FEBS Letters 579 (2005) 4138–4144 FEBS 29751

ASF/SF2 and SC35 regulate the glutamate receptor subunit 2 alternative flip/flop splicing

Trine Elkjær Crovato*, Jan Egebjerg

Department of Molecular Biology, University of Aarhus, C.F. Møllers Alle 130, DK-8000 Aarhus C, Denmark

Received 25 April 2005; revised 17 June 2005; accepted 21 June 2005

Available online 6 July 2005

Edited by Horst Feldmann

Abstract The properties of the glutamate receptor subunits 1–4 (GluR1–4) are influenced by the alternative splicing of two homologous and mutually exclusive exons flip and flop. The flip form is most abundant during early development, while the flop form is dominant in adults. From transfections with a GluR2 mini-gene we show that flip is the preferred splice form in all tested cell lines, but coexpression of the SR-proteins ASF/SF2 and SC35 increases the flop to flip splice ratio. The increased flop incorporation depends on ASF/SF2- and SC35-dependent enhancer elements located in the flop exon, which stimulate the splicing between the flop exon and the preceding exon 13.

Keywords: Alternative splicing; Glutamate receptor; GluR2; SR-proteins; Splicing enhancer

1. Introduction

Alternative pre-mRNA splicing plays a central role in regulation of gene-expression in higher eukaryotes. More than half of the human gene products undergo alternative splicing [1], considerably enlarging the complexity of the proteome [2].

Regulation of alternative splicing is complex, and a single alternative splicing event is in many cases under the control of several exonic and intronic *cis*-elements, termed enhancers and silencers, which typically function as recognition sites for *trans*-acting splicing factors that stimulate or repress splicing (e.g. [3–6] or reviewed in [7]). One of the best characterised classes of splicing factors is the family of serine/arginine-rich (SR) proteins, which are involved in both constitutive and alternative splicing [8,9]. SR-proteins activate splicing of many regulated exons via binding to exonic enhancer elements (see, for example, [10–13]) from where they are proposed to recruit factors of the splicing machinery to the adjacent splice sites [9,14]. Individual SR-proteins exhibit distinct substrate specificities, but since RNA recognition sequences for the different SR-proteins are degenerate and overlapping [15–18], the prediction of SR-protein dependent enhancers is uncertain.

The glutamatergic system is by far the most abundant excitatory neurotransmitter system in the mammalian brain. There are different subtypes of ionotropic glutamate receptors, and the AMPA- (α-amino-3-hydroxy-5-methyl-4-isoazolepropionic acid) subtype plays a central role in the fast transmission. The AMPA receptors are glutamate gated ion channels formed as tetrameric complexes of the subunits GluR1–4 (or GluRA-D) (reviewed in [19,20]). The genes encoding the subunits GluR1–4 all contain a pair of highly homologous exons, flip and flop, which are spliced in a mutually exclusive manner (Fig. 1) [21]. Receptors formed of subunits of either the flip or flop splice variants exhibit different kinetic and pharmacological properties, where the flip variants desensitise slower than the flop forms and are also more sensitive to compounds, which block desensitisation, such as cyclothiazide [21,22]. Furthermore, the flip/flop alternative splicing is regulated in a tissue and developmental stage specific manner with subunit specific variations [21,23–25]. For the GluR2 subunit, the flip splice form dominates at the embryonic state and in early life, while the flop form takes over in a tissue-specific manner at later stages [25]. So far, very little is known about the molecular basis for regulation of the complex flip/flop splicing.

In this report, we examine the importance of the SR-proteins ASF/SF2 and SC35 in regulation of the GluR2 flip/flop splicing by transient transfections in the neuroblastoma cell line N2a. We show that both proteins shift the flip/flop splicing balance, inducing a higher flop to flip ratio. Furthermore, by site-directed mutagenesis, we identify *cis*-regulatory elements within the flop exon that respond to the splicing factors.

2. Materials and methods

2.1. Plasmid construction and mutagenesis

The R2L mini-gene sequence was amplified from genomic DNA purified from rat liver by methods previously described [26] using the primers 5′ CGC GCG CTC GAG CTT TTT CTG CAG AGA TCT AAA ATC GCA GTG and 5′ CGC GCG CTC GAG CCT CGT TCC TAC CCC CTA AAT TTT AAC and the Expand™ Long Template PCR System (Boehringer Mannheim). The PCR-product was cloned as an *Xho*I fragment into pcDNA3 (Invitrogen). Point mutations of R2L were made by site-directed mutagenesis by consecutive overlapping PCRs.

