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

Enrique Álvarez [1,*], Alfredo Castelló [1,2], Luis Carrasco, José M. Izquierdo

Centro de Biología Molecular Severo Ochoa (CSIC-UAM), Nicolás Cabrera, 1 Universidad Autónoma de Madrid, Cantoblanco, 28049 Madrid, Spain

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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

* Corresponding author. Fax: +34 911964420.
E-mail address: ealvarez@cbm.uam.es (E. Álvarez).
[1] These authors contributed equally to this work.
[2] Present Address: European Molecular Biology Laboratory (EMBL), Meyerhofstrasse 1, 69117 Heidelberg, Germany.

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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.

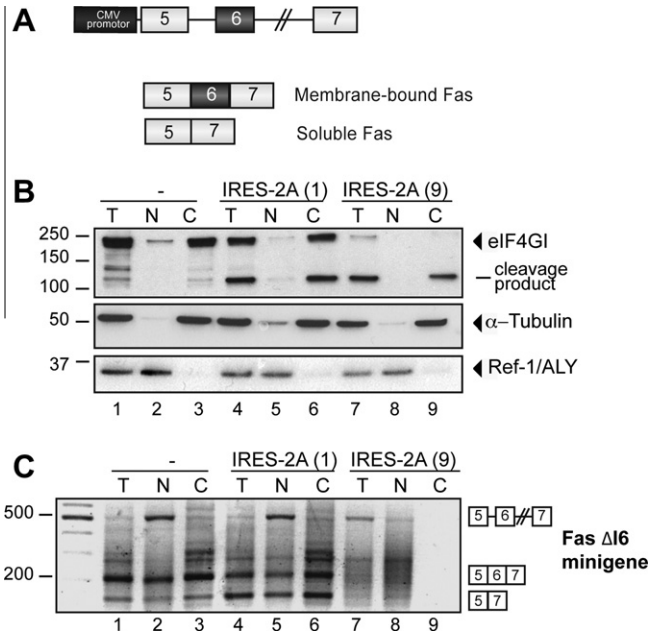

Fig. 1. Fas exon 6 splicing is modulated in PV 2A^{pro}-expressing HeLa cells. (A) Schematic representation of human Fas minigene. Exons 5, 6 and 7 are represented by boxes and introns by lines. The vertical line in intron 6 represents a deletion that facilitates the detection of unspliced RNA but that 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

