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Splicing transitions of the anchoring protein ENH during striated muscle development

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ABSTRACT

The ENH (PDLIM5) protein acts as a scaffold to tether various functional proteins at subcellular sites via PDZ and three LIM domains. Splicing of the ENH primary transcript generates various products with different repertories of protein interaction modules. Three LIM-containing ENH predominates in neonatal cardiac tissue, whereas LIM-less ENHs are abundant in adult hearts, as well as skeletal muscles. Here we examine the timing of splicing transitions of ENH gene products during postnatal heart development and C2C12 myoblast differentiation. Real-time PCR analysis shows that LIM-containing ENH1 mRNA is gradually decreased during postnatal heart development, whereas transcripts with the short exon 5 appear in the late postnatal period and continues to increase until at least one month after birth. The splicing transition from LIM-containing ENH1 to LIM-less ENHs is also observed during the early period of C2C12 differentiation. This transition correlates with the emergence of ENH transcripts with the short exon 5, as well as the expression of myogenin mRNA. In contrast, the shift from the short exon 5 to the exon 7 occurs in the late differentiation period. The timing of this late event corresponds to the appearance of mRNA for the skeletal myosin heavy chain MYH4. Thus, coordinated and stepwise splicing transitions result in the production of specific ENH transcripts in mature striated muscles.

1. Introduction

PDLIM proteins constitute a family of anchoring proteins that contain a PDZ domain and one to several LIM domains [1]. PDZ and LIM domains are protein interaction modules with distinct characteristics. PDZ domains consist of 80–90 amino acid residues and in many cases bind to a C-terminal peptide motif of cytoskeletal and membrane proteins. On the other hand, LIM domains are composed of two zinc-finger motifs and mediate interaction with various signaling molecules and transcription factors. Thus, PDLIM proteins are considered to act as scaffolds to assemble cellular signal-controlling molecules at certain subcellular sites.

ENH, Enigma homologue, is a member of PDLIM family (PDLIM5) containing a PDZ domain at the N-terminal region and three LIM domains in the C-terminal portion [2]. The PDZ domain of ENH binds to alpha-actinin and actin [3,4], whereas the LIM domains interact with protein kinases, such as several PKCs and PKD1 [2,5], as well as the transcription factor Id2 [6] and the neuronal postsynaptic protein SPAR [7]. ENH has been shown to sequester Id2 in the cytosol to prevent its transcriptional activity and neural differentiation [6], whereas ENH appears to alter the spine morphology of neuronal dendrites [7]. We have also shown that regulation of L-type Ca^{2+} channel by PKD1 requires scaffolding of these proteins by ENH [5]. Thus, ENH controls diverse cellular functions in neurons and cardiac myocytes by interacting with various molecules.

Although the identified cellular functions are based on the presence of both PDZ and LIM domains, the ENH gene generates splicing isoforms that lack LIM domains. In particular, the splicing variants ENH3 and ENH4 are predominant in cardiac and skeletal muscles in adult animals [3,4,8]. These two variants, as well as ENH2, are generated by inclusion of the exon 11, resulting in the insertion of a termination codon before the C-terminal region containing three LIM domains. Our previous work also revealed the important role of ENH splicing in the development of cardiac hypertrophy [8]. Aortic banding-induced hypertrophied myocardium appears to contain more ENH1 with three LIM domains and less ENHs without LIM domains. Furthermore, overexpression of ENH1 in neonatal myocytes results in hypertrophy, whereas LIM-less ENH4 prevents myocyte hypertrophy induced by various extracellular stimuli. These findings indicate that the splicing transition of ENH is a key event

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in the development of cardiac hypertrophy. They also suggest a unique role of LIM-less ENHs in striated muscles.

We wish to elucidate molecular mechanisms underlying the splicing transitions of ENH. In this report, we examine splicing transitions of ENH during postnatal heart development and C2C12 myoblast differentiation *in vitro*. We show here that the generation of cardiac and skeletal muscle-selective ENH variants is controlled by splicing events that occur at distinct differentiation periods.

2. Materials and methods

2.1. Animals

Care and handling of animals were in accordance with institutional guidelines and were approved by the Animal Care and Use Committees of the Tokyo Institute of Technology and the Nagaoka University of Technology. Experiments were performed using Sprague Dawley rats. Adult tissues were obtained from 250 to 300-g male animals (12–15 weeks old), whereas postnatally-developing heart tissues were from animals in both sexes at various postnatal days from multiple littermates. Ventricles were obtained by eliminating the top part of the isolated hearts containing atrial and aortic tissues. The obtained tissues were washed with ice-cold phosphate-buffered saline, cut into small pieces and frozen on dryice.

2.2. Cell culture

Mouse skeletal C2C12 cells were obtained from RIKEN BRC Cell Bank (Ibaraki, Japan). Cells were maintained in Dulbecco's Modified Eagle Medium supplemented with 10% fetal bovine serum (Gibco, Auckland, NZ), 50 U/ml penicillin and 50 μg/ml streptomycin (Nacalai Tesque, Kyoto, Japan) under 5% CO_2 atmosphere at 37 °C. For differentiation, cells were passed on plastic dishes at ~50% and cultured until semi-confluence (~90%). Culture medium was then switched to the low serum medium containing 2% fetal bovine serum. The medium was changed once every three days during the entire experimental period.

