates amplified E1A DNA. The data are representative of three independent experiments. Quantitative digital image analysis was performed after electrophoresis. A Western blot was introduced in order to evaluate the respective amounts of proteins expressed in HeLa cells after CDC2L5 or N-ter transfections. Twenty micrograms of total proteins were loaded per lane on a 10% SDS–PAGE and the Western blot was probed with anti-HA antibodies.

TABLE II. CDC2L5 Constructs Inhibit Constitutive Splicing of TNF β

% of transcripts	T23	T1	T0
PCDNA	9	42	49
	11	36	53
	10	44	46
CDC2L5	12	41	46
	12	36	52
	17	44	39
N-ter	22	48	30
	21	51	29
	19	56	24
KD	10	45	44
	10	44	46
	12	46	42
Cyc L	16	62	22
	14	57	29
	16	59	25

NIH3T3 cells were transiently cotransfected with a TNF β reporter plasmid and either the empty pCDNA3 vector or the plasmid containing the HA-tagged full-length CDC2L5, its N-terminal (N-ter) or kinase (KD) domain or cyclinL (Cyc L) as a positive control. The resulting TNF β splicing products were analyzed by RNase mapping as detailed in legend of Figure 5A and Materials and Methods. Bands were quantified by Phosphorimager and results were expressed in percentage of transcripts, the data corresponding to three independent experiments are reported below and in Figure 5A.

shown). Although both mRNA are expressed at similar levels, an immunoblot confirmed that the full-length protein was produced at a lower level than the truncated form (Fig. 5B). The numerous PEST sequences located in the CDC2L5 C-terminus possibly reduce the stability of the full-length protein. We presume that the lower expression level of CDC2L5 obscures possible effects on TNF β splicing, but we cannot exclude that CDC2L5 has no effect on the splicing of the TNF β gene in vivo. Interestingly, cyclinL/Ania6, which has been shown to stimulate constitutive β-globin splicing in vitro [Dickinson et al., 2002], has an inhibitory effect on TNF β splicing in this in vivo assay. ASF/SF2 also shows such paradoxical behavior, as it functions as an activator of HIV tat pre-mRNA in an in vitro assay while the gene depletion stimulates tat RNA splicing in vivo [Wang et al., 1998].

As SR-proteins participate in the regulation of alternative as well as constitutive splicing, we subsequently investigated the potential effect of CDC2L5 on alternative splicing in vivo, using the adenovirus *E1A* gene. The adenovirus E1A pre-mRNA is spliced into three predominant mRNA variants termed 13S, 12S, and 9S mRNAs, through the use of three alternative 5′ splice sites and a single 3′ splice site [Bourgeois et al., 1999]. HeLa cells were cotransfected with the *E1A* gene and either the full-length CDC2L5 or the N-terminal domain. Spliced products were then analyzed by RT-PCR. The overexpression of the CDC2L5 full-length protein or its N-terminal domain increased the use of the most distal 5′ splice site, which gives rise to the 9S isoform (Fig. 5B). However, full-length CDC2L5 had a smaller effect than its N-terminal domain, probably due to the weaker overexpression as previously mentioned. These results demonstrate that CDC2L5 affects alternative splicing events in vivo, possibly through protein–protein interactions involving the CDC2L5 N-terminal domain.

DISCUSSION

Proteins containing a domain rich in alternating arginine and serine dipeptides (RS domain) are mainly involved in pre-mRNA splicing. CDC2L5 and CrkRS are the only kinases of the Cdk type that contain such an RS domain [Marques et al., 2000; Ko et al., 2001]. This structural feature led us to consider the potential role of CDC2L5 in the control of mRNA processing. Here, we demonstrate that firstly, CDC2L5 is a 200 kDa nuclear protein with a higher concentration in speckles, the storage sites for splicing components, and secondly, CDC2L5 physically interacts with p32, a regulating partner of the ASF/SF2 splicing factor. In agreement with these results that argue for an involvement of CDC2L5 in splicing, the overexpression of CDC2L5 constructs containing the RS domain disturbs constitutive as well as alternative splicing in vivo. These data strongly suggest that the regulation of pre-mRNA splicing is one of the cellular functions of CDC2L5.

