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Alternative splicing, a new target to block cellular gene expression by poliovirus 2A protease

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ABSTRACT

Viruses have developed multiple strategies to interfere with the gene expression of host cells at different stages to ensure their own survival. Here we report a new role for poliovirus $2A^{pro}$ modulating the alternative splicing of pre-mRNAs. Expression of $2A^{pro}$ potently inhibits splicing of reporter genes in HeLa cells. Low amounts of $2A^{pro}$ abrogate Fas exon 6 skipping, whereas higher levels of protease fully abolish Fas and FGFR2 splicing. In vitro splicing of MINX mRNA using nuclear extracts is also strongly inhibited by $2A^{pro}$, leading to accumulation of the first exon and the lariat product containing the unspliced second exon. These findings reveal that the mechanism of action of $2A^{pro}$ on splicing is to selectively block the second catalytic step.

1. Introduction

The genome of poliovirus (PV) comprises a single-stranded positive-sense RNA molecule of about 7.5 kb. This RNA has a single open reading frame, encoding a large polyprotein that is hydrolyzed by the two virus-encoded proteases: $2A^{pro}$ and $3C^{pro}$ [1]. In addition, these proteases also target several cellular proteins, leading to the inhibition of host gene expression in infected cells [2]. Thus, both proteases are involved in the viral hijacking of host protein synthesis machinery by cleaving eukaryotic translation initiation factors (eIFs). PV $2A^{pro}$ cleaves eIF4GI and eIF4GII, whereas $3C^{pro}$ proteolyzes poly(A)-binding protein (PABP) [3]. During PV infection, several transcription factors are also cleaved by $2A^{pro}$ and $3C^{pro}$, including the TATA-binding protein (TBP) [4,5], cyclic AMP-responsive element binding protein (CREB) [6] and octamer binding protein (Oct-1) [7]. PV $2A^{pro}$ also cleaves the nucleoporins (Nups) Nup98, Nup153 and Nup62, which are components of the nuclear pore complex (NPC) essential for RNA and protein trafficking between nucleus and cytoplasm [8–10]. Therefore, PV $2A^{pro}$ abrogates cellular gene expression at many different stages.

Splicing of pre-mRNAs represents a fundamental regulatory step in the expression of all multiexon genes, expanding the information content of eukaryotic genomes [11]. During this multistep process, introns are spliced out and exons are ligated generating the mature mRNAs. This process involves large RNA/protein dynamic machinery known as a spliceosome [12]. This nanomachine consists of five small ribonucleoprotein particles (snRNPs) U1, U2, U4, U5 and U6, and a large number of splicing-associated protein factors. Proteomic approaches have identified about 140–300 different proteins co-purifying with spliceosomes [13,14]. In most cases, the exact role of these proteins in the splicing reaction remains unclear. This complex machinery is finely tuned in response to physiological and pathological conditions [15]. For instance, during PV infection, $2A^{pro}$ cleaves Gemin-3, which is a component of the survival of motor neurons (SMN) complex, affecting the assembly of U snRNPs [16]. Nevertheless, no clear effect on the splicing of cellular pre-mRNAs was detected after Gemin-3 proteolysis [16]. The goal of this work was to examine the action of PV $2A^{pro}$ on three well-defined pre-mRNA splicing events.

2. Materials and methods

2.1. Cell culture

HeLa cells were grown at 37 °C in Dulbecco's modified Eagle's medium supplemented with 2 mM L-glutamine, non-essential amino acids and 10% fetal calf serum.

2.2. Plasmids and electroporation and transfection assays

The plasmid pTM1-2A, which encodes PV $2A^{pro}$ has been described previously [17]. The minigenes Fas ΔI6 and RK97 were generated as described [18–21].

The in vitro transcription reactions as well as electroporation and transfection assays of RNA and DNA, respectively, in HeLa cells were carried out as described [22]. Alternatively spliced RNA

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products from Fas and FGFR-2 reporter minigenes were analyzed by semiquantitative RT-PCR and agarose gel electrophoresis [23].

