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SF2/ASF Regulates Proteomic Diversity by Affecting the Balance Between Translation Initiation Mechanisms

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ABSTRACT

Post-splicing activities have been described for a subset of shuttling serine/arginine-rich splicing regulatory proteins, among them SF2/ASF. We showed that growth factors activate a Ras-PI 3-kinase-Akt/PKB signaling pathway that not only modifies alternative splicing of the fibronectin EDA exon, but also alters in vivo translation of reporter mRNAs containing the EDA binding motif for SF2/ASF, providing two co-regulated levels of isoform-specific amplification. Translation of most eukaryotic mRNAs is initiated via the scanning mechanism, which implicates recognition of the m7G cap at the mRNA 5′-terminus by the eIF4F protein complex. Several viral and cellular mRNAs are translated in a cap-independent manner by the action of *cis*-acting mRNA elements named internal ribosome entry sites that direct internal ribosome binding to the mRNA. Here we use bicistronic reporters that generate mRNAs carrying two open reading frames, one translated in a cap-dependent manner while the other by internal ribosome entry site-dependent initiation, to show that in vivo over-expression of SF2/ASF increases the ratio between cap-dependent and internal ribosome entry site-dependent translation. Consistently, knocking-down of SF2/ASF causes the opposite effect. Changes in expression levels of SF2/ASF also affect alternative translation of an endogenous mRNA, that one coding for fibroblast growth factor-2. These results strongly suggest a role for SF2/ASF as a regulator of alternative translation, meaning the generation of different proteins by the balance among these two translation initiation mechanisms, and expand the known potential of SF2/ASF to regulate proteomic diversity to the translation field. J. Cell. Biochem. 107: 826–833, 2009. © 2009 Wiley-Liss, Inc.

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Serine/arginine-rich (SR) proteins, together with heterogeneous nuclear ribonucleo-proteins (hnRNPs) are the best-characterized constitutive and alternative splicing regulatory factors. Alternative splicing is a powerful mechanism in terms of expanding protein diversity derived from a given genome as well as in regulating gene expression and cellular function. There is a growing research field exploring the influence of extracellular cues on alternative splicing regulation through diverse signaling pathways [Blaustein et al., 2007; Stamm, 2008].

SR proteins constitute a family of evolutionarily conserved polypeptides that comprise one or two N-terminal RNA-recognition motives (RRMs), and a region of variable length that contains repetitive Arg-Ser dipeptides (RS domain) [Graveley, 2000]. The RS domain is extensively phosphorylated on Ser residues by a number of SR protein kinases [Gui et al., 1994; Colwill et al., 1996; Rossi et al., 1996; Ko et al., 2001; Hernandez et al., 2004; Blaustein et al., 2005].

Even though they were originally isolated as splicing regulators, it is now known that certain SR proteins, in particular some of them capable of nucleo-cytoplasmic shuttling [Cáceres et al., 1998], also play important roles in mRNA export [Huang and Steitz, 2001], mRNA stability [Lemaire et al., 2002], nonsense-mediated decay [Zhang and Krainer, 2004], and even translation [Sanford et al., 2004, 2005; Blaustein et al., 2005; Swartz et al., 2007]. In particular, SF2/ASF stimulates translation of reporter mRNAs, and this activity is increased by the presence of an exonic-splicing enhancer that

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functions as a binding site for this SR protein [Sanford et al., 2004]. The phosphorylation state of SF2/ASF influences its binding to cellular mRNAs, as well as its function in translation [Sanford et al., 2005]. We have reported that Akt/PKB phosphorylates SF2/ASF and postulated that phosphorylation of SF2/ASF in response to extracellular stimuli that activate the Ras-phosphatidylinositol 3-kinase (PI 3-kinase)-Akt/PKB pathway leads to a concerted regulation of alternative splicing and translation, resulting in an increased production of the fibronectin (FN) mRNA isoform containing the alternative exon EDA, and an enhanced translation of reporter mRNAs bearing SF2/ASF binding sites [Blaustein et al., 2004, 2005].

Whether SR proteins, in particular SF2/ASF, stimulate translation initiation, elongation or perhaps mRNA recruitment to translation active sites, has been an intriguing question. Translation of most eukaryotic mRNAs is initiated via the scanning mechanism, which implicates recognition of the mRNA 5′ end and its m7G-cap structure by the translation initiator complex eIF4F. Translation initiation can also be mediated by *cis*-acting mRNA elements named internal ribosome entry sites (IRESs) that target ribosome binding to an initiation codon without recognition of the m7G-cap by eIF4F [Hellen and Sarnow, 2001]. Several viral RNAs, are translated when cap-dependent initiation within the infected host cell is down-regulated by viral proteins [Pelletier and Sonenberg, 1988]. Many eukaryotic cellular mRNAs can also be translated via a cap-independent, IRES-mediated mechanism [Arnaud et al., 1999; Hellen and Sarnow, 2001].

