ZNF265—a novel spliceosomal protein able to induce alternative splicing

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The formation of the active spliceosome, its recruitment to active areas of transcription, and its role in pre-mRNA splicing depends on the association of a number of multifunctional serine/arginine-rich (SR) proteins. ZNF265 is an arginine-serine-rich (RS) domain containing zinc finger protein with conserved pre-mRNA splicing protein motifs. Here we show that ZNF265 immunoprecipitates from splicing extracts in association with mRNA, and that it is able to alter splicing patterns of Tra2-β1 transcripts in a dose-dependent manner in HEK 293 cells. Yeast two-hybrid analysis and immunoprecipitation indicated interaction of ZNF265 with the essential splicing factor proteins U1-70K and U2AF35. Confocal microscopy demonstrated colocalization of ZNF265 with the motor neuron gene product SMN, the snRNP protein U1-70K, the SR protein SC35, and with the transcriptosomal components p300 and YY1. Transfection of HT-1080 cells with ZNF265-EGFP fusion constructs showed that nuclear localization of ZNF265 required the RS domain. Alignment with other RS domain-containing proteins revealed a high degree of SR dipeptide conservation. These data show that ZNF265 functions as a novel component of the mRNA processing machinery.

Introduction

Gene transcription and pre-mRNA splicing are dynamic and highly coordinated processes that occur in a spatially organized manner in the nucleus (Singer and Green, 1997). Splicing takes place in the spliceosome, a large RNA-protein complex composed of various small nuclear ribonucleoprotein particles (snRNPs),* and many other protein factors that include members of the highly conserved serine/arginine-rich (SR) protein family. SR proteins, by RNA-protein and protein-protein interactions, coordinate the passage of the spliceosomal complex though the splicing reaction (reviewed in Fu, 1995; Manley and Tacke, 1996; Cáceres et al., 1997). SR protein recruitment to active areas of transcription and RNA processing involves their signature arginine-serine-rich (RS) domains and an interaction with RNA polymerase II through its COOH-terminal domain (Yuryev et al., 1996; Du and Warren, 1997; Kim et al., 1997; Misteli and Spector, 1999).

RS domains mediate protein–protein interactions with other general splicing factors during the formation of the spliceosome. By yeast two-hybrid for example, interactions of the SR proteins SC35 and SF2/ASF with both U1-70K and U2AF35 have been documented, the latter two proteins functionally binding to the 5′ and 3′ splice sites, respectively, in early splicing complexes (Wu and Maniatis, 1993; Cao and García-Blanco, 1998). Binding of SR proteins to exonic splicing enhancers generally stimulates splicing (Sun et al., 1993; Dirksen et al., 1994; Liu et al., 1998, 2000; reviewed in Blencowe, 2000), but antagonism of splice site recognition has also been observed (Labourier et al., 1999; Barnard and Patton, 2000). Many of the functions of SR proteins are facilitated by a meshwork of interacting factors that promote the passage of the splicing reaction and participate in postsplicing processes such as mRNA transport, which appears to be coupled to splicing (Cáceres et al., 1998; Belsham et al., 2000).

ZNF265 (formally termed “Zis”) is a zinc finger– and RS domain–containing protein (Karginova et al., 1997; Adams et al., 2000) that was first identified, along with renin, because of its modulated expression in differentiating renal...
juxtaglomerular cells (Karginova et al., 1997); it is now known to be expressed by most tissues, especially early in development (Adams et al., 2000). We have also found that the nuclear magnetic resonance solution structure of the zinc fingers accords with RNA binding (Plambeck, C.A., D.J. Adams., L. van der Weyden., J.P. Mackay, and B.J. Morris.

