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TDP43 depletion rescues aberrant CFTR exon 9 skipping

Youhna M. Ayala, Franco Pagani, Francisco E. Baralle*

Department of Molecular Pathology, International Centre for Genetic Engineering and Biotechnology (ICGEB), Padriciano 99, 34012 Trieste, Italy

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Abstract **CFTR exon 9 presents a 3′ splice site polymorphism, $(UG)_mU_n$, whose composition influences splicing. TDP43 specifically binds the UG tract of the transcript and inhibits splicing in vitro. We report that depletion of TDP43 through RNA interference removes splicing inhibition caused by unfavorable $(UG)_mU_n$ sequences, indicating that TDP43 exerts a potent inhibitory effect in vivo. We also show that the UG–TDP43 interaction has a dominant role over other exon 9 splicing regulatory elements. These results suggest that TDP43 association near a splice site has determined the evolution of positive splicing regulatory elements to contrast this inhibition.**

Keywords: TDP43; Alternative splicing; hnRNP; CFTR exon 9; Cystic fibrosis; Splicing regulation

1. Introduction

Cystic fibrosis (CF) is caused by mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene [1,2]. One of the causes for CF is the skipping of CFTR exon 9, which results in a nonfunctional protein [3]. Exon 9 recognition is modulated by a characteristically high number of exonic and intronic elements with enhancer and silencer splicing properties, among these is the $(TG)_mT_n$ polymorphism at the 3′ splice site. Unfavorable $(TG)_mT_n$ compositions are linked to the occurrence of non-classical or mild forms of CF and particularly to the development of male sterility. When compared to healthy individuals, patients more often carry a high number of TG repeats (11–13) and a short T tract ($\leqslant 5$) [4]. In addition, various mutations within the exon may have unexpected deleterious effects on splicing due to the intrinsically weak definition of this exon. Among these are mutations in composite exonic regulatory elements of splicing (CERES) that disrupt positive regulatory elements and thus result in exon skipping (e.g., A455E) [5]. Other regulatory sequences are located in the downstream intron and act as enhancer and silencer elements by recruiting the splicing factor TIA-1 (T cell intracellular antigen-1) [6], and the splicing factor 2/alternative splicing factor (SF2/ASF) [7], respectively.

We have earlier shown that the TAR DNA binding protein (TDP43) regulates exon 9 splicing, most likely through the association with the UG repeats at the 3′ splice site [8]. TDP43 belongs to the family of the heterogeneous nuclear ribonucleoproteins (hnRNPs) and is highly conserved among metazoans both in sequence and function [9,10]. The proteins from human, *Drosophila* and *Caenorhabditis elegans* bind UG repeats with affinities in the low nanomolar range [9,11]. In human cells, overexpression of TDP43 decreases exon 9 recognition and specifically causes exon skipping in vitro [8,12].

Here, we investigate the effect of TDP43 depletion on exon 9 recognition in vivo with the use of RNA interference. Our results indicate that TDP43 is responsible for CFTR exon 9 skipping in the presence of unfavorable $(UG)_mU_n$ sequences and that the protein plays a dominant role over other exonic or intronic regulatory elements. Consistent with these observations, TDP43 downregulation in patient cells carrying the $(TG)_{13}T_3$ locus significantly improves the levels of exon 9 inclusion. Moreover, removal of TDP43 results in proper exon 9 recognition despite disruption of enhancer elements or addition of inhibitory factors.

2. Materials and methods

2.1. Cell culture and transfection

Duplex RNA oligonucleotide was synthesized (Dharmacon) against the target sequence AAGCAAAGCCAAGAUGAGCCU (siRNA^{TDP43}+). siRNA against the firefly luciferase gene (siCONTROL Non-Targeting siRNA #2) was used as a nonspecific control (siRNA^{TDP43}−). Cells were transfected as recently described [13]. One microgram of minigene constructs were cotransfected during the second siRNA transfection. EBV transformed lymphocytes from control and patient [8] were electroporated as described elsewhere [14]. Cells were collected 36 h after transfection with RNAwiz and total RNA was isolated according to manufacturer instructions. The TDP43 recognizing antibody was described earlier [8] and mouse monoclonal antibody was used to detect tubulin as control (T5168 Sigma). SF2/ASF expression vectors were described earlier [7].

2.2. Minigene constructs

The hCF-(TG)(T) minigenes and mutant C1496A, C1496G, and C1496T constructs have been previously described [5,7]. The mutation in the intronic splicing enhancer used was earlier referred to as M1, 2, 3 [6].

2.3. Splicing analysis

Splicing efficiency was carried out as previously described [7]. Quantification of PCR products was carried out according to Pagani et al. [5]. In the case of endogenous CFTR transcript analysis, the products were amplified with primers specific for exons 8, 10, and 11. Allele specific amplification from patient cells was carried out with reverse primers described in Fig. 3. The relative levels of exon inclusion were visualized on 2% agarose gels.

2.4. Sequence similarity of CFTR exon 9 and surrounding introns from different organisms

Nucleotide sequence alignment of the CFTR exon 9 including flanking introns from different mammals was performed. We analyzed sequences (NCBI) from human, macaca, chimpanzee, baboon, lemur, rabbit, armadillo, cat, horse, cow, hedgehog, pig, dog, mouse, and rat.

*Corresponding author. Fax: +39 040 3757361.
E-mail address: baralle@icgeb.org (F.E. Baralle).