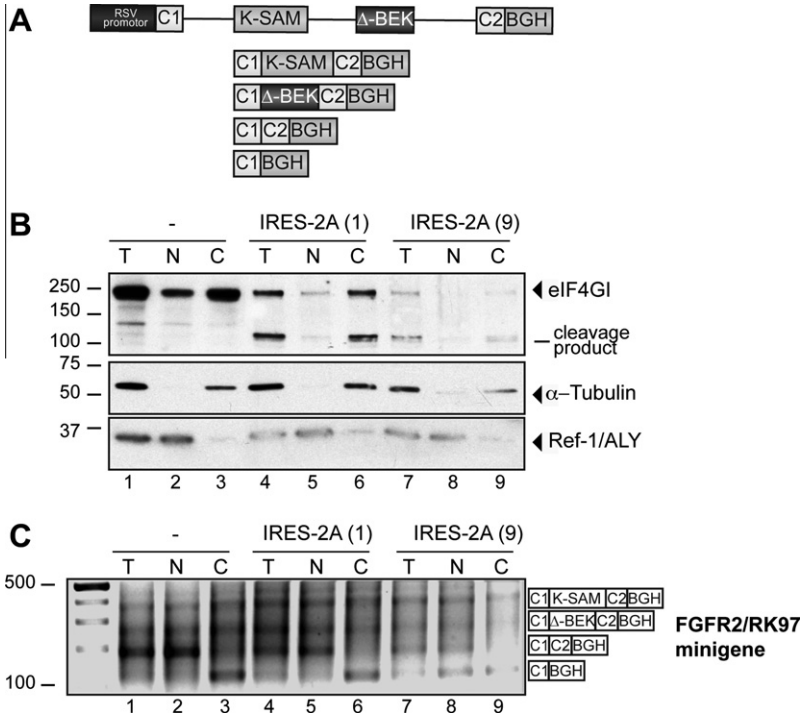

Fig. 2. FGFR-2 splicing pattern is altered in PV 2A^{pro}-expressing HeLa cells. (A) Schematic representation of human FGFR-2 minigene. The FGFR2/RK97 minigene encodes the K-SAM and BEK exons as well as the upstream flanking exon Cl, part of the downstream flanking exon C2 and the β-globin exon. Sequence coding of FGFR-2 is also represented. Alternative splicing products are shown below. (B and C) Effect of PV 2A^{pro} expression in HeLa cells on alternative splicing of FGFR-2 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 FGFR-2 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 FGFR-2 alternatively spliced isoforms are indicated on the right by boxes.

forms decreased. Moreover, electroporation with 9 μg of the same mRNA resulted in a stronger inhibition of FGFR-2 splicing and therefore, in a significant reduction of the steady-state levels of RNA isoforms (Fig. 2C, compare lanes 1–6 with 7–9). Interestingly, C1-BGH mRNA, which was the prevalent mature form in control cells, accumulated in the nucleus after electroporation of 9 μg of IRES-2A, suggesting that PV 2A independently affects splicing and mRNA export machineries. These results therefore indicate that PV 2A^{pro} also alters the FGFR-2 splicing pattern, in agreement with the above observations obtained with Fas minigene. Overall, these findings demonstrate that PV 2A^{pro} inhibits the splicing process even when present in low concentrations.

3.2. PV 2Apro abolishes the splicing reaction at the second catalytic step

To further assess the action of PV 2A^{pro} on the splicing machinery, in vitro splicing assays were carried out. Given that Fas and FGFR-2 pre-mRNAs derived from corresponding Fas ΔI6 and RK97 minigenes are not efficient substrates for such experiments and that the effect of PV 2A^{pro} expression seems to be general on splicing machinery, we decided to carry out this analysis by using a universal and highly efficient RNA substrate such as MINX [26]. HeLa nuclear extracts competent to perform in vitro splicing reactions [21] were treated with buffer, maltose binding protein (MBP), recombinant MBP-2A^{pro} or recombinant human immunodeficiency virus type-1 (HIV-1) protease. Untreated and treated HeLa nuclear extracts were incubated with ^{32}P-labeled MINX RNA and the resulting spliced RNA products were analyzed by denaturing gel electrophoresis. In the absence of ATP the MINX RNA substrate is not processed (Fig. 3A, lane 1), whereas accumulation of the spliced product containing exons A and B of MINX was observed in presence of ATP (Fig. 3A, lane 2). Treatment of HeLa nuclear extracts with purified recombinant PV 2A^{pro} abolished the appearance of the A–B spliced product (Fig. 3A, lane 4), leading to accumulation of two immature products: (i) the exon A alone and (ii) the lariat product containing the unspliced exon B. As control, incubation with recombinant MBP protein alone did not interfere with splicing reaction, giving rise to A–B spliced product (Fig. 3A, compare lane 3 with 4). HeLa nuclear extracts treated with HIV-1 protease blocked the production of each unspliced or spliced product of MINX, indicating that this protease, unlike PV 2A^{pro}, blocks in vitro splicing from the initial step. The conclusion of these results is that PV 2A^{pro} selectively and specifically inhibits the second step of the splicing reaction. This is an interesting and unexpected observation because that step of the splicing process is poorly understood.