The ASF/SF2, SC35, and U2AF65 expression plasmids were kindly provided by G.R. Screaton: The cDNAs were cloned into the multiple cloning site of the pCDM8 vector and expressed from the CMV promoter. The U1 70k expression plasmid (pBC-U170k [27]) was a gift from D.J. Keene.

*Corresponding author. Fax: +45 86 19 65 00.
E-mail address: tel@mb.au.dk (T.E. Crovato).

Abbreviations: ASF/SF2, alternative splicing factor/splicing factor 2; SC35, Spliceosome component of 35 kDa; AMPA, α-amino-3-hydroxy-5-methyl-4-isoazolepropionic acid; GluR, glutamate receptor; SR-protein, serine/arginine-rich protein

0014-5793/$30.00 © 2005 Published by Elsevier B.V. on behalf of the Federation of European Biochemical Societies.
doi:10.1016/j.febslet.2005.06.044

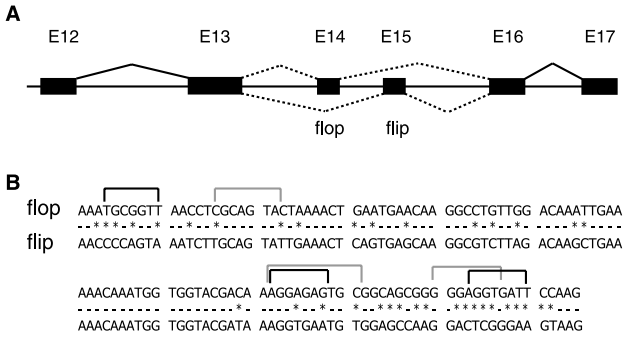

Fig. 1. (A) Schematic representation of the genomic organisation of the alternative exons of the glutamate receptors subunits. Exons 12 to 17 are shown (not drawn to scale). Exons are represented by boxes and introns by lines. The mutually exclusive splicing pathways are shown by dotted lines. (B) Alignment of the sequences of the flip and flop exons of the rat GluR2. Putative recognition sites for ASF/SF2 (grey) and SC35 (black) are indicated by brackets.

2.2. Cell culturing, transfections and RNA purification

The mouse neuroblastoma cell line N2a was cultured at 37 °C and 7.5% CO_2 in Dulbecco's modified Eagle's medium with glutamax (Invitrogen) supplemented with 5% (v/v) fetal bovine serum (FBS) and 1% penicillin-streptomycin (100 units/ml–100 μg/ml). Transfection was performed in 6-well plates at 70–90% confluence with 1 μg of mini-gene and 1 μg of SR-protein expression plasmid using Lipofectamine™ 2000 (Invitrogen) as recommended by the manufacturers. RNA was purified after 48 h by the TRIzol method (Invitrogen).

2.3. Reverse transcription (RT) and PCR

Superscript RT II (Invitrogen) was used for reverse transcription of the RNA with a GluR2 exon 16 specific primer (Ex16anti; 5′ CCG GGA CTT GTA GCA GAA CTC) at 42 °C for 1 h. cDNA was amplified by PCR using a T7 primer and the Ex16anti primer. The flip and flop PCR-products are equal in length and differ at only 36 of 590 positions, suggesting a linearity of the flip/flop ratio during the RT-PCR amplification. To verify this assumption, various ratios of GluR2 flip and flop cDNAs were analysed by PCR and primer extension, where we found a linear correlation between the ratios measured by the assay and the actual ratio, and we obtained the same results independently of the number of cycles in the amplification (data not shown).

2.4. Primer extension

A PCR-product of 590 nucleotides corresponding to either flip or flop containing splice products was purified after agarose gel electrophoresis and used as template for extension of primer 5′ [^{32}P]ACA CCT AAA GGA TCC TCA T, which was radiolabelled with [γ-^{32}P]ATP (3000 Ci/mmol; Pharmacia Biotech) using T4 polynucleotide kinase (New England Biolabs). The primer was extended by Thermo Sequenase (Amersham) as recommended by the manufacturers in the presence of 0.3 mM ddCTP and 0.03 mM dGTP, dATP, and dTTP. The extension products were separated on a 15% denaturing polyacrylamide gel and the bands were quantified by PhophorImaging. The level of flop inclusion was determined as the intensity of the signal obtained from extension by 10 nucleotides (flop) relative to the sum of the intensities of the signals obtained from primers extended by 10 and 7 (flip) nucleotides. For samples containing PCR-products of splice variants including both flip and flop (e.g. when cotransfected with SC35) special caution must be taken in the primer extension to correct for contamination of the double inclusion product, which otherwise will result in an overestimate of flop including products. The contamination was quantified using a primer annealing in flop and extending into flip (for the double inclusion product) or exon 16 (for the flop splice form).