2.3. Subcellular fractionation and Western blot analyses

At indicated times post-transfection, HeLa cells were collected and fractionated as described [9]. Western blot analysis was carried out as described [9].

2.4. Purification of recombinant proteins

The MBP and MBP-2A^{pro} recombinant proteins were purified by affinity chromatography, as described previously [24]. Recombinant HIV-1 protease was provided by I. Pichova (Centralized Facility for AIDS Reagents).

2.5. In vitro MINX splicing assay

In vitro transcription and splicing assays using capped MINX RNA were carried out as described [18].

3. Results and discussion

3.1. PV 2A^{pro} inhibits the splicing of foreign genes in HeLa cells

To study the effect of PV 2A^{pro} ectopic expression on the splicing process, standard reporter minigenes were employed. Splicing of these minigenes was analyzed by detection of both precursors of unspliced pre-mRNAs and alternatively spliced products. To this end, the first experimental approach involves a well-characterized alternative splicing event such as the inclusion/exclusion of exon 6 in apoptosis receptor Fas (Fig. 1A). This splicing event is of biological relevance because alternative splicing of Fas pre-mRNA can regulate the sensitivity of Fas-expressing cells to Fas-induced apoptosis. The protein isoform encoded by the mRNA lacking exon 6 corresponds to the soluble form of the receptor able to inhibit Fas signaling whereas the protein isoform containing exon 6 encodes the membrane-bound Fas apoptosis receptor (Fig. 1A). The activity of PV 2A^{pro} in regulating Fas splicing was tested by co-expression of different amounts of IRES-2A mRNA with a Fas ΔI6 minigene, which comprises human Fas genomic sequences from exons 5 to 7 with a deletion of around 1000 nt in intron 6 to enable visualization of unspliced Fas pre-mRNA (Fig. 1A) [20,25]. Thus, HeLa cells were electroporated with transcription buffer, 1 or 9 μg of IRES-2A mRNA and then transfected with the Fas ΔI6 minigene at 4 h post-electroporation (hpe). Cells were collected and fractionated at 16 h post-transfection (hpt), giving rise to the corresponding total (T), cytoplasmic (C) and nuclear (N) fractions (Fig. 1B). As previously reported, dose-dependent cleavage of eIF4GI was detected in electroporated cells [22]. The effectiveness of fractionation was checked by Western blot analysis against a cytoplasmic protein such as α-tubulin or a nuclear protein such as Ref1/Aly. As expected, α-tubulin was only detected in the cytoplasmic fraction, whereas Ref1/Aly was present only in the nuclear fraction (Fig. 1B). Next, total RNA from the different fractions was isolated and analyzed by semiquantitative reverse transcriptase and polymerase chain reaction (RT-PCR) analyses to detect changes in isoform expression and to visualize unspliced and spliced products (Fig. 1C). After electroporation of HeLa cells with 1 μg of IRES-2A mRNA, a significant and reproducible increase in Fas exon 6 skipping was observed (Fig. 1C, compare lanes 1–3 with 4–6). However, when a higher 2A^{pro} dose (electroporation of 9 μg of IRES-2A mRNA) was expressed, Fas splicing was fully abolished since no mature forms of this mRNA were detected. In addition, high levels of PV 2A^{pro} might target both splicing (see accumulation of unspliced Fas pre-mRNA in Fig. 1C, lane 7) and mRNA export to the cytoplasm (see disappearance of the Fas spliced isoforms in the cytoplasm, Fig. 1C, lane 9), which is in good agreement with our previous report [9]. However, under these conditions, PV 2A^{pro} did not block mRNA transcription [9]. Taken together, these results suggest that PV 2A^{pro} modulates the splicing machinery in a dose-dependent manner.