3.3. High specificity of the PV 2A^{pro} on proteins associated with the splicing machinery

Splicing of pre-mRNA in the nucleus requires the spliceosome machinery, which is composed of a large number of components. Proteolysis of some of these components takes place during apoptosis, which is a cellular process that is largely steered by proteases. PV 2A^{pro} has some substrates in common with cellular caspases. In this regard, eIF4GI and eIF4GII, which are hydrolyzed by PV 2A^{pro}, are cleaved during apoptosis leading to inhibition of cap-dependent translation [27,28]. Moreover, Nup153, another well-known substrate of PV 2A^{pro}, is targeted by caspases [29]. These findings suggest common strategies between PV infection

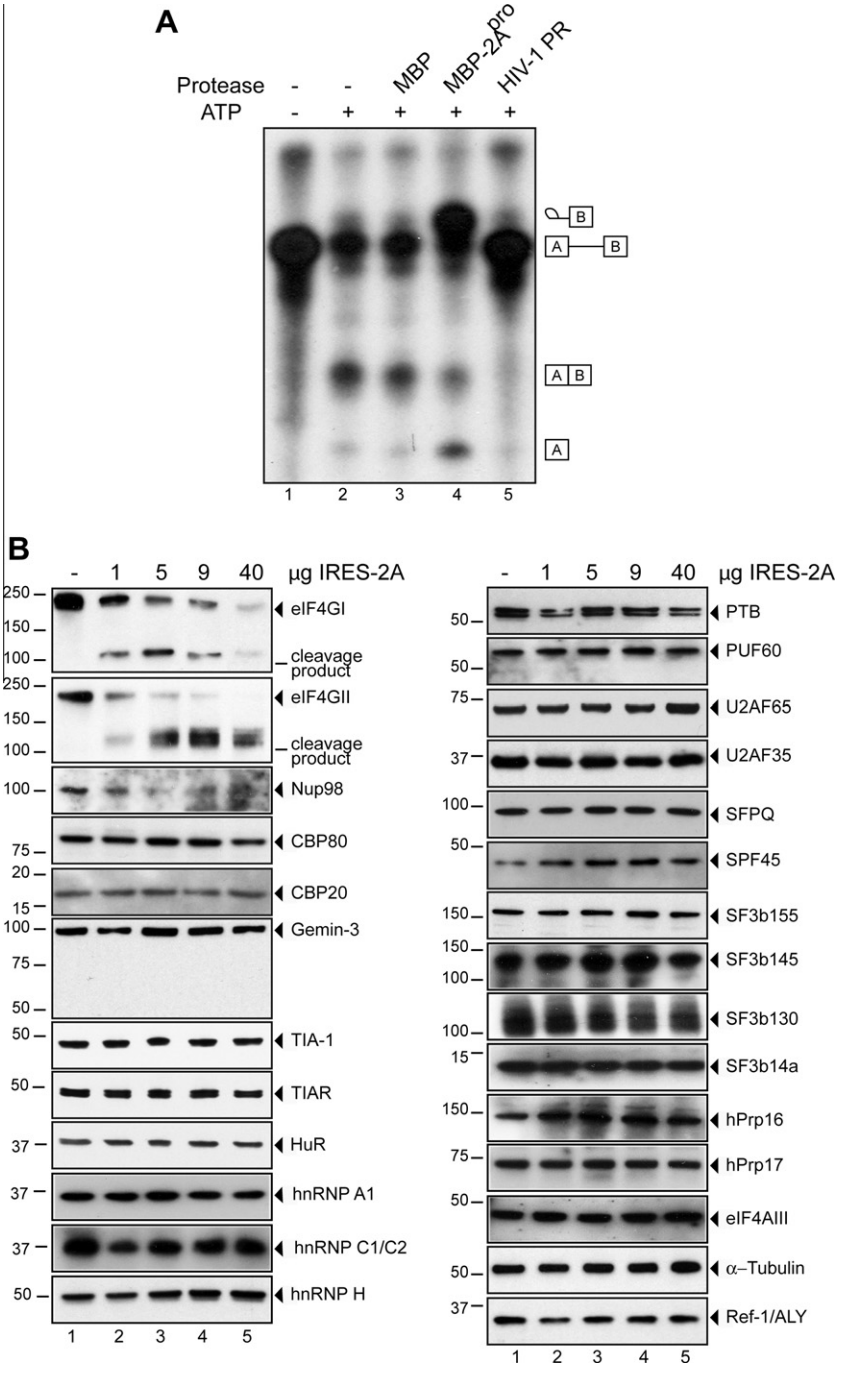

Fig. 3. Splicing inhibition at the second catalytic step by PV 2A^{pro}. (A) Differential inhibition of MINX splicing by viral 2A^{pro} and HIV proteases. Splicing reactions using ^{32}P-labeled MINX pre-mRNA as substrate RNA were supplemented with HeLa nuclear extracts treated with buffer minus ATP (lane 1), buffer plus ATP (lane 2) MBP protein plus ATP (lane 3, 5 µg), recombinant MBP-2A^{pro} protein plus ATP (lane 4, 5 µg) and recombinant HIV protease plus ATP (lane 5, 50 ng). RNA products of splicing were purified and analyzed on a denaturing (8 M urea) 10% polyacrylamide gel and visualized by autoradiography. Positions of the unspliced transcripts, splicing intermediates and the spliced products are displayed schematically. Exons are represented by boxes and the intron by a line. (B) Analysis of the integrity of several splicing factors in PV 2A^{pro}-expressing cells. HeLa cells were electroporated with 1, 5, 9 or 40 µg IRES-2A mRNA. At 8 hpe, samples were analyzed by Western blotting with antibodies raised against different host proteins as indicated in each panel. Molecular mass markers (kDa) for protein and the identities of protein bands are shown on the left and the right, respectively.