IRES-mediated translation has been usually considered an alternative translation mechanism, in opposition to the canonical cap-dependent one. We refer here to ''alternative translation'' as the generation of different proteins or protein isoforms by the balance among these two translation initiation mechanisms.

In order to test whether the stimulatory effect of SF2/ASF on protein synthesis is dependent on the mechanism of translation initiation, we analyzed SF2/ASF activity in cap- and IRES-dependent translation. Moreover, we wondered whether this SR protein may not only function as a regulator of alternative splicing but also of alternative translation, expanding its already described potential to regulate protein diversity even further.

We transfected bicistronic vectors, carrying either cellular or viral IRESs, into cultured cells to show that over-expression of SF2/ASF but not of the non-shuttling SR protein SC35, increases cap-dependent over IRES-dependent translation ratio. We demonstrate that knock-down of endogenous SF2/ASF has the opposite effect. These effects on alternative translation rely on the presence of any of the SF2/ASF modular domains. Furthermore, we show that changes in expression levels of SF2/ASF also affect translation patterns of the endogenous fibroblast growth factor-2 (FGF-2) mRNA, strongly suggesting a role for SF2/ASF as a regulator of alternative translation.

MATERIALS AND METHODS

CELL CULTURE

Hep3B and HeLa cells were grown in DMEM (Invitrogen), supplemented with 10% (v/v) FBS. HeLa cells were used for FGF-2 Western blot analysis, since Hep3B produced very low quantities of this protein.

CELL TREATMENTS

Amino acid starvation of Hep3B cells was performed as described [Yaman et al., 2003].

PLASMIDS, SMALL INTERFERING RNAs (siRNAs), AND TRANSFECTIONS

Bicistronic vectors [Yaman et al., 2003; Kalliampakou et al., 2005]; EDA minigene plasmid, pSVEDA/mFN [Cramer et al., 1997]; pCMV6-HA-AKT Myr [Ahmed et al., 1997]; pCGT7-SF2/ASF and mutants, as well as pCGT7-SC35 [Cáceres et al., 1997; Sanford et al., 2005] were previously described.

SF2/ASF siRNA and non-related siRNAs used were already described [Blaustein et al., 2005]. hnRNP A1 siRNA used (sense strand) was 5′-CAGCUGAGGAAGCUCUUCA-3′ (Invitrogen).

In every case, 40 pmol of siRNA were added per 35-mm tissue culture well. Transfections were performed using Lipofectamine or Lipofectamine 2000 (Invitrogen) according to manufacturer instructions. RNA and protein extracts were prepared simultaneously, 72 h after transfection in knock-down experiments or 48 h after transfection in the rest of the experiments.

LUC AND CAT ASSAYS

Firefly luciferase (LUC) activity in cell lysates was measured using the Luciferase Assay System (Promega) as described [Blaustein et al., 2004]. To measure chloramphenicol acetyl-transferase (CAT) activity, 40 μl of the supernatant obtained from cell lysates were heated at 60°C for 10 min and assayed in a total volume of 100 μl containing 0.1 μCi of ^{14}C-chloramphenicol and 25 μg of *n*-butyryl CoA, at 37°C for 2 h. The product, *n*-butyrylated chloramphenicol, was quantified with a liquid scintillation counter following extraction with mixed xylenes.

WESTERN BLOT ANALYSIS

Protein extract preparation and Western blot analysis were performed as described [Blaustein et al., 2004]. The antibodies used were anti-extracellular signal-regulated kinase 2 (ERK2, Santa Cruz Biotechnology), anti-FGF-2 (provided by C. Lanari), anti hnRNP A1 (4B10, Sigma), anti-SF2/ASF (mAb 103, provided by A.R. Krainer), anti-HA (Covance), and anti-T7 (Novagen).

RNA ISOLATION AND RADIOACTIVE RT-PCR AMPLIFICATION

Total RNA purification from cultured cells and radioactive RT-PCR analysis of FN EDA minigene-derived transcripts were carried out as described [Cramer et al., 1999].

Bicistronic mRNA levels were quantified by quantitative RT-real time PCR using a Realplex^2 (Eppendorf), Taq Polymerase (Invitrogen), 1:30,000 SYBR green dye (Molecular Probes) and the following oligonucleotide primers that anneal to LUC sequences: ''ffLUC-For'': 5′-CGGTCGGTAAAGTTGTTCC-3′ and ''ffLUC-Rev'': 5′-TGTAGCCATCCATCCTTGT-3′. PCR cycles were: 94°C, 15 s; 67°C, 20 s and 72°C, 30 s.

Regular RT-PCR amplification of the CAT/cat-1IRES/LUC bicistronic mRNA was carried out with the following oligonucleo-

tide primers annealing to sequences corresponding to the 5′end of the CAT ORF and the 3′ end of the LUC ORF: "CAT-For": 5′-ATCCCAATGGCATCGTAAAG-3′ and "LUC-Rev": 5′-GATCTTT-CCGCCCTTCTTG-3′. PCR cycles were: 94°C, 30 s; 58°C, 30 s and 72C°, 4 min.

