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Identification and characterization of RED120: A conserved PWI domain protein with links to splicing and 3′-end formation

Puri Fortes[a,d,*], Dasa Longman[b], Susan McCracken[c], Joanna Y. Ip[c], Raymond Poot[d], Iain W. Mattaj[d], Javier F. Cáceres[b], Benjamin J. Blencowe[c,*]

[a] *Division of Gene Therapy and Hepatology, CIMA, University of Navarra, Pamplona 31008, Spain*
[b] *MRC Human Genetics Unit, Edinburgh EH4 2XU, Scotland, UK*
[c] *Banting and Best Department of Medical Research, Centre for Cellular and Biomolecular Research, University of Toronto, Toronto, Ontario, Canada M5S 3E1*
[d] *Gene Expression Programme, European Molecular Biology Laboratory, Heidelberg D-69117, Germany*

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Abstract **Precursor (pre)-mRNA splicing can impact the efficiency of coupled steps in gene expression. SRm160 (SR-related nuclear matrix protein of 160 kDa), is a splicing coactivator that also functions as a 3′-end cleavage-stimulatory factor. Here, we have identified an evolutionary-conserved SRm160-interacting protein, referred to as hRED120 (for human Arg/Glu/Asp-rich protein of 120 kDa). hRED120 contains a conventional RNA recognition motif and, like SRm160, a PWI nucleic acid binding domain, suggesting that it has the potential to bridge different RNP complexes. Also, similar to SRm160, hRED120 associates with snRNP components, and remains associated with mRNA after splicing. Simultaneous suppression in *Caenorhabditis elegans* of the ortholog of hRED120 with the orthologs of splicing and 3′-end processing factors results in aberrant growth or developmental defects. These results suggest that RED120 may function to couple splicing with mRNA 3′-end formation.**

Keywords: Splicing; 3′-End processing; RNAi; snRNPs; SRm160

1. Introduction

The processing of precursor (pre-) mRNA to mature mRNA involves a series of highly integrated and coupled steps, including the addition of a 5′-m7G cap, intron removal by splicing, and 3′-end cleavage and polyadenylation. Most pre-mRNAs in higher eukaryotes contain at least one intron that must be excised by a spliceosome. The major spliceosome consists of the five small nuclear ribonucleoprotein particles (snRNPs) U1, U2, U4/U6, and U5, as well a large number of non-snRNP protein splicing factors (for reviews see [1–3]). Members of the SR (Serine/Arginine) family of splicing factors, as well as SR-related proteins, are among the best characterized non-snRNP splicing factors and have well defined roles in splice site selection, and in the coupling of splicing to other steps in gene expression (reviewed by [4–7]).

Splicing influences coupled steps in gene expression by several mechanisms. For example, several splicing factors have been described that affect 3′-end processing [8,9]. The 3′-end processing machinery is relatively well-conserved from yeast to higher eukaryotes, and is composed of five different factors: Poly(A) polymerase, cleavage and polyadenylation specific factor (CPSF), cleavage stimulation factor (CstF) and cleavage factors I and II (CFI and CFII). These factors associate with the bipartite poly (A) signal in the nascent transcript and catalyze the coupled 3′-end cleavage and polyadenylation reaction (reviewed by [10,11]). Direct interactions between splicing factors and 3′-end processing factors may result in the stimulation of cleavage or polyadenylation. These interactions include the binding of the U2 snRNP auxiliary factor 65 kDa subunit to poly(A) polymerase and CFI and interactions between U2 snRNP and CPSF [12–14].

The carboxyl terminal domain (CTD) of the largest subunit of RNA polymerase II (pol II) and the mRNA binding exon-junction complex (EJC) also mediate effects of splicing on coupled steps in gene expression [8,15]. The EJC is composed of a set of factors that associate with pre-mRNA during or soon after transcription and which remain bound to mRNA after splicing [16]. The EJC is deposited 20–24 nucleotides upstream of spliced exon junctions [17–19], and is known to contain factors implicated in mRNA export, 3′-end formation, mRNA turnover via nonsense-mediated decay (NMD), and translation [20–24].

SRm160 (the SR-related nuclear matrix protein of 160 kDa) is a splicing coactivator that associates with snRNP components, assembled splicing complexes, and the EJC [18,19,25–28]. In addition to its role in the splicing of specific pre-mRNAs, SRm160 promotes 3′-end cleavage via its conserved N-terminal PWI domain [28]. In order to understand how SRm160 functions, we have recently used mass spectrometry to identify factors that associate with this protein [49]. This resulted in the identification of hRED120 (human Arg/Glu/Asp-rich protein of 120 kDa), which shares several similarities with SRm160. In addition to interacting with SRm160, hRED120 associates with snRNPs in the absence of pre-mRNA, with assembled splicing complexes containing the pre-mRNA

*Corresponding authors. Fax: +34 948 194717 (P. Fortes); +1 416 978 8287 (B.J. Blencowe).
E-mail addresses: pfortes@unav.es (P. Fortes), b.blencowe@utoronto.ca (B.J. Blencowe).