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3. Results

3.1. TDP43 knockdown removes splicing inhibition through UG repeats

To test the effect of TDP43 on exon 9 splicing in transient expression in vivo, we depleted the protein through RNA interference in the presence of minigene constructs containing variations of the $(TG)_mT_n$ sequence at the 3′ splice site. These sequences are associated with non-classical or mild CF ($TG_{11-13}T_{3-5}$). Protein silencing resulted in >90% removal of TDP43 as indicated by Western blotting (Fig. 1A). CFTR exon 9 inclusion was detected using hybrid minigenes (Fig. 1B) previously described to successfully mimic the splicing pattern of endogenous exon 9 CFTR [7,8,15]. The minigenes were transfected into HeLa cells in the presence of control and TDP43-targeted small interfering (si)RNA and the relative levels of exon inclusion were monitored by RT-PCR. In agreement with previous studies, we obtained a high proportion of exon skipping in the presence of high number of UG repeats and a short U tract. In addition, $UG_{13}U_3$ leads to the activation of a cryptic 3′ splice site [8]. In the absence of TDP43, however, exon skipping was significantly reduced even in the presence of the extreme $UG_{13}U_3$ sequence, and splicing through the cryptic 3′ splice site no longer occurred (Fig. 1C, upper panels). In fact, specific amplification of the transcript containing the aberrant exon, showed the absence of cryptic site usage in TDP43− cells (Fig. 1C, lower left panel). As previously observed, deletion of the UG tract (ΔTG) resulted in complete exon 9 inclusion and as expected, removal of TDP43 had no effect on splicing (Fig. 1C, upper right panel). To rule out a non-specific effect of TDP43 RNAi depletion on splicing regulation, we analyzed the splicing of an unrelated minigene construct that lacks UG repeats near 3′ splice sites and normally shows exon skipping. A variant of exons 34–38 of the neurofibromin 1 gene containing a mutation in exon 37 results in the skipping of exon 37 and exons 36 and 37 (M. Baralle, personal communication). Unlike the case of CFTR exon 9, the pattern and relative proportion of exon skipping of the NF1 mutant was not influenced by the depletion of TDP43 (Fig. 1C, lower right panel).

3.2. Downregulation of TDP43 rescues endogenous exon 9 skipping from wild type and patient cells

Next, we asked whether TDP43 removal by RNAi would similarly affect the splicing of the chromosomal CFTR exon 9. We used wild type HeLa, Hep3B, and lymphoblasts. These cells generate 10–20% exon 9− transcript, consistent with the variability in the level of exon exclusion observed among

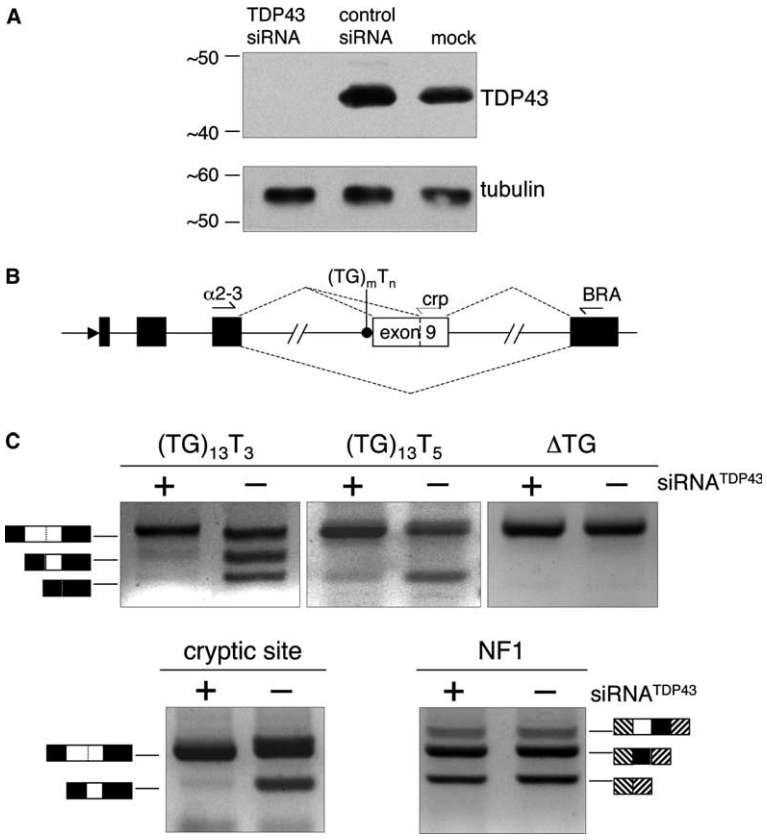

Fig. 1. In vivo depletion of TDP43 by RNAi restores exon 9 inclusion despite unfavorable $(TG)_mT_n$ sequences. (A) Western blot analysis of HeLa cells treated with TDP43 siRNA, control firefly luciferase siRNA, and mock transfection. (B) Schematic representation of the hybrid minigene constructs. The large arrow represents the promoter site, black boxes represent non-CFTR exons, and small arrows depict primers used for amplification. (C) α2-3 and BRA primers were used for transcript amplification from $(TG)_{13}T_3$,$(TG)_{13}T_5$, and ΔTG constructs upon RNAi treatment. Top and lower bands correspond to complete exon 9 inclusion and exclusion, respectively. The middle band present in $(TG)_{13}T_3$ control lane corresponds to the activation of a cryptic 3′ splice site. Primers α2-3 and crp were used to specifically monitor 3′ cryptic splice site activation of the $(TG)_{13}T_3$ construct. As a control, the effect of TDP43 siRNA was tested on an unrelated hybrid minigene system containing exons 34–38 of the neurofibromin 1 (NF1) gene carrying a mutation that leads to the skipping of one or two exons.