RESULTS

SF2/ASF, BUT NOT SC35, STIMULATES CAP- OVER IRES-DEPENDENT TRANSLATION RATIO OF BICISTRONIC REPORTERS IN VIVO

To test a possible role for SF2/ASF as an alternative translation regulator we made use of a bicistronic reporter that contains two open reading frames (ORFs), CAT at the 5′end and LUC at the 3′end (Fig. 1a). Translation of the first ORF is cap-dependent, while translation of the second one is cap-independent but relies on the presence of the cationic amino acid transporter (cat1) IRES, a cellular IRES placed between the two ORFs that becomes activated by aminoacid deprivation [Yaman et al., 2003]. In this system, CAT and LUC are translated from the same mRNA, ruling out effects due to different transfection efficiencies or different mRNA levels and allowing data analysis of cap- over IRES-dependent translation in terms of CAT/LUC ratios. We first validated that this construct can effectively report alternative translation regulation when transiently transfected into Hep3B cells by exposing the cells to aminoacid starvation or, in contrast, by over-expressing Akt Myr, a constitutively active mutant of Akt/PKB. Aminoacid starvation

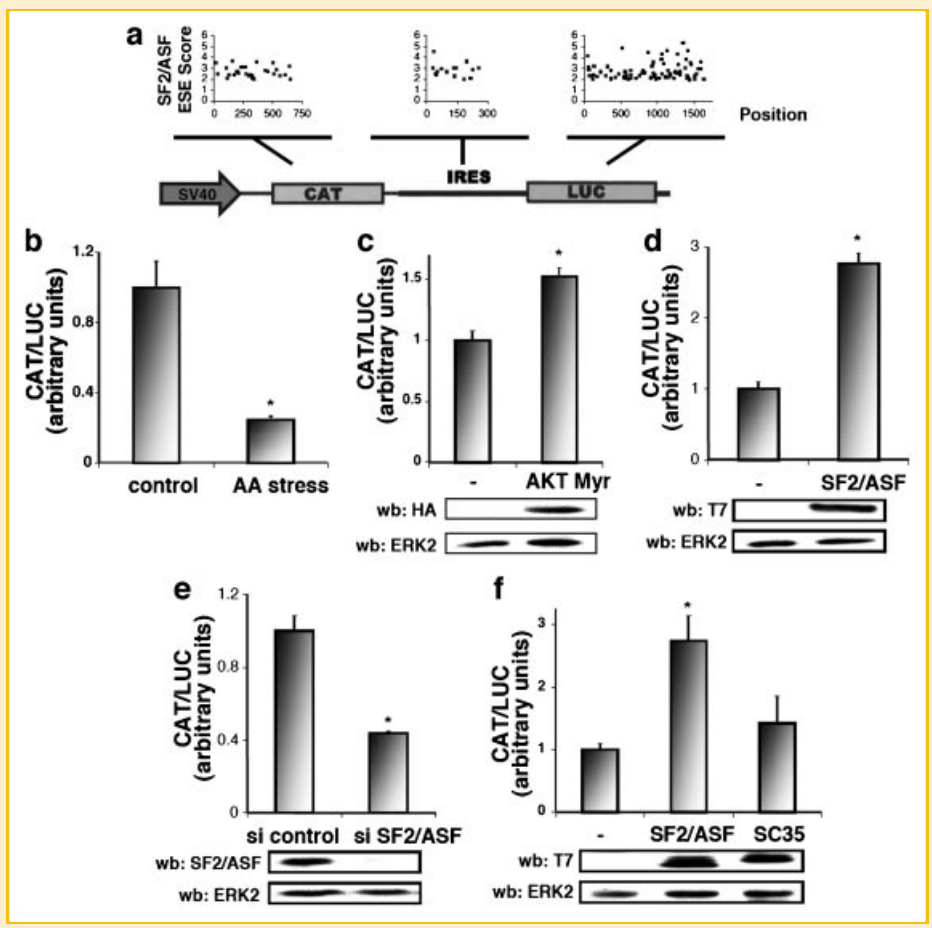

Fig. 1. SF2/ASF regulates alternative translation of a bicistronic reporter in vivo. a: Scheme of the bicistronic CAT/LUC reporter containing the cat1 IRES sequence, used for in vivo translation assays. Potential SF2/ASF binding sites are shown for each region of the reporter according to their position and ESEfinder Score. b: Hep3B cells were transfected with the CAT/cat1-IRES/LUC bicistronic reporter and 5 h later were aminoacid deprived (AA stress) or left untreated (control). CAT and LUC activities were measured and CAT/LUC ratios calculated. c: Hep3B cells were co-transfected with the bicistronic reporter and 500 ng of pCMV6-HA-Akt Myr (AKT Myr) or empty vector (–). Western blot analysis with an antibody against HA epitope tag confirmed Akt/PKB expression from the transfected construct. Western blot against ERK2 is shown in every case as a loading control. d: Hep3B cells were co-transfected with the bicistronic reporter and 200 ng of pCGT7-SF2/ASF (SF2/ASF) or empty vector (–). Over-expression of T7-SF2/ASF was confirmed by Western blot with an antibody against T7. e: Hep3B cells were co-transfected with the bicistronic reporter and siRNA against SF2/ASF (si SF2/ASF) or control (si control). Endogenous SF2/ASF knock-down was confirmed by Western blot analysis with an antibody against SF2/ASF. f: Hep3B cells were co-transfected with the bicistronic reporter and 200 ng of pCGT7-SF2/ASF (SF2/ASF), pCGT7-SC35 (SC35) or empty vector (–). Over-expression of T7-SF2/ASF and T7-SC35 was confirmed by Western blot. Asterisk (*) indicates $P < 0.05$ (Student's t-test). CAT and LUC activities as well as different protein levels were measured after 48 h in over-expression assays and after 72 h in knock-down experiments.