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substrates, intermediates and products of in vitro splicing reactions, and remains preferentially bound to spliced mRNA. We further show that simultaneous repression by RNA interference (RNAi) in the nematode *Caenorhabditis elegans* of orthologs of hRED120 and the SR family splicing factor SRp20, or of the orthologs of hRED120 and the 3′-end processing factors CstF50 and Clp1, results in growth impairment or embryonic lethality. Together, our results provide evidence for the importance of interactions between RED120 and factors involved in pre-mRNA processing and further suggest a possible role for RED120 at the interface of splicing and 3′-end formation.

2. Materials and methods

2.1. Cell extracts and antibodies

HeLa nuclear extract was purchased from C4 (Belgium). The following antibodies were used in this study: αSRm160 murine monoclonal (mAb-B1C8; [29,30]), αSRm160 rabbit polyclonal (αSRM160; [25]), αSRm300 rabbit polyclonal (αSRm300; [31]), rabbit polyclonal α116 kDa-U5 snRNP-specific protein [32], rabbit polyclonal αCBP80 [33], polyclonal αRNPS1 [34], polyclonal αUAP56 [35], monoclonal αY14 (mAb-4C4 from G. Dreyfuss), monoclonal αSm proteins (Y12; [36]) and monoclonal αSC35 [37]. Antibodies specific for hRED120 were raised by immunization of two New Zealand white rabbits with 200 μg of nhRED120 or chRED120 proteins (see below) per immunization. Five immunizations were administered.

2.2. Database searches

Putative RED120 family members were identified using Blast searches [38]. Proline-rich regions, RNA recognition motifs and PWI domains [39] were identified using the Prosite database [40]. Low complexity regions were identified using the SEG program [41]. Multiple sequence alignment and a phylogenetic tree were calculated using ClustalX [42].

2.3. DNA constructs

RNA isolated from HeLa cells (ATCC) was reverse transcribed with an oligonucleotide complementary to the 3′UTR of hRED120 and hRED120 cDNA was amplified by PCR using oligonucleotides (Sigma) ''5′hRED120'' and ''3′hRED120''. 5′hRED120 contained a SpeI recognition site and hRED120 translation initiation sequences. 3′hRED120 contained a KpnI recognition site and sequences complementary to the hRED120 translation termination region. The resulting PCR product was digested with SpeI and KpnI (New England Biolabs) and cloned into the multiple cloning site of pBluescript IIKS (Stratagene). Positive pBShRED120 clones were verified by sequencing. hRED120 5′ end sequences were isolated from pBShRED120 after digestion with SpeI and HindIII. The resulting fragment was subcloned into the PstI and HindIII sites of pQE30 (Qiagen) to produce pQEnhRED120. SauIIIA–KpnI digestion of pBShRED120 served to isolate hRED120 3′ end sequences which were then subcloned into pQE31 (Qiagen) to generate pQEchRED120.

2.4. Protein expression and antibody production

E. coli strain BL21/pREP4 (Qiagen) was transformed with pQEnhRED120 and pQEchRED120 and grown in LB medium supplemented with ampicillin at 37 °C to an OD_{600} of 0.06 and at room temperature to an OD_{600} of 0.8. Protein expression was induced with 0.5 mM IPTG (Sigma) for 3 h. Cell pellets were sonicated in lysis buffer (500 mM NaCl, 5 mM magnesium acetate, 1 mM PMSF and 50 mM Tris–HCl, pH 7.5) and cell lysates were recovered after 20 min of centrifugation at 9000 rpms with a SS34 rotor. Extracts were adjusted to 20 mM imidazole (Sigma) and mixed with 200 μl of buffer-washed packed Nickel-NTA beads (Invitrogen) per liter of bacterial culture. The mixture was incubated for 90 min at 4 °C and the beads were recovered in an Econo-Column (Biorad). The beads were washed twice in 10 vol. of PBS-20% Glycerol (PBS-G) and protein was eluted in the same buffer supplemented with 0.4 M imidazole and dialyzed against PBS-G.

2.5. Protein detection and immunolocalization

hRED120 was detected by Western blotting using rabbit αnhRED120 or αchRED120, diluted 1:1000. Detection was performed using a goat αrabbit secondary antibody coupled to peroxidase, diluted 1:2000 (Sigma). Western blots with antibodies to SRm160, RNPS1, UAP56, Y14, U5-116 kDa protein, CBP80, and Sm proteins were performed as described previously [25,32–36].