**Results and discussion**

Using a polyclonal ZNF265 antibody (Fig. 1 A) and antibodies directed against specific components of the spliceosome, we observed nuclear colocalization of ZNF265 with the survival of motor neuron (SMN) protein, the authentic SR protein SC35 (at the periphery of the SC35-staining aggregates), and the snRNP protein U1-70K, but none with the common snRNP protein antigen Sm (Fig. 1 B). As expected, SMN showed some cytoplasmic localization (Pagliardini et al., 2000), but this did not overlap with the trace amount of cytoplasmic ZNF265 localization (Fig. 1 B). ZNF265 also colocalized with the transcription factors YY1 and p300 (Fig. 1 B), both of which have been shown to colocalize within active transcriptional compartments and, in the case of p300, with RNA polymerase II (Bannister and Kouzarides, 1996; Ogryzko et al., 1996; Yang et al., 1996; von Miekisz et al., 2000). These colocalizations are consistent with a role for ZNF265 in transcription and/or splicing. In this regard, ZNF265 may be cotranscriptionally recruited with RNA polymerase II to pre-mRNA transcripts, as has been reported for other RS domain–containing proteins (Corden and Patturajan, 1997).

To determine the region of ZNF265 necessary for its nuclear localization, cDNA expression plasmids were generated from which specific domains were deleted. Compared with the nuclear localization of the wild-type ZNF265 fusion protein (C2-ZNF265), fusions containing the zinc finger with (C2-Mut3) or without (C2-Mut2) the putative nuclear localization signal (NLS) showed a predominantly cytoplasmic distribution (Fig. 2). In contrast, nuclear localization was preserved when the RS domain was retained, either with (C2-Mut4) or without (C2-Mut5) the NLS. Consistent with this observation, nuclear localization was not affected by mutation of the NLS (C2-Mut6). Thus, nuclear localization is dictated by the RS domain of ZNF265, consistent with the behavior of other RS proteins such as SC35 (Hedley et al., 1995), SF2/ASF, SRp20, and 9G8 (Cáceres et al., 1997, 1998).

To test whether ZNF265 could interact with other RS domain–containing proteins we conducted a yeast two-hybrid screen against representative spliceosomal proteins that included many with RS domains, namely U1-70K, U2AF35, U2AF65, SC35, p80 Colin, WT1, 9G8, SF2/ASF, SRp20, SRp30c, and SRp40. Interaction was seen with U1-70K and U2AF35, as determined by growth on SD-L-W-A-H plates, and the production of a blue precipitate on a β-gal filter assay (Fig. 3 A). Interaction of ZNF265 with U1-70K and U2AF35 was confirmed by coimmunoprecipitation (Fig. 3 B). Liquid β-gal assay, which provides a semiquantitative estimation of interaction strength, showed that ZNF265 interacted more strongly with U1-70K than with U2AF65 (Fig. 3 C). A U1-70K cDNA clone was also isolated in a yeast two-hybrid screen against a human fetal brain cDNA library using ZNF265 as "baits." Analysis of this clone revealed that residues 180–437 of U1-70K were responsible for mediating the interaction of U1-70K with the RS domain of ZNF265 (unpublished data). It is notable that this region contains the residues necessary for the binding of SF2/ASF to U1-70K (Cao and García-Blanco, 1998). Several cDNA clones for the SR protein kinase Clk1 were also isolated from this screen. Because ZNF265 contains the Clk1 consensus phosphorylation site R/KXR/KXR/KXSXR (Colwill et al., 1996; Moselein et al., 1999), there may be a role for phosphorylation in the regulation of ZNF265.

The fact that ZNF265 interacts with U1-70K and U2AF35 points to its early commitment to the spliceosome, as the latter factors are necessary for the first detectable association between splice sites during formation of the E complex (Michaud and Reed, 1993; Wu and Maniatis, 1993; Xiao and Manley, 1998). Based on the composition of affinity-purified E complex, this association has been proposed to occur through direct or indirect interaction of U1snRNP and U2AF35 bound to the 5' and 3' splice sites, respectively (Michaud and Reed, 1993). One model has suggested that SF2/ASF or SC35 was subsequently able to form a bridge between U1-70K and U2AF35 (Wu and Maniatis, 1993). In contrast to the interaction of SF2/ASF and SC35 with U1-70K and U2AF35 (Wu and Maniatis, 1993), our finding that ZNF265 interacts more strongly with U1-70K than with U2AF35 (Fig. 3 C) suggests earlier binding of ZNF265 to U1-70K, as opposed to U2AF35, during recruitment to the spliceosome.