decreased CAT/LUC ratio in Hep3B cells (Fig. 1b), consistently with the already reported enhancement of cat1 IRES activity and inhibition of cap-dependent translation in other cell lines upon this culture condition [Fernandez et al., 2001; Yaman et al., 2003]. On the contrary, Akt Myr over-expression increased CAT/LUC ratio (Fig. 1c), which is predictable given that Akt/PKB enhances translation at the level of cap-dependent recruitment of RNAs to polysomes by modifying translation initiation factor activity [Gingras et al., 1998]. As predicted by ESEfinder© [Smith et al., 2006], both cistrons as well as the IRES sequence contain several SF2/ASF binding sequences (Fig. 1a). After over-expression of SF2/ASF we found a consistent enhancement of CAT/LUC ratio (Fig. 1d). In this context, SF2/ASF effect seems to be achieved by stimulating cap-dependent translation, while leaving IRES-dependent translation unaffected, as indicated by the analysis of absolute values of CAT and LUC activities normalized to bicistronic mRNA levels for each culture condition (Supplementary Fig. 1). Furthermore, knock-down of endogenous SF2/ASF by siRNA caused the opposite effect than its over-expression (Fig. 1e). Over-expression of SC35, a non-shuttling SR protein, failed to alter the CAT/LUC ratio (Fig. 1f), which is consistent with its inability to leave the cell nucleus. Therefore, SF2/ASF effects on alternative translation cannot be extended to all SR proteins and most likely this regulation may depend on its shuttling capacity. Moreover, the effect of SF2/ASF on alternative translation is not restricted to a particular IRES, as demonstrated by using other bicistronic vectors containing either the immunoglobulin heavy chain binding protein (BiP) cellular IRES or the Hepatitis C virus (HCV) IRES between the two ORFs (Fig. 2).

We have shown previously that increased levels of either Akt/PKB or SF2/ASF exert similar effects not only on the regulation of alternative splicing but also on cap-dependent translation of reporter mRNAs carrying SF2/ASF binding sites [Blaustein et al., 2005]. The pool of cellular proteins can vary according to alterations in the balance between cap- and IRES-mediated translation initiation upon different cellular physiological or pathological conditions [Pickering and Willis, 2005]. Thus, we extend those results suggesting that SF2/ASF could be considered a regulator of alternative translation, defined here as the generation of different proteins by the balance among these two translation initiation mechanisms.

SF2/ASF POTENTIAL AS AN ALTERNATIVE TRANSLATION REGULATOR CORRELATES WITH ITS ABILITY TO INFLUENCE ALTERNATIVE SPLICING

To compare SF2/ASF ability to regulate alternative splicing with its capacity to control alternative translation, we designed a set of experiments to test a possible correlation between both processes. Alternative translation was analyzed with the bicistronic reporter described above (Fig. 1a), while alternative splicing was evaluated with an FN EDA splicing reporter minigene (Fig. 3a), measuring the proportion of EDA-containing over EDA-lacking mRNAs by RT-PCR as already described [Cramer et al., 1997]. We analyzed not only the outcome of changing SF2/ASF expression levels but also the consequences of deleting any of the modular domains from SF2/ASF. Interestingly, we found a correlation between SF2/ASF effects on alternative splicing and alternative translation, suggesting that