Immunofluorescence localization was performed as described previously [43] with pre-immune serum or αchRED120 diluted 1:250 and αSC35 diluted 1:3. Secondary antibodies were α mouse and α rabbit coupled to Texas red or FITC, respectively, diluted 1:100 (Sigma). Preparations were analyzed using a Zeiss LSM 510 confocal microscope.

2.6. Immunoprecipitations

Interactions between hRED120, SRm160, SRm300, RNPS1, Y14, and UAP56 were analyzed as follows: 30 μl of packed protein A Sepharose beads were loaded with a mixture of αchRED120 and αnhRED120 sera (14 μl), antigen affinity-purified rabbit polyclonal αSRm160 (50 μg), αSRm300 serum (25 μl), and, as a control for non-specific immunoprecipitation, with rabbit αmouse IgG and IgM (72 μg). Antisera were cross-linked to protein A beads with 20 mM dimethylpimelidate (Harlow and Lane). Rabbit αmouse IgG and IgM (150 μg) was coupled to protein A-Sepharose prior to coupling of B1C8, which is an IgM monoclonal. Beads were mixed with 1.5 mg of nuclear extract preincubated for 15 min at 30 °C under splicing conditions (2 mM $MgCl_2$, 1.5 mM ATP, 5 mM phosphocreatine, 16 ng/μl RNase cocktail (Boehringer), DNase I (0.3 U/μl), 1 mM potassium fluoride, 0.1 mM sodium pyrophosphate, and 1 mM sodium β-glycerophosphate). The extract was incubated with the beads for 3 h at 4 °C with gentle rotation in 60 mM NaCl, 13 mM HEPES, pH 7.9, 1.4 mM $MgCl_2$, 14% glycerol, 0.5 mM DTT, 0.7 mM β-glycerol phosphate, 0.7 mM NaF, 0.07 mM Na^+ pyrophosphate in a final volume of 750 μl. The beads were washed three times with 1.5 ml of IPWB100 (100 mM NaCl, 50 mM Tris–HCl, pH 7.5, 2 mM $MgCl_2$, 0.1% NP40) and eluted with 2 M NaCl, 10 mM HEPES, pH 7.5, 1 mM EDTA (200 μl). These pooled eluates were back-bound with Protein A Sepharose or Protein A Sepharose coated with rabbit αmouse IgG and IgM for 30 min at 4 °C with rotation. After elution, the samples were precipitated with trichloroacetic acid (TCA) (20%) and sodium deoxycholate (1.5 mg/ml), washed with 10% TCA, and with acetone, resuspended in SDS sample buffer and analyzed by SDS–PAGE. To co-immunoprecipitate other splicing factors, 8 mg of HeLa nuclear extract was incubated overnight at 4 °C with 400 U of HPRI, 22 μg of *E. coli* tRNA, 100 μg of heparin and 4 mg of preimmune or αchRED120-coupled protein A beads. Beads were recovered in an econo-column and washed three times with 1 ml of buffer D/0.1% NP40 [44]. The beads were eluted with 150 μl of buffer D/0.1% NP40 mixed with NaCl as indicated, or with 150 μl of Glycine 0.1 M, pH 3. Before elution, beads were incubated 15 min at 4 °C. After each elution beads were washed three times with 150 μl of elution buffer. Two percent of the input and one-third of the eluates were analyzed by SDS–PAGE and immunoblotting. Two percent of the input and two-third of the eluates were analyzed by Northern blot (see below). When immunoprecipitations were performed with RNase-treated extract, 6.5 mg of HeLa extracts were incubated for 1 h in a splicing reaction (see below) in the presence of buffer alone, 200 μg of RNase A (Boehringer), or 36 U of RNaseH (Gibco) mixed with 11.4 nmol of 2b oligo (5′CAGATACTACACTTG3′).

To immunoprecipitate from splicing reactions, 200 μg of protein A Sepharose beads (Amersham) were bound to 20 μl of antibody for 1 h at room temperature in buffer D. Then, beads were washed extensively with buffer D and buffer D diluted 1:3 (buffer D/3) and mixed with 20 μl splicing reactions incubated for 1 h (see below). 10 μg of competitor *E. coli* tRNA (Boehringer), 1 U/μl of HPRI (Amersham) and 50 μg/ml of heparin (Sigma) were also added. 50000 cpm of labeled U3 snoRNA were included where indicated (see below). The mix was incubated for 2 h at 4 °C with rotation in a final volume of 200 μl of buffer D/3. After incubation, beads were washed 3 times with 10 vol. of buffer D/3 and twice with 10 vol. of buffer D-200 mM KCl-0.1% NP40. RNA was analyzed from the beads after proteinase K treatment as described [45]. Quantifications were done in a Cyclone Phosphorimager (Perkin Elmer). Percentage of binding was calculated after subtracting the preimmune background from the signal obtained in the RED120 antibody immunoprecipitation.