We first showed that human CDC2L5 is a 200 kDa protein expressed in various cell lines, a result in agreement with the ubiquitous expression of CDC2L5 mRNA in human tissues [Marques et al., 2000]. The endogenous protein is present throughout the nucleoplasm with a particular enrichment in discrete clusters. The N-terminal domain and a 15 aa sequence at the beginning of the C-terminal domain, both containing putative NLS, are critical for nuclear localization of CDC2L5.

Most RS domain-containing proteins accumulate in nuclear speckles. Here, we found that the HA- or GFP-tagged full-length CDC2L5, which was distributed in the whole nucleoplasm

of U2OS and HeLa cells, was further enriched in compartments where SC35 was visualized. In addition, the CDC2L5 N-terminus with or without the KD accumulates in SC35-stained loci. These results suggest that CDC2L5, like other splicing factors, can shuttle between nucleoplasm and speckles and that the CDC2L5 N-terminus, containing the RS motif, is critical for localization of this kinase in nuclear speckles. The CDC2L5 nuclear distribution is reminiscent of the localization of another kinase of the CDK type, $CDK11^{p110}$ and of proteins of the Clk family. $CDK11^{p110}$ localizes to both splicing factor compartments and to the nucleoplasm and data collected over a decade demonstrate its role in transcription and pre-mRNA splicing [Loyer et al., 1998]. As CDK11, CDC2L5 might also play several functions in the regulation of gene expression. The Clks are only distantly related to the Cdk family despite their name of Cdc2-like kinases, [Guo and Stiller, 2004]. However, they contain, like CDC2L5, an RS domain at the N-terminus of their KDs. When overexpressed, wild-type Clks distribute throughout the nucleus, whereas catalytically inactive mutants, unable to autophosphorylate on their RS motifs, are located in nuclear speckles [Colwill et al., 1996; Nayler et al., 1998]. This emphasizes the role of the RS motif and its phosphorylation status in Clk localization. In a similar way, the state of phosphorylation of the CDC2L5 RS domain could also play a role in the kinase localization.

The presence of CDC2L5 in nuclear speckles suggests that this protein could associate with splicing components. Using yeast two-hybrid assay, coIP, GST-pull down, and cellular colocalization experiments, we showed that CDC2L5 interacts with the ASF/SF2-associated protein p32, the CDC2L5 N-terminal domain being sufficient to support this interaction. The p32 protein was originally found to be tightly associated and copurified with ASF/SF2 [Krainer et al., 1991]. It was later shown to interact with non-splicing-related proteins, thereby regulating their intracellular localization [van Leeuwen and O'Hare, 2001 and references therein]. However, the role of p32 in splicing regulation is better documented. The p32 protein hampers the action of ASF/SF2 as a splicing enhancer/repressor by preventing stable ASF/SF2 interaction with RNA targets and by inhibiting its phosphorylation [Petersen-Mahrt et al., 1999]. Moreover, p32 is an essential splicing inhibitor for HIV replication in human [Zheng et al., 2003]. Therefore, the interaction between the SR-related protein CDC2L5 and the ASF/SF2 cofactor p32, in addition to the speckled localization of this kinase, favor the involvement of CDC2L5 in splicing, possibly through ASF/SF2 regulation. However, this does not exclude additional CDC2L5 functions such as in transcriptional elongation or in other key pre-mRNA processing reactions.