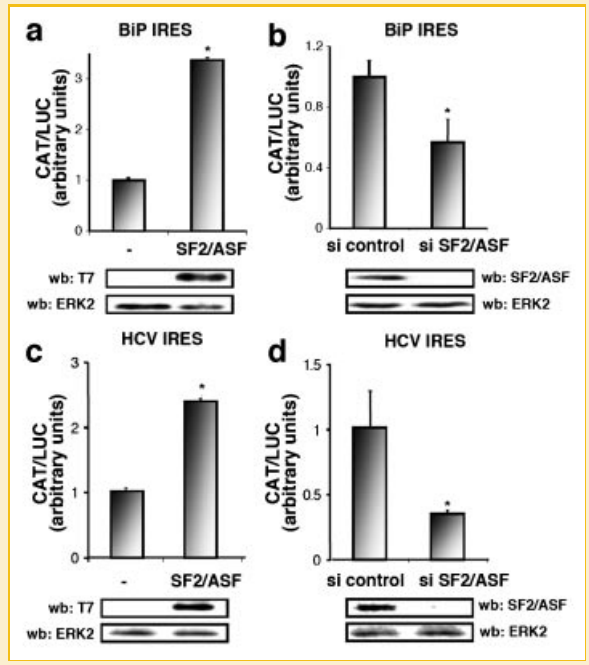

Fig. 2. SF2/ASF effect on alternative translation can be extended to other IRES. a: Hep3B cells were co-transfected with the CAT/BiP-IRES/LUC bicistronic reporter and 200 ng of pCGT7-SF2/ASF or empty vector (−). CAT and LUC activities were measured after 48 h. Over-expression of T7-SF2/ASF was confirmed as already indicated. b: Hep3B cells were co-transfected with the bicistronic CAT/BiP-IRES/LUC reporter and siRNA against SF2/ASF (si SF2/ASF) or control (si control). CAT and LUC activities were measured after 72 h. Endogenous SF2/ASF knock-down was confirmed by Western blot analysis with an antibody against SF2/ASF. c,d: Idem a and b but using a bicistronic reporter carrying the viral HCV IRES. Asterisk (*) indicates $P < 0.05$ (Student's *t*-test).

the abundance of this protein may provide a strong coordination of both processes that results in regulating proteome diversity (Fig. 3b, left and middle panels). In this cellular context and with the reporters used here, despite the fact that deletion of each SF2/ASF domain (RRM1, RRM2, or RS) affects alternative splicing and alternative translation to different extents, all of them seem to be crucial for SF2/ASF full function in both analyzed processes. Moreover, over-expression of SC35 has similar consequences to the over-expression of SF2/ASF on alternative splicing patterns of the FN EDA exon, it favors the production of the FN EDA containing mRNA isoform (Fig. 3b, right panel) [Cramer et al., 1999]. However, as already mentioned it has no effect on cap- over IRES-dependent translation ratio. These results suggest that a given signaling pathway influencing SF2/ASF abundance and/or activity would impact both, alternative splicing and alternative translation, relying on the same SF2/ASF modular domains.

SF2/ASF MODULATES ALTERNATIVE TRANSLATION OF ENDOGENOUS FGF-2 mRNA

We then asked whether SF2/ASF could also modulate alternative translation from an endogenous target. We worked with the human FGF-2 mRNA, which contains five in-frame translation initiation