and apoptosis to interfere with cell gene expression at different steps. The integrity of several splicing factors was analyzed to demonstrate that PV 2A^{pro} functions as a highly specific and selective protease and also to try to identify the splicing factor(s) targeted by this protease. Some splicing factors that are cleaved during apoptosis such as U2AF65 [25,30], Hu-antigen R (HuR) [31], heterogeneous nuclear ribonucleoprotein (hnRNP) A1 [32], hnRNP C1/C2 [33], polypyrimidine tract-binding protein (PTB) [34], splicing factor 45 (SPF45) [30] and SF3b145 [30] were included in this analysis. HeLa cells were electroporated with different amounts of IRES-2A mRNA (1–40 µg) and cell extracts were obtained. The integrity of several splicing factors implicated in or related to the first step of the splicing process such as nuclear cap-binding protein (CBP)80, CBP20, Gemin-3, TIA-1, TIAR, HuR, hnRNP A1, hnRNP C1/C2, hnRNP H, PTB, poly(U)-binding-splicing factor (PUF60), U2AF65 and U2AF35 as well as to the second step

such as splicing factor proline- and glutamine-rich (SFPQ), SPF45, SF3b155, SF3b145, SF3b130, SF3b14a, pre-mRNA processing factor (hPrp)17, hPrp16 and eIF4AIII was analyzed. The results obtained indicate that none of the splicing factors tested were hydrolyzed by PV $2A^{pro}$ under our experimental conditions (Fig. 3B), including Gemin-3, which had been previously described as a specific target of this protease [16]. The absence of Gemin-3 cleavage using our $2A^{pro}$ expression system has been previously observed [9]. Furthermore, none of the caspase-3 substrates were cleaved during our experiments, suggesting that apoptosis was not induced under these conditions. These results were validated by testing the cleavage of well-known substrates of PV $2A^{pro}$. As expected eIF4GI, eIF4GII and Nup98, which were previously reported to be targets of PV $2A^{pro}$ [9], were efficiently cleaved in HeLa cells, whereas Ref-1/ALY and α-tubulin remained intact under these conditions (Fig. 3B). These observations support the idea that this protease exhibits a high specificity and selectivity and therefore might be useful as a molecular tool for elucidating the mechanism of the second step of the splicing reaction.

