Biochimica et Biophysica Acta 1839 (2014) 537–545

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbagrm

Refinement of the spectra of exon usage by combined effects of extracellular stimulus and intracellular factors

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ARTICLE INFO

Article history:
Received 20 February 2014
Received in revised form 29 April 2014
Accepted 12 May 2014
Available online 17 May 2014

Keywords:
Membrane depolarization
Alternative splicing
hnRNP L/LL
PTBP1
hnRNP K

ABSTRACT

Finely tuned differential expression of alternative splice variants contributes to important physiological processes such as the fine-tuning of electrical firing or hearing frequencies; yet the underlying molecular basis for the expression control is not clear. The inclusion levels of four depolarization-regulated alternative exons were measured by RT-PCR in GH_3 pituitary cells under different conditions of stimulation and/or RNA interference of splicing factors. The usage of the exons was reduced by membrane depolarization to various extents and was differentially modulated by the knock-down of splicing factors hnRNP L, L-like, I (PTBP1) or K or their combinations. A spectrum of each exon's level was produced under six knock-down conditions and was significantly shifted by depolarization. When all these conditions were considered together, a more refined or expanded spectrum of exon usage was obtained for each of the four exons. As a proof of principle for the molecular basis of the fine-tuning of exon usage, we show in the cases of hnRNP L and LL that their differential effects through the same element or different combinations of RNA sequences by the same factor hnRNP L are critical. The results thus demonstrate that the combined effect of varying extracellular stimuli and intracellular factors/RNA sequences refines or expands the spectra of endogenous exon usage, likely contributing to the fine-tuning of cellular properties.

1. Introduction

Alternative splicing generates a large number of variant transcripts from the relatively small number of protein-coding genes, contributing greatly to the proteomic complexity in metazoans [1,2]. For some cellular processes, dramatically different or even antagonistic functions of the resulting protein isoforms are important; however, for other cases such as electrical firing of neurons or the tuning of hearing frequencies in cochlear hair cells, finely-tuned function is critical [3–7]. For example, gradient expression of *Slo1* splice variants has been found along the tonotopic map of the cochlear hair cells [4,6,7]. How such a fine spectrum of the different levels of splice variants is determined and refined in cells remains unknown.

Alternative splicing is regulated by both *cis*-acting pre-mRNA elements and *trans*-acting factors, whose important roles have been demonstrated in the control of spatial or temporal expression of splice variants [8,9]. It can also be regulated in many cases by extracellular factors and cellular signaling such as the protein kinase C or Ca^{2+} pathways [10,11]. We thus attempted to determine the combined effect of both the extra- and intracellular factors/elements in refining the spectrum of the levels of an endogenous exon usage in an experimental system.

We have chosen to use our system primarily based on the regulation of the stress axis-regulated exon (STREX) of the *Slo1* BK potassium channel gene by membrane depolarization and its splicing regulators [11–17]. The exon expresses in gradient along the tonotopic map of turtle cochlear hair cells [4], and is important for the fine-tuning of hearing frequencies [3,4,6,7]. Using the exon as a model to study the control of alternative splicing by external factors, we have found that hnRNP L [16,17], its paralogous L-like (hnRNP LL) [17], and the polypyrimidine tract binding protein 1 (PTBP1, or hnRNP I) [16] are intracellular factors involved in its regulation by membrane depolarization. Particularly, hnRNP L binds to a CaMKIV (Ca^{++}/calmodulin-dependent protein kinase IV)-responsive RNA element (CaRRE1) [16], and mediates the depolarization effect on STREX with hnRNP LL [17]. HnRNP L is phosphorylated at serine 513 by the depolarization-activated CaMKIV [14,17].

Besides phosphorylation, different ways of regulation of splicing factors by depolarization/calcium signaling have also been observed in other systems [11]. Therefore, a robust approach to dissecting the role of endogenous splicing factors in the refinement of exon usage would

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http://dx.doi.org/10.1016/j.bbagrm.2014.05.002
1874-9399/

be to examine their loss-of-function effects. Here we examined the effect of depolarization in combination with RNA interference of the involved splicing factors on the expression spectrum of STREX, as well as other depolarization-regulated CaRRE1-type exons [18].

2. Results

2.1. Differential regulation of a group of alternative exons by depolarization and hnRNP proteins in GH_3 pituitary cells

To determine the role of both extra- and intracellular factors in creating a spectrum of exon usage, we measured the inclusion levels of the STREX as well as 10 other reported CaRRE1-type exons [18], in GH_3 pituitary cells without or with membrane depolarization by a high concentration of KCl (50 mM) for 6 h. Using semi-quantitative RT-PCR, we found that STREX was included at 39% in non-treated cells and reduced to 25% upon KCl treatment, as previously reported [17]. Nine of the ten other gene transcripts were detectable; five alternatively spliced with their levels of exon inclusion ranging from 51% to 78% (Table 1 and Fig. 1A), suggesting differential control of their splicing. Three of the exons were repressed upon KCl addition (net reductions from 9% to 24%). They are the exons 11 (E11) of the *phosphatidylinositol transfer protein beta* (*Pitpnb*) gene, E2 of the *Szrd1* (*SUZ RNA binding domain containing 1* or *D4Ertd22*) gene and E36 of the *spectrin alpha 2* (*Spna2*) gene. Thus, these four exons are differentially expressed and regulated by membrane depolarization in GH_3 pituitary cells.