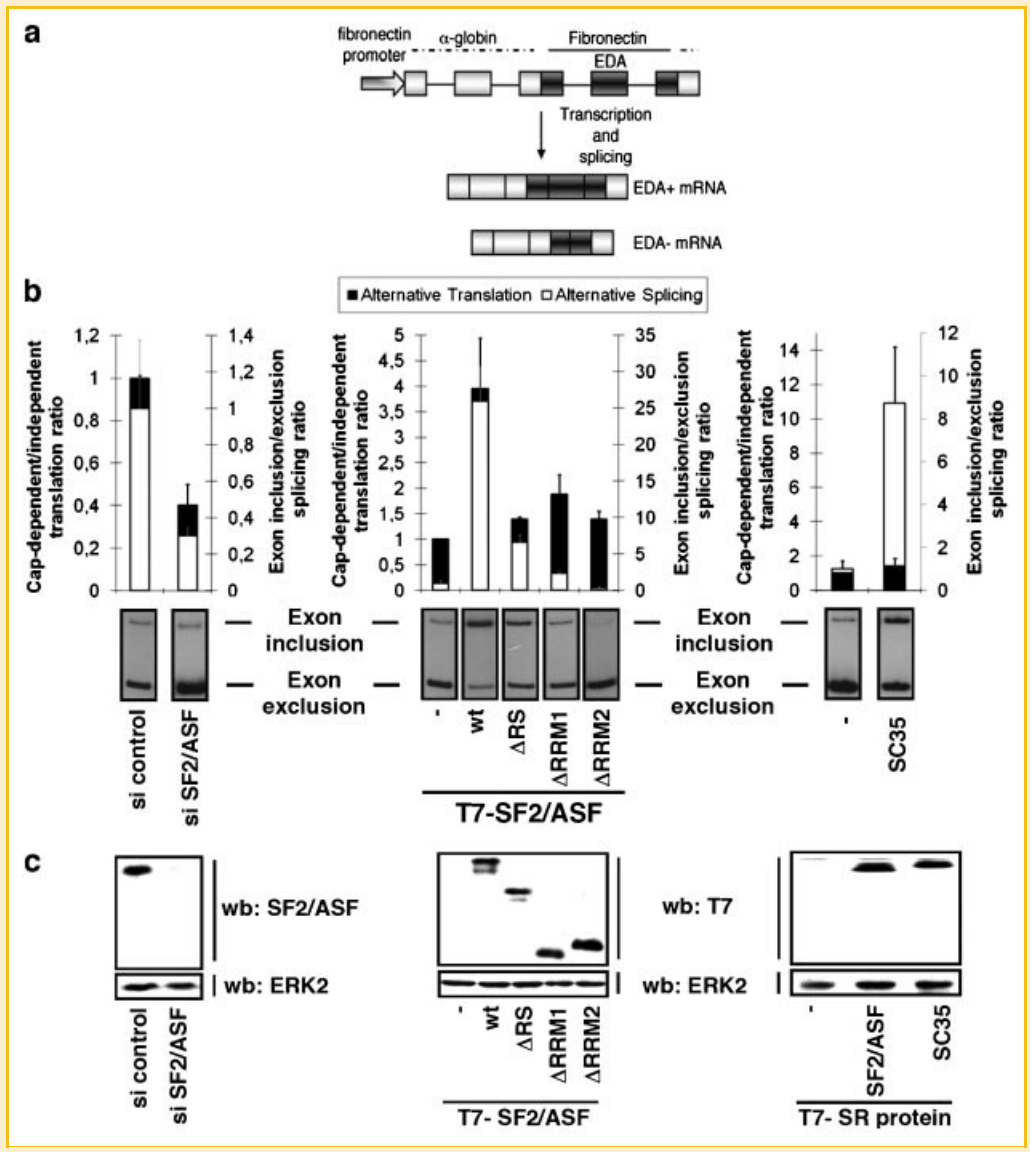

Fig. 3. SF2/ASF potential as an alternative translation regulator correlates with its ability to influence alternative splicing. a: FN EDA splicing reporter minigene used for transient transfections and mRNAs derived from it, either containing (EDA+) or lacking (EDA−) the alternative exon EDA. b: Analysis of cap- over IRES-dependent translation (CAT/LUC ratios; black bars) versus alternative splicing (exon inclusion/exon exclusion ratio; white bars). Hep 3B cells were transfected with either the bicistronic reporter or the FN splicing reporter. For each case, cells were co-transfected with either siRNA against SF2/ASF, siRNA control, pCGT7-SF2/ASF (wt), its deletion mutants (ΔRS, ΔRRM1, or ΔRRM2), pCGT7-SC35 or empty vector (−). Cells were harvested 48–72 h later and processed simultaneously for CAT and LUC analysis and for RNA purification and RT-PCR analysis to evaluate EDA+ and EDA− mRNA isoforms (exon inclusion/exon exclusion ratio). c: SF2/ASF knock-down as well as similar expression levels of T7-SC35, T7-SF2/ASF and its mutants were confirmed by Western blot.

codons (four CUGs and one AUG) that give rise to five FGF-2 protein isoforms that vary in their amino-terminal extensions. While the 34-kDa isoform is initiated by a cap-dependent translation mechanism, the smaller isoforms are initiated by an IRES-mediated process (Fig. 4a) [Arnaud et al., 1999]. These different FGF-2 isoforms have different sub-cellular localizations and functions, and their expression is translationally regulated depending on the cell type or cell physiological status [Bonnal et al., 2005].

We analyzed the expression pattern of FGF-2 by Western blot with an antibody that recognizes all FGF-2 protein isoforms and found that either over-expression of SF2/ASF or knock-down of the endogenous protein changed translation patterns. Particularly, when SF2/ASF was depleted the proportion of isoforms translated by an IRES-dependent mechanism increased compared to those translated by cap-mediated initiation, as reflected by a decrease in cap/IRES-dependent translation ratio (Fig. 4b, left panel). Over-expression of SF2/ASF caused the opposite effect, as reflected by an increased in cap/IRES-dependent translation ratio (Fig. 4b, middle panel). As already reported, hnRNP A1, a known antagonist of SF2/ASF within the context of splicing regulation [Cáceres et al., 1994], stimulates translation of FGF-2 IRES-dependent isoforms. Consistently, knocking-down hnRNP A1 also