3.4. Why is the splicing process shut down during PV infection?

Hydrolysis of splicing regulatory components during PV infection could be an efficient mechanism to down-regulate the expression of antiviral defense programs of host cells. Cleavage of Gemin-3 resulted in reduced assembly of U snRNPs that could affect downstream reactions associated with pre-mRNA splicing [16]. However, no significant effect was observed on splicing of an exogenous reporter construct after reducing the intracellular amounts of Gemin-3 by small interfering RNA (siRNA) and after PV $2A^{pro}$ expression. This lack of inhibitory effect on splicing when Gemin-3 cleavage occurs could be explained by the presence of strong splice-site sequences within the pre-mRNA rendering it insensitive to early effects on spliceosomal activity. Our present findings reveal for the first time that ectopic expression of PV $2A^{pro}$ can modulate two alternative splicing events associated with the Fas and FGFR-2 pre-mRNAs at low concentrations of this viral protease. Extensive expression of $2A^{pro}$ completely abolishes these splicing reactions. Interestingly, the target of this viral protease might be associated with the second step of the splicing reaction, highlighting PV $2A^{pro}$ as a potentially powerful tool for elucidating this still poorly understood process just as it was used in the past to reveal the translation initiation mechanism.

During the course of viral infection, host cells induce responses directed at inhibiting viral replication and expression. For this reason, viruses have developed and evolved diverse strategies to evade the antiviral program of host cells. For instance, PV interferes with host gene expression at different steps of RNA metabolism including DNA-dependent transcription, import/export of RNAs and proteins as well as stability and translation of mRNAs (Fig. 4) ([9] and references therein). During the past 10 years, PV $2A^{pro}$ has emerged as a viral component that interferes with cellular functions in order to establish an optimal intracellular environment for viral replication. This protease cleaves at least 7 different host proteins with functional consequences for cell homeostasis ([2,9] and references therein and Fig. 4). We now demonstrate that PV $2A^{pro}$ can impair RNA splicing by specifically affecting the second step of splicing reaction. Perhaps PV needs to ensure avoidance of host interference, which in most cases requires synthesis of mRNAs encoding proteins with apoptotic, antiviral or pro-inflammatory activities. Indeed, there are many examples of signaling components and executors of both intrinsic and extrinsic pathways of apoptosis which are alternatively spliced factors with simultaneous pro- and anti-apoptotic properties [35]. Deregulation of alternative splicing in host cell emerges as an important target for blocking gene expression of infected cells and for evading antiviral responses

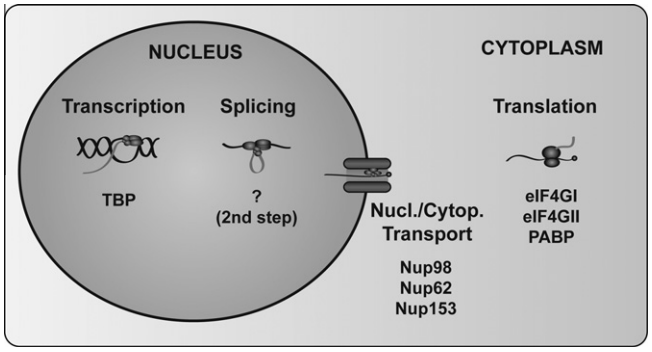

Fig. 4. PV $2A^{pro}$ interferes with cellular RNA metabolism. PV $2A^{pro}$ can mediate proteolysis of transcription factors (TBP), unknown components of the splicing machinery (at the second catalytic step), structural components of nuclear pore involved in import/export of RNAs and proteins (NUPs 62, 98 and 153) and canonical translation initiation factors (eIF4GI, eIF4GII and PABP). These cleavages by this viral protease would block and reprogram the host antiviral gene responses.

to ensure the progression of viral infection and to impair the establishment of a hostile cellular environment. Moreover, our present observations add further support to the notion that successful PV infection is based on modification of RNA metabolism. This can be accomplished by the action of $2A^{pro}$ on post-transcriptional regulatory events involving pre-mRNA splicing, mRNA transport and its translation (Fig. 4). Further efforts are needed to explore in detail this new role of PV $2A^{pro}$ in controlling alternative splicing in infected cells and to identify the host splicing factor(s) targeted by this viral protein.

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