Alignment of the RS domain of ZNF265 with that of other RS domain–containing spliceosomal proteins (Fig. 4) showed strong SR dipeptide conservation; this was particularly evident between ZNF265, SC35, and SRp40. The aligned region of SC35 contains the putative RS domain NLS, RRRRRRSRRSRSSRSTRYRSTRSKRSR-TRRSSRTSKSRS (Hedley et al., 1995).

The specific recognition of splice sites within pre-mRNA precursors has been proposed as a control point for splicing. RS domain proteins could play a major role in such regulation, as they are required for the early recognition of splice sites during spliceosome assembly. Thus, the structural features of ZNF265, its nuclear localization, and its association with U1-70K and U2AF35 prompted us to investigate a functional role for this protein in pre-mRNA splicing.

In vitro splicing reactions showed that ZNF265 is immunoprecipitated in a complex that includes spliced mRNA (Fig. 5 A). This result indicates that ZNF265 binds directly or indirectly to mRNA, but much less to pre-mRNA. This property is shared with other splicing factors, such as SF2/ASF and RNPS1 (Hanamura et al., 1998; Mayeda et al., 1999), both of which synergistically stimulate general splicing. Here we show in splicing assays in cultured cells that ZNF265 can regulate alternative splicing in a concentration-dependent manner (Fig. 5 B). Namely, overexpression of ZNF265 resulted in exclusion of exons 2 and 3 from the
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Tra2-β1 pre-mRNA, which led to an increase in the production of the β3 alternatively spliced isoform. Our in vivo splicing result suggests that ZNF265 may have the ability to antagonize the alternative splicing activity of SR proteins on Tra2-β1 pre-mRNA. Splicing factor SR protein-mediated antagonism of alternative 5′ splice site selection has been reported for human hnRNP A1 protein in that hnRNP A1 causes activation of distal alternative 5′ splice site and exon exclusion in vitro and in vivo (Mayeda and Krainer, 1992; Mayeda et al., 1993; Cáceres et al., 1994; Yang et al., 1994). In contrast to hnRNP A1 that does not cause inhibition of general constitutive splicing, we have shown that addition of recombinant ZNF265 to SR protein-deficient HeLa cell S100 extracts supplemented with recombinant SF2/ASF may antagonize constitutive splicing of a β-globin pre-mRNA substrate and repress its splicing (our unpublished data). In Drosophila, RSF1 protein antagonizes and represses splicing by binding to SF2/ASF and preventing it from interacting with U1-70K (Labourier et al., 1999). It is possible that ZNF265 may also interfere with SF2/ASF-mediated constitutive splicing by binding directly to U1-70K.

The suggestion that ZNF265 binds directly to mRNA is supported by the association of the zinc finger region of the Xenopus homologue C4SR with cyclin 1B mRNA (Ladomery et al., 2000). Furthermore, our nuclear magnetic resonance studies of the first zinc finger of ZNF265 indicate a structure capable of binding zinc ions, which in turn induce conformational changes in the finger to expose RNA binding side chains (Plambeck, C.A., D.J. Adams, L. van der Weyden, J.P. Mackay, and B.J. Morris. 22nd Ann. Conf. Org. Express. Genome. 2001. Abstr. 2–28).

In conclusion, we have shown that ZNF265 colocalizes with the spliceosome, associates with mRNA and essential splicing factors U1-70K and U2AF35, and can regulate alternative splicing of the Tra2-β1 pre-mRNA. Therefore, ZNF265 is a functional component of the RNA processing machinery.
Figure 2. **Role of the RS domain of ZNF265 in nuclear localization.** (Left) EGFP fusion protein constructs used for the expression of ZNF265. Wild-type ZNF265 sequence (1st row) and 5 mutant sequences (2nd–6th row) were used. (Right) EGFP fluorescence (green) and DAPI (blue) detection in HT-1080 cells at 48 h posttransfection. Bar, 10 μm.