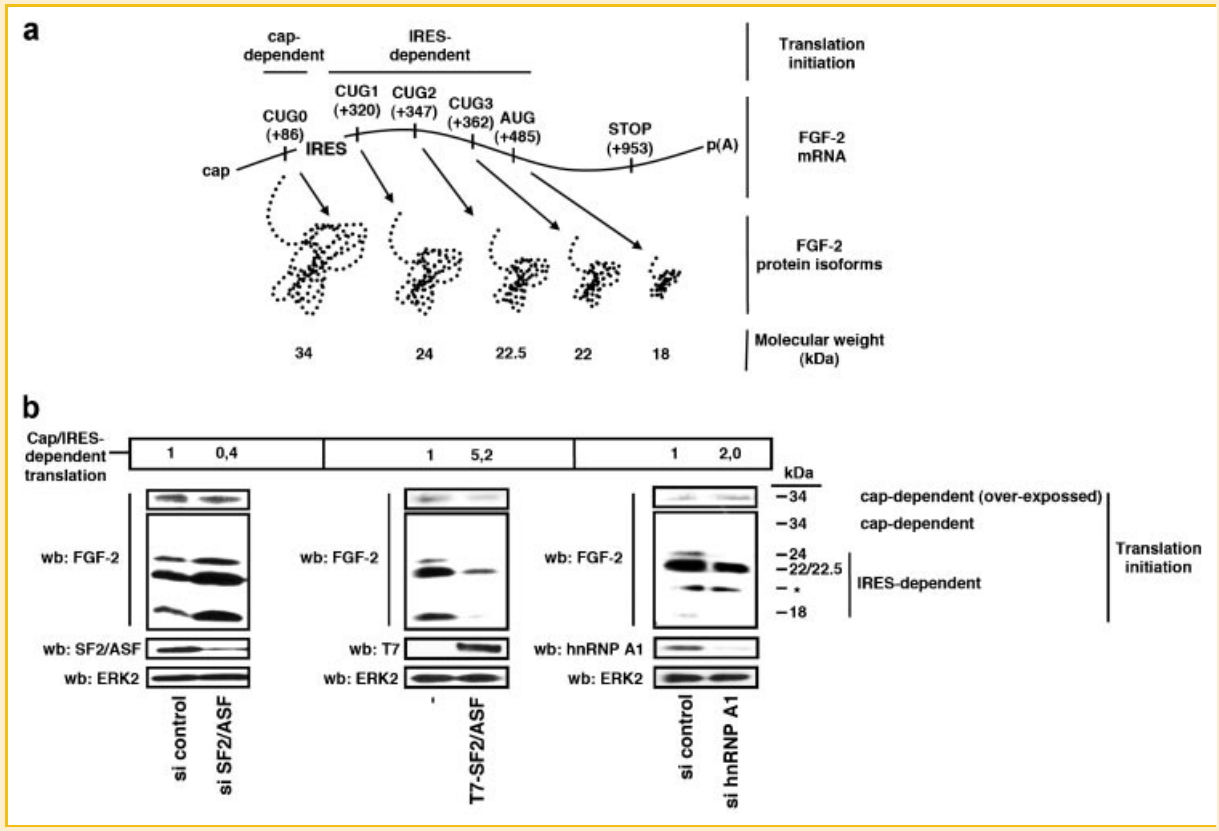

Fig. 4. SF2/ASF changes alternative translation of endogenous FGF-2 mRNA. a: Scheme of the human FGF-2 mRNA and protein isoforms derived from it by alternative translation. IRES position is indicated. b: HeLa cells were transfected with either siRNA against SF2/ASF (si SF2/ASF), siRNA against hnRNP A1 (si hnRNP A1), control siRNA (si control), pCGT7-SF2/ASF or control vector and harvested 72 h later. Western blot analysis with antibodies against FGF-2, SF2/ASF, hnRNP A1 and ERK2 is shown. For quantification of cap- over IRES-dependent translation, for each lane the signal of the cap-dependently translated isoform was divided by the sum of the signals of all IRES-dependently translated isoforms, achieved by Image J 1.14, NIH. Asterisk (*) indicates $P < 0.05$ (Students *t*-test).

increases cap/IRES-dependent translation ratio (Fig. 4b, right panel) in this cellular context. These results are consistent with those obtained with bicistronic reporters, altogether showing that alterations in SF2/ASF expression levels modulate the ratio between cap- and IRES-dependent translation in the same manner. Furthermore, they add a fourth example in which alternative translation of an IRES-containing mRNA is regulated by SF2/ASF, with the advantage that in the case of FGF-2 we are not measuring two different enzymatic activities. More important, they show that SF2/ASF can indeed affect alternative translation of endogenous mRNAs. SF2/ASF abundance and/or activation may thus be, not only a way to change alternative splicing patterns, but also to change alternative translation patterns of IRES-containing mRNAs.

DISCUSSION

We describe here that SF2/ASF can regulate alternative translation, meaning the generation of proteomic diversity by the balance between two different translation initiation mechanisms. Over-expression of SF2/ASF increases the ratio between cap- and IRES-dependent translation of mRNAs derived from transfected bicistronic vectors, carrying either different eukaryotic cellular IRES or a viral IRES. A similar effect is observed on translation patterns from the FGF-2 endogenous mRNA. Interestingly, a pool of endogenous mRNAs associated with SF2/ASF not only in the nucleus but also in the cytoplasm and in actively translating ribosomes has been recently identified suggesting a coordination between splicing and translation by this protein [Sanford et al., 2008]. Our results also show that knocking-down SF2/ASF decreases cap- over IRES-dependent translation ratio for all transcripts tested. Whether these observed changes are achieved by exactly the same molecular mechanism awaits further investigation. In this respect, considering our current results together with recently published ones from other laboratories, we can speculate about two possible regulatory scenarios not necessarily mutually exclusive. SF2/ASF could be favoring cap-dependent translation by releasing the interaction between eIF4E and 4E-binding protein, as proposed by Cáceres and coworkers [Michlewski et al., 2008]. Alternatively, SF2/ASF can be exerting its effect by competing out its known antagonist hnRNP A1 [Cáceres et al., 1994], which can function as a stimulator of IRES-dependent translation of FGF-2 mRNA ([Bonnal et al., 2005] and Fig. 4b, right panel). Interestingly, it has been