To examine the effect of the hnRNP proteins L, LL and PTBP1 on the usage of these exons, we knocked down their expression individually or in combination using gene-specific shRNA-expressing lentiviruses, as verified by Western blots of the respective proteins (Fig. 1B). Another factor, hnRNP K, was included in the assay since its potential binding site UCCCA, as reported by others [19], is also present in the upstream 3′ splice site of STREX [14,15] (and see below). The inclusion levels of the four depolarization-regulated exons in these samples were measured by RT-PCR.

To allow for comparison between the inclusion levels of different exons under different conditions, we set the level of each exon's inclusion in cells without depolarization (NT) and mock-transduced (Mock) as 100 (Fig. 1C). The levels of the same exon under other conditions were scaled relative to this level.

For the STREX exon, its average level of inclusion was increasingly enhanced by the knockdown of hnRNP L, PTBP1 or L and PTBP1, but reduced by the knockdown of hnRNP LL, L and LL, or hnRNP K in untreated (NT) cells. Upon depolarization, its inclusion was reduced, ranging from 10 to 31% relative decrease on average ($p = 0.0002$ in paired t-test, Fig. 1C).

For the *Pitpnb* exon 11, its average level of inclusion was enhanced by the knockdown of the splicing factors except PTBP1 alone, but reduced by depolarization to various extents in all cases (3% to 29% relative reduction, $p = 6E-04$ in paired t-test, Fig. 1C).

For the *Szrd1* exon 2, its average level of inclusion was slightly enhanced except in the shPTBP1 sample, which showed no change, and the shhnRNP K sample, which showed a decrease (to 68%). The inclusion was reduced by depolarization in all samples (11 to 19% relative reduction, $p = 7.9E-06$ in paired t-test, Fig. 1C).

For the *Spna2* exon 36, its average level of inclusion was enhanced by the knockdown of PTBP1, L and LL or L and PTBP1 but reduced by the knockdown of hnRNP L, LL or hnRNP K. Depolarization reduced the average levels of inclusion in all samples (8 to 27% relative reduction, $p = 0.0002$ in paired t-test, Fig. 1C).

Taken together, the average levels of inclusion of the four exons are differentially regulated by depolarization and the hnRNP factors in GH_3 cells.

2.2. Spectra of exon usage are refined by the combined effect of depolarization and change of splicing factors

An interesting point from the above experiment is that the differential regulation of exon inclusion by the extra- and intracellular factors appeared to make the levels of exon inclusion span a range with various intermediate states for each exon. To determine the effect of these factors on the spectra of exon inclusion, we compared the average levels of exon inclusion in each knockdown samples among treatment groups NT, KCl, or NT- and KCl-combined pools in bar-code graphs (Fig. 2).

The average index levels of exon inclusion of each group as single-row barcodes displayed a spectrum in the shRNA knockdown samples without depolarization (NT). This spectrum is significantly left-shifted overall by KCl treatment ($p < 0.001$, $n = 7$, Fig. 2A, upper panel). On average, the interval between adjacent indexes of exon inclusion levels is not different between the NT and KCl groups but is significantly lower when the NT and KCl groups are combined (Fig. 2A, $p < 0.05$ compared to those of the two individual groups, lower panel). Therefore, the combined effect of KCl treatment and knockdown of different hnRNPs creates a more refined spectrum of STREX exon levels.

Analysis of the other three exons also showed significant overall left-shift of exon inclusion levels by KCl (Fig. 2B, $p < 0.001$, $n = 7$ for each exon). For the *Pitpnb* exon 11 and *Szrd1* exon 2, the hnRNP knockdowns mostly shifted up the levels but KCl treatment shifted most of them down to below the original levels, thus expanding the range of the spectrum. Additionally, for the *Szrd1* exon, the KCl-shifted levels appear to also fill a gap in the spectrum of the NT samples. For the *Spna2* exon 36, the range is also expanded. Besides, it has more numbers of different levels within the overlapping range where the total number of bars were increased from 4 in the NT or KCl groups to 8 when the two groups were combined. Therefore, the combined effect of KCl and hnRNP knockdown refines or expands the spectrum of exon usage depending on the target exon.

Taken together, this analysis of fours exons indicates that the combined effects of extracellular stimulation by depolarization and varied

Table 1
List of CaRRE1-harboring alternative exons screened for their expression and regulation by membrane depolarization in GH3 pituitary cells. These exons were selected for their regulation by depolarization in a mouse cell line P19, as identified by Lee et al. [18]. The gene/exon names, CA-rich CaRRE1 motif sequence and location are listed. The alternative exons are either repressed or no change upon depolarization.

Gene/exon	Predicted CaRRE1/CA	Location of CaRRE1/CA	Expression detected	Alternatively spliced	Change by depolarization
Adcyap1r1/E14	cacaccca	Upstream intron			
Szrd1/E2	cacuuuua	Upstream intron	Yes	Yes	Repressed
Nf2/E16	cucauuua	Upstream intron	Yes		
Pitpnb/E11	uacauuua	Upstream intron	Yes	Yes	Repressed
Shc1/E6	gacauuua	Upstream intron	Yes		
Atp2b1/E21	CACAUGUA	Exon	Yes		
Rnf14/E4	CACACUUA	Exon	Yes	Yes	No change
Ktn1/E41	CACAGUUA	Exon	Yes	Yes	No change
Spna2/E36	cacuuuua	Downstream intron	Yes	Yes	Repressed
U26/E2	cacacuua	Downstream intron	Yes		