Because ASF/SF2, as other SR proteins, is known to regulate constitutive as well as alternative splicing, we assessed the involvement of CDC2L5 both in constitutive and alternative splicing events using in vivo splicing assays. The full-length CDC2L5 had no effect on TNF β pre-mRNA processing. As stated in the results section, the low expression of the transfected full-length CDC2L5, likely due to protein instability, may account for the absence of effect on splicing. However, the CDC2L5 expression level is sufficient to promote a small increase in the 9S product of *E1A* gene by alternative splicing. In contrast, the CDC2L5 N-terminal domain, containing the RS protein–protein recognition motif, decreases constitutive splicing of TNF β and promotes a larger increase in E1A 9S mRNAs, whereas the CDC2L5 KD alone has no effect. Unexpectedly, CDC2L5 constructs disturb pre-mRNA processing in a KD independent manner. Similarly, splicing inhibition, independent of a functional catalytic domain, was also reported for the protein kinase MELK in an in vitro assay [Vulsteke et al., 2004]. There are several potential explanations for the behavior of CDC2L5. One is that the splicing inhibition is independent of phosphorylation by CDC2L5 in these particular assays. Indeed, the relevant kinase substrate or a downstream splicing factor might be absent from host cells, or the reporter transcripts we used may not be natural targets regulated by CDC2L5. Thus, a systematic splicing disturbance would be observed, due to interaction between CDC2L5 RS domain and SR proteins. Alternatively, the full-length CDC2L5 (or the truncated form containing the KD) could lead to a hyperphosphorylation of SR proteins, either directly or through a kinase cascade, while the CDC2L5 N-terminal domain, acting by a dominant negative mechanism, could prevent the phosphorylation of natural CDC2L5 substrates thereby promoting a hypo-

phosphorylation of SR proteins. Prasad et al. [1999] demonstrated, in particular using Clk/Sty derivatives that both under and overphosphorylation of SR proteins repress constitutive splicing and switch alternative splice site selection. As we showed for CDC2L5, Clk1, 2, and 3 [Duncan et al., 1997, 1998] reduced E1A 13S 5′ splice site selection and enhanced the far upstream 9S 5′ splice site. Moreover, phosphorylation of the RS domain, not only directly affects the activity of splicing factors but also induces their sub-cellular redistribution [Colwill et al., 1996], modifying their accessibility to the spliceosome. CDC2L5 kinase activity might also modulate splicing activity through localization of downstream targets.

CDC2L5 kinase substrates remain unknown thus far. CDC2L5 neither phosphorylates the C-terminal domain of RNA polymerase II (CTD) nor the classical substrates of cell-cycle-related CDKs, H1 histone, and Rb (data not shown). As CDC2L5 was shown to physically interact with the splicing regulator p32, we wondered if p32 or the associated splicing factor ASF/SF2 would be CDC2L5 substrates. Neither p32 nor ASF/SF2 was directly phosphorylated by CDC2L5 in vitro (data not shown). Several kinases have been shown to phosphorylate ASF/SF2 on RS motifs [reviewed in Soret and Tazi, 2003], such as the Clk/Sty and the SRPK family kinases and the topoisomerase I. The activity, localization, or substrate affinity of these kinases, that is, the Clk proteins, is in turn regulated by phosphorylation [Duncan et al., 1997]. These data lead the authors to propose that Clk kinase activity may be controlled by signal transduction pathways, which would in turn regulate pre-messenger RNA splicing [Duncan et al., 1998]. Recent studies have highlighted examples where transducing signals can differentially affect alternative splicing of specific genes through the regulation of specific SR protein phosphorylation status [Shin and Manley, 2004]. CDC2L5 might belong to such a phosphorylation cascade. Our preliminary results, showing that CDC2L5 could phosphorylate in vitro the C-terminal domain of Clk2 (data not shown), argue in favor of CDC2L5 being part of a kinase cascade. Several data are in agreement with this hypothesis: CDC2L5-directed antisense oligonucleotides were previously shown to inhibit megakaryocyte differentiation of mouse bone marrow cells [Lapidot-Lifson et al., 1992] suggesting that CDC2L5 could participate in cell-line induction in response to transducing signals during hematopoiesis. Moreover, a significant induction of transcripts encoding a putative CDC2L5 homologue has been observed in mosquitoes *A. aegypti* after bacterial infection, a process that triggers activation of intracellular transducing signals [Chiou et al., 1998].

In conclusion, we believe that CDC2L5 could be activated by a signal transduction pathway to regulate splicing activity in a time and/or tissue-specific dependent way. However, more investigation, such as in vitro splicing assays, are needed to determine the precise role of CDC2L5 in splicing and the mechanisms involved in this regulation.

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