proposed that hnRNP A1 is not a general enhancer of IRES-dependent translation but its effect is dependent on the particular IRES-containing mRNA analyzed [Bonnal et al., 2005], which is consistent with the fact that different IRES may recruit different IRES trans-acting factors and/or different canonical translation initiation factors, as explored by Cáceres and co-workers [Michlewski et al., 2008]. Despite the fact that over-expression of SR proteins SC35 and SF2/ASF triggers similar changes in alternative splicing patterns of FN EDA exon, neither SC35, which is incapable of nucleo-cytoplasmic shuttling, nor SF2/ASF mutants lacking any of the modular domains, can mimic the full effect elicited by SF2/ASF on the regulation of alternative translation described here. The fact that deletion of each SF2/ASF domain differentially impacts alternative splicing and alternative translation may reflect either the ability of each of these domains to establish different protein-protein and protein-RNA interactions, or the requisite of differential phosphorylation levels for this factor activities [Cáceres and Krainer, 1993; Huang et al., 2004; Sanford et al., 2005; Tintaru et al., 2007]. Further investigation would be required to address this topic. In this respect, SF2 RRM2 domain has been shown to be critical for the activity of this factor in alternative splicing [Dauksaite and Akusjarvi, 2002; Chiodi et al., 2004], as well as for its interaction with PP2A and mTOR associated with the activation of cap-dependent translation initiation [Michlewski et al., 2008].

Our results do not address whether SF2/ASF exerts its effects on alternative translation by direct binding to the mRNA. However, insertion of SF2/ASF binding sites into a LUC reporter enhances cap-dependent translation both in vivo and in vitro, proving a direct role for SF2/ASF in translation [Sanford et al., 2004]. Interestingly, SF2/ASF has been shown to bind to FGF-2 mRNA by RNA-affinity chromatography [Bonnal et al., 2005]. As shown in Figure 1a, several putative SF2/ASF binding sites can be found in the bicistronic reporters used here. Nevertheless, effects of SF2/ASF on translation that could be independent from its binding to these reporters cannot be ruled out. In this way, it has been shown that over-expression of SF2/ASF regulates the splicing pattern of *RPS6KB1* and *MNK2* genes, both of which are involved in translation regulation [Karni et al., 2007].

Viral strategies of infection are normally associated with the inhibition of different steps of host cell gene expression and/or metabolism in order to maximize viral replication. It has been reported that several SR proteins become inactivated by dephosphorylation during certain viral infections suggesting that pre-mRNA splicing may be a common target of viral interference [Kanopka et al., 1998; Huang et al., 2002; Sciabica et al., 2003]. Furthermore, different virus inhibit host cell cap-dependent translation as a key infection strategy [Vagner et al., 2001]. Thus, it is possible to speculate that SR protein inactivation after viral infection could be important not only to interfere with the splicing machinery but also to repress host cell cap-dependent translation machinery favoring IRES translation of viral RNAs.

Cap-independent translation is not restricted to viral RNAs. Several eukaryotic mRNAs possess IRES sequences. Alternative translation appears to be physiologically regulated during mitosis, quiescence, differentiation, and under stress conditions [Vagner et al., 2001]. Thus, alteration of SR protein activity, which is associated with changes in alternative splicing in all these processes [Blaustein et al., 2007], should be also investigated within the field of translation regulation. A role for the SR protein SRp38 in translation repression in response to heat shock has been proposed [Shin et al., 2005]. Furthermore, increased hnRNP A1 phosphorylation upon stress stimuli leads to cytoplasmic accumulation of hnRNP A1 bound to mRNAs that are recruited to stress granules [Guil et al., 2006], cytoplasmic domains that harbor translationally arrested mRNAs. A physiological coupling between alternative splicing and alternative translation has been revealed by experiments showing that inclusion of alternatively spliced sequences in the ornithine decarboxylase mRNA 5′ UTR facilitates cap-independent, IRES-mediated translation initiation in pancreatic tumor cells, leading to accumulation of ornithine decarboxylase protein that may contribute to cellular transformation [Pyronnet et al., 2005].

As already mentioned, hnRNP A1 enhances IRES-dependent translation of cellular FGF-2. More recently, it has been described that the shuttling SR protein SRp20 favors poliovirus IRES translation [Bedard et al., 2007]. Thus, the expression level, together with the phosphorylation/activation balance of certain SR proteins and hnRNPs, might be key objectives not only for splicing regulation but also for alternative translation regulation, and even for viral and antiviral strategies.

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