2.3. Real-time PCR analysis

Total RNA was isolated from heart tissues and C2C12 cells using a commercial reagent (TRIzol reagent, Invitrogen). The first-strand cDNA was synthesized using a commercial kit (ReverTra Ace qPCR RT Kit, Toyobo, Tokyo, Japan) according to the manufacturer's instructions. Real-time PCR was done with synthesized cDNA (50 ng of RNA content), 250 nM primers, and commercial master mix (KAPA SYBR FAST qPCR Kit, Kapa Biosystems, Woburn, MA) in a final volume of 25 μl. The PCR reaction was carried out in 96-well plates using Stratagene Mx3000P (Agilent Technologies, Santa Clara, CA).

Primers were designed to detect individual ENH variants (see Fig. 1A and B), as previously published [8], except that detection of mouse ENH3 mRNA was done with the reverse primer that corresponded to the exon 8: 5′-GTGGGCGTTTGGGTGGAATTTTC-3′. We used clones (T-Vector pMD20, Takara, Otsu, Shiga, Japan) each containing individual target PCR products as standards for quantitation. We also used skeletal marker genes for analysis: skeletal myosin heavy chain (GenBank Accession#: NM_010855), 5′-CTGACCGAGCA GATTGCTGAG-3′ (Nucleotide 4636-4656) 5′-ATCCTCAGAGCAT-CATTCCTGCTG-3′ (Nucleotide 4943-4920); and myogenin (GenBank Accession#:NM_031189), 5′-TCACATAAGGCTAACAC CCAGC-3′ (Nucleotide 876-896), and 5′-GCACTCATGTCTCTCAAACG GT-3′ (Nucleotide 1090-1069).

2.4. Semi-quantitative PCR analysis

We used semi-quantitative measurement of PCR products to estimate exon usages in the region encompassing the exon 5–8. For this analysis, standard PCR was performed in a final volume of 25 μl containing synthesized cDNA (50 ng of RNA content), 400 nM primers and commercial master mix (EmeraldAmp MAX PCR Master Mix, Takara, Otsu, Shiga, Japan). Primers corresponded to a part of the exon 3 and 9. The 5′ primer sequence was 5′-GAAGCCCAGAACAAGATTAAGGC-3′ for both species, whereas the 3′ primers were the ones used to detect ENH4 (see Fig. 1A and B). PCR conditions were 95 °C for 5 s, 64 °C for 10 s, and 72 °C for 60 s for 25–30 cycles with the final extension at 72 °C for 4 min. The PCR products were resolved by electrophoresis on a 3.5% agarose gel and stained with 0.5 μg/ml ethidium bromide. We cloned several PCR products obtained by this process into T vector (T-Vector pMD20) and sequenced.

For semi-quantitation of the PCR products, images were captured, and band intensity was measured using a charged coupled device-based camera system (Ultra-Violet Products, Upland, CA). Briefly, background was eliminated using the joint valley procedure, and the integral optical density was determined by the perpendicular drop method.

2.5. Data analysis

The mRNA levels were determined with a cDNA standard with or without normalization using cyclophilin mRNA as a control. We found that cyclophilin mRNA level substantially reduced during differentiation of C2C12 cells. Therefore, we used the data without normalization for C2C12 cells, whereas normalization with cyclophilin mRNA was used for the animal samples unless otherwise stated. Statistical analysis was performed by one-way *ANOVA*, followed the layered *Bonferroni*'s test. Data are presented as the mean ± SEM. $^{*}p < 0.05$, $^{**}p < 0.001$ and $^{***}p < 0.0001$ compared to day 1 for postnatal heart development and day 0 for C2C12 differentiation.

3. Results

3.1. Expression of ENH splicing variants during postnatal heart development

Previous studies have shown that LIM-less ENHs are abundant in the heart and skeletal muscle of adult rodents [3,4,8]. We first tested whether different adult organs might selectively express individual ENH splicing products. We designed primers that target the regions each unique for a subset of splicing variants (Fig. 1A and B). Specifically, ENH1 primers were targeted to the LIM region, whereas ENH3 and ENH4 primers detected transcripts containing the exons 5–8 and 6, respectively. Real-time PCR analysis revealed that different ENH splicing variants are predominant in adult rat organs. In the ventricle of the heart, ENH3 mRNA was more than 10 and 100 times abundant than ENH1 and ENH4 transcripts, respectively (ENH variant/cyclophilin mRNA ratio (%): ENH1 = 0.18 ± 0.09, ENH3 = 1.98 ± 0.55, ENH4 = 0.016 ± 0.007, $n = 3$). In contrast, ENH4 mRNA is the most abundant in the skeletal muscle (ENH1 = 0.07 ± 0.04, ENH3 = 0.13 ± 0.10, ENH4 = 8.3 ± 2.1, $n = 3$), whereas only ENH1 transcript was significant in the brain (ENH1 = 0.15 ± 0.010, ENH3 = 0.001, ENH4 < 0.001, $n = 3$). Thus, although the absolute levels of ENH variants largely differ among adult rat organs, fully developed organs selectively express individual ENH splicing variants.

Our previous study indicated that the levels of ENH splicing variants markedly differ between newborn and adult heart tissues [8]. To determine the timing of this splicing transition, we measured