Figure 3. **Interaction of ZNF265 with the essential spliceosomal factors U1-70K and U2AF35.** (A) Activation-domain plasmids (pACT) p80 coilin (1), 9G8 (2), SC35 (3), SRp20 (4), SRp30c (5), SRp40 (6), U1-70K (7), U2AF35 (8), U2AF35 (9), WT1 (10), ASF/SF2 (11), and negative control SNF4 (12), were transformed into AH109 yeast containing the pGBK-ZNF265 binding-domain plasmid, cultured on SD-L-W-A-H plates, and transferred to filters. The ability of the yeast containing pGBK-ZNF265 and either pACT-U1-70K or pACT-U2AF35 to grow on autotrophic media (I: brown) and produce β-gal (II: blue) was observed. The inability of yeast containing pACT plasmids alone to produce β-gal (III) was shown as a control. (B) Results of coimmunoprecipitation performed using anti-ZNF265 to pulldown U1-70K and U2AF35 in association with ZNF265 from HeLa cell nuclear extracts. Immunoprecipitates were analyzed by Western blotting using antibodies against U1-70K or U2AF35 (arrow points to band of predicted size). (C) Relative strength of interaction of ZNF265 with U2AF35 or U1-70K, shown as β-gal activity relative to that for interaction of T-antigen with p53 (mean ± SE, n = 4, *P < 0.0001). (Control) β-galactosidase activity of AH109 yeast containing pGBK-ZNF265 alone.
Materials and methods

Cell culture

Cell lines were obtained from The American Type Culture Collection (ATCC). Calu-6 cells (ATCC HTB-56) were cultured at 37°C, 5% CO₂ in MEM (GIBCO BRL) as described previously (van der Weyden et al., 2000). HT-1080 human fibrosarcoma cells (ATCC CCL 121), HeLa cervical carcinoma cells (ATCC CCL 2), HepG2 hepatocellular carcinoma cells (ATCC HB 8065), and HEK293 (ATCC CRL 1573) were maintained at 37°C, 5% CO₂ in DME (GIBCO BRL) supplemented with 10% FCS (GIBCO BRL), penicillin/streptomycin (5,000 U/ml; GIBCO BRL).

Antibodies

ZNF265 polyclonal antibodies (produced for us by Alpha Diagnostics) were generated by inoculating New Zealand white rabbits with a keyhole limpet hemocyanin–tagged peptide (CEDEDLSKYKLDEDED) correspond-

Figure 4. Conservation of serine and arginine residues in ZNF265 and other RS domain proteins. Alignment of the RS domain of ZNF265 (NP_005446) with RS domains of the spliceosomal proteins SF2/ASF (NP_008855), SC35 (A42634), SRp20 (NP_003008), SRp40 (S59042), SRp55 (S59043), SRp75 (A48133), U1-70K (A25707), U2AF35 (Q01081), U2AF65 (NP_009210), 9G8 (A57198), and p54 (XP_001835). Sequence alignment was performed using “Pileup” and “Prettybox” (Australian Genomic Information Service). Residues conserved in the majority of the aligned proteins are shaded. Numbers indicate the amino acid position of each protein.

Figure 5. Function of ZNF265 in the spliceosome. (A) In vitro splicing reactions were performed using labeled β-globin pre-mRNA and immunoprecipitated with the indicated antibodies, pre-immune serum, anti-SF2/ASF/anti-hnRNP A1, and increasing amounts of anti-ZNF265 (shown by triangle), immobilized on protein G-Sepharose. The immunoprecipitated complexes were washed extensively and RNA was extracted and analyzed by denaturing PAGE followed by autoradiography. 1/20 of total RNA recovered from the supernatant of an immunoprecipitation with control preimmune serum reflects the initial relative abundance of predicted pre-mRNA, intermediates and products, which are schematically depicted on left hand side. (B) Ability of ZNF265 to stimulate exon exclusion of alternatively spliced Tra2 pre-mRNA. At top is schematic diagram of the Tra2--pre-gene construct and splice products (introns: A, B, C; exons: 1, 2, 3, 4). HEK 293 cells were transfected with 3 μg of total plasmid DNA and, as indicated below abscissa, an increasing proportion of the expression plasmid C2-ZNF265. Representative ethidium bromide stained gel is shown, with schematic diagram of β1, β3, and β4 isoforms detected by this assay depicted on the right. (M) 100-bp marker. Relative abundance of the β4, β1, and β3 isoforms from each lane (mean ± SD) from three experiments is shown in the panels.
ing to amino acids 160–174 of ZNF265 (GenBank/EMBL/DDJB database accession no. NP005446). Antiserum was affinity purified by column chromatography using antigen peptide immobilized on Sepharose-4B beads. Monoclonal SC35, p300, and YY1 antibodies were from PharMingen, Calbiochem, and Santa Cruz Biotechnology, Inc., respectively. Monoclonal SMN antibody (clone 11F3) was provided by Dr. G.E. Morris (MIRC Biotechnol- 
yogy Group, North East Wales Institute, Wrexham, UK), monoclonal ZF2/ 
sibody (Lerner et al., 1981) was provided by Dr. A.I. Lamond (University of 
onsense exhibited the same morphological pattern.

Fluorescence, indirect immunofluorescence, and imaging

In preparation for visualization of fluorescence, cells were cultured on Lab-Tek chamber slides (Nunc) and fixed with 2% vol/vol paraformaldehyde in 

Plasmid constructs and subcloning

Full-length ZNF265 cDNA was amplified by RT-PCR and subcloned into pGEM-T-Easy (Promega) to create the plasmid pZNA (Adams et al., 2000). pZNA was used as a template for PCR using primers ZNF-5'A (ctcgagagaatcagagggaaagcagttgg) and NLS3

Yeast culture and two-hybrid assay

Saccharomyces cerevisiae strain AH109 (James et al., 1996) and pGBK7–ZNF265, then with the appropriate activation domain plasmid. The yeast were then plated onto synthetic defined medium deficient in leucine, tryptophan, histidine, and adenine (SD-L-W- 

In vitro and in vivo splicing and immunoprecipitation of splicing complexes

mGppppG-capped 32P-labeled pre-mRNA substrates were made by runoff transcription of linearized template DNA with SP6 RNA polymerase (Mayeda et al., 1999), first with pGBKT7–ZNF265, then with the appropriate activation domain plasmid. The RNA products of the immunoprecipitates were analyzed by electrophoresis on a 5.5% polyacrylamide/7 M urea gel, followed by autoradiography. Additional splicing assays were performed essentially as described in Stoss et al. (1999). Human transformer-2-B (Tra2-β1) minigene (Nayler et al., 1998) and C2-ZNF265 expression plasmids were transfection into HEK293 cells. After RNA isolation and reverse transcription (Hartmann et al., 1999), PCR to amplify minigene products was performed thus: 35 cycles of 94°C for 1 min, 60°C for 1 min each, followed by 10 min at 

We gratefully acknowledge the assistance of the following: Michelle Pedler in transfection and antibody staining, Dr. Guy Cox (Electron Microscope Unit, University of Sydney, Sydney, Australia) for help with confocal microscopy, Drs. Tom Maniatis, Derek Kennedy, Gideon Dreyfuss, Angus Lamond, and Glen Morris for donation of antibodies, and Drs. Rachel Davies, Nick Hastie, and David Elliott for yeast two-hybrid constructs. We wish to express our gratitude to the reviewers of this paper for many helpful comments and suggestions that significantly improved the manuscript. This work was funded by grants from the Australian Research Council (to B.J. Morris), the Lucille P. Markey Trust (to A. Mayeda), the Deutsche Forschungsgemeinschaft (to S. Stamm), and the National Health and Medical Research Council of Australia (to J.E. Rasko).