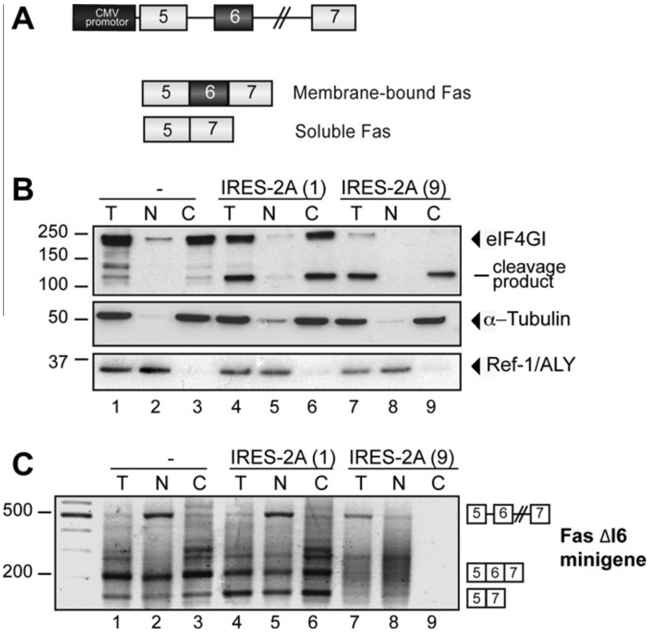

Fig. 1. Fas exon 6 splicing is modulated in PV 2A^{pro}-expressing HeLa cells. (A) Schematic representation of human Fas minigene. Exons 5, 6 and 7 are represented by boxes and introns by lines. The vertical line in intron 6 represents a deletion that facilitates the detection of unspliced RNA but that does not affect Fas splicing or its control. Alternative isoforms of Fas mRNAs generated by inclusion or skipping of exon 6 are indicated below. The protein isoform encoded by the mRNA lacking exon 6 corresponds to the soluble form of the receptor able to inhibit Fas signaling whereas the protein isoform containing exon 6 encodes the membrane-bound Fas apoptosis receptor. (B and C) Effect of PV 2A^{pro} expression in HeLa cells on alternative splicing of Fas minigene. (B) Nuclear-cytoplasmic distribution and Western blot analysis against eIF4GI, α-tubulin and Ref-1/ALY proteins. HeLa cells were electroporated with transcription buffer, 1 or 9 μg of IRES-2A mRNA. At 4 hpe, HeLa cells were transfected with Fas minigene. At 16 hpt, protein samples from post-transfected HeLa cells were isolated, fractionated and analyzed by Western blotting with antibodies raised against different host proteins as indicated. Molecular mass markers (kDa) for protein are indicated on the left. The identities of protein bands are indicated on the right by arrowheads. (C) Analysis of alternatively spliced products derived from Fas reporter minigene. Total RNAs isolated from HeLa cells in (B) were analyzed by RT-PCR. Molecular mass marker (bp) is indicated on the left. PCR amplification products corresponding to the Fas alternatively spliced isoforms are indicated on the right by boxes.

To further assess the interference of PV 2A^{pro} in the splicing process, we used the FGFR2 (fibroblast growth factor receptor type 2) minigene RK97 corresponding to a partial genomic DNA sequence of FGFR2 gene containing the Rous sarcoma virus promoter (RSV), the bovine growth hormone polyadenylation signal (BGH), FGFR-2 C1, C2 and K-SAM exons and the partially deleted BEK exon (ΔBEK) (Fig. 2A). Following the protocol described above, HeLa cells were electroporated with transcription buffer, 1 or 9 μg of IRES-2A mRNA and at 4 hpe, plasmid containing the FGFR-2/RK97 minigene was transfected. Cellular samples were fractionated and cytoplasmic and nuclear protein extracts were obtained and analyzed by Western blotting against eIF4GI, α-tubulin and Ref-1/ALY as described above, indicating the good quality of the fractions obtained (Fig. 2B). Once again, 1 μg of IRES-2A mRNA inhibits the alternative splicing of FGFR-2, giving rise to a different ratio between FGFR-2 isoforms (Fig. 2C, compare lanes 1–3 with 4–7). In particular, levels of unspliced pre-mRNA increased whereas the mature mRNA