2.5. Q-PCR

Q-PCR was performed on a Q-PCR machine (Biorad) in the presence of the fluorescent CYBR green. Each cycle was 30 s at 95 °C, 30 s at 58 °C and 30 s at 72 °C. The primers were 1: 5′ CAA TGA ACG AGT ACA TCG AGC AGA, 2: 5′ ACT GTG CAT CGC TGC TGC TTA CTT TGT, 3: 5′ CAG TAA CTC TGC AGG CAT TAC CAA, 4: 5′ AAT CTG CTC ACC CTG TCT GAC AAG, 5: 5′ CTC ACC GTT TGC TTA GGC CAA ATG, 6i: 5′ ACT GGT CTT TTC CTT ACT TCC CGA, 6o: 5′ ACT GGT CTT TTC CTT GGA ATC ACC. PCRs were performed with four primer pairs, 1 + 2, 1 + 2, 4 + 6o and 4 + 6i. To determine the relative efficiency of the primer pairs, each set of primers was normalized to a standard primer pair: Inserts corresponding to the four PCR products were cloned into pcDNA3. Q-PCR reactions were performed with either the splice specific primer pair or a primer pair annealing to the amp gene in pcDNA3. The ratios between the two amplifications were used as the efficiency of the splice specific primers pair.

3. Results

3.1. Flip is preferably included in most cell types

To study the regulation of the alternative splicing of the flip and flop exons in GluR2, we PCR amplified and isolated a fragment of the rat GluR2 gene, which contains exon 13 through exon 16, and cloned it into the expression-vector pcDNA3. The resulting construct, pR2L (Fig. 2), was transfected into different cell lines, and RNA was isolated. By RT-PCR, using primes annealing to a vector specific sequence and to exon 16, we selectively amplified RNA transcribed from the pR2L

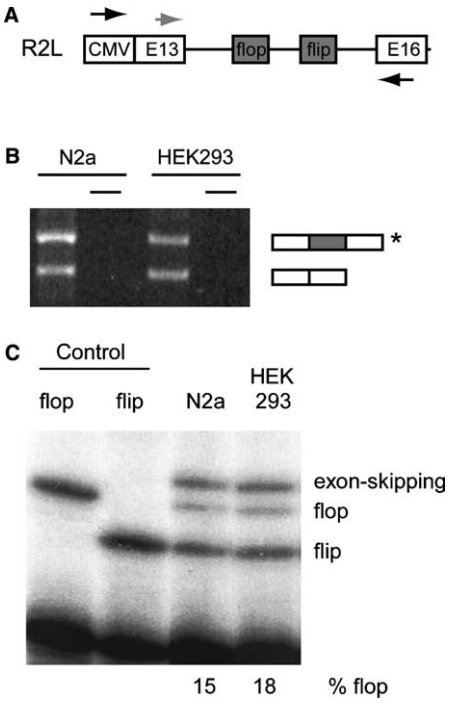

Fig. 2. Flip is preferentially included in N2a and HEK 293 cells. (A) Schematic representation of the R2L mini-gene including exon 13 to exon 16 of the rat GluR2 gene. Black arrows show the approximate position of primers used for RT-PCR and the grey arrow shows the position of the primer used in the primer extension assay. (B) RT-PCR performed on RNA from pR2L transfected N2a or HEK293 cells. The positions of PCR-products corresponding to splice products containing either flip or flop and to splice products excluding both exons are indicated. Minus lanes are reactions performed in the absence of reverse transcriptase. (C) Primer extension assay using the PCR-product marked with an asterisk (Fig. 2B) as template for extension of a ^{32}P labelled primer in the presence of ddCTP to distinguish between flip and flop products. The positions of the primer extension products corresponding to flip, flop and exon-skipping products are shown on the right. The percentage of flop inclusion was calculated from the intensity of the flop signal relative to the sum of the intensities of the flop and flip signals, as quantitated by PhosphorImaging.