主論文の要旨

Competitive regulation of alternative splicing and alternative polyadenylation by hnRNP H and CstF64 determines acetylcholinesterase isoforms

（hnRNPH と CstF64 によるスプライシングとポリアデニル化の競合的制御が、アセチルコリンエステラーゼのアイソフォームを決定する）

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Background

Acetylcholinesterase (AChE), encoded by the *ACHE* gene, is a serine-specific hydrolase that hydrolyzes the neurotransmitter acetylcholine to terminate synaptic transmission. In vertebrates, the 3' end of *ACHE* pre-mRNA is alternatively processed to generate three different isoforms: (i) $AChE_T$ – which can form a tetrameric structure and anchors to proline-rich membrane anchor PRiMA (in brain) or trimeric collagen Q tail (in muscle), (ii) $AChE_H$ - that dimerizes itself and anchors to the hematopoietic cell membrane using glycophosphatidylinositol (GPI), and (iii) $AChE_R$ - an unspliced and rare 'readthrough' subunit, which remains as a soluble monomer (Figure 1). In this study, we dissected the molecular mechanism of alternative splicing (AS) and alternative polyadenylation (APA) of the human *ACHE* gene.

Methods

We employed RT-PCR, RNA affinity purification, mass spectrometry, siRNA-mediated knockdown, cDNA overexpression, tethered function assay, 3' RACE, antibody-mediated depletion of an RNA-binding protein, immunoblotting, and bioinformatic analysis of CLIP-seq and RNA-seq data to dissect the molecular mechanism of alternative RNA processing of the human *ACHE* gene.

Results

In order to dissect *cis*-elements governing isoform-specific alternative splicing of *ACHE*, we sequentially deleted seven 100-bp blocks and one 49-bp block in exon 5a, in the *ACHE* minigene (Figure 2A). We found that deletion of E5a-Δ4 and E5a-Δ5 reduced the $AChE_T$ isoform, indicating presence of splicing regulatory *cis*-elements in these blocks. To further dissect *cis*-elements, we sequentially deleted eight 25-bp blocks in E5a-Δ4 and E5a-Δ5 (Figure 2B). We found that blocks E5a-Δ4d, E5a-Δ5b, and E5a-Δ5c potentially harbor splicing regulatory *cis*-elements. Further analysis revealed that blocks E5a-Δ5b and E5a-Δ5c harbor the essential branch point and polypyrimidine tract, respectively (data not shown). As there was no known splicing regulatory *cis*-element(s) in E5a-Δ4d, we searched for *trans*-factors that bind to E5a-Δ4d and regulate alternative 3' ss selection. We synthesized biotinylated wild-type (Wt-E5a) probe as well as a mutant (Mut-E5a) RNA probe with mutations disrupting all the putative RNA binding motifs (Figure 2C) predicted by SpliceAid 2 database. RNA affinity purification assay using nuclear extracts of SH-SY5Y cells followed by coomassie blue staining detected a distinct band at ~55 kDa with Wt-E5a but not with Mut-E5a probe (Figure 2D). Mass spectrometry revealed that the excised band was for hnRNP H. We also confirmed by immunoblotting that the bound proteins were hnRNP H and its paralog hnRNP F, although the amount of hnRNP F was much lower (Figure 2E).

We next examined the effect of siRNA-mediated downregulation of hnRNP H and F on alternative 3'ss usage. Immunoblotting confirmed that the siRNAs efficiently downregulate hnRNP H and F in SH-SY5Y cells (Figure 3A). Individual downregulation of hnRNP H slightly decreased the selection of distal 3' ss, whereas downregulation of hnRNP F had no effect (Figure 3B). However, downregulation of both hnRNP H and F significantly reduced the selection of the distal 3' ss, suggesting that hnRNP H and F are able to compensate for the lack of the counterpart. We also confirmed that overexpression of siRNA-resistant hnRNP H (si-res-hnRNP H) rescued the effect of the downregulation of hnRNP H/F (Figure 3C). HnRNP H/F proteins are known to bind to G-runs, and we identified two G-runs in E5a-Δ4d (Figure 3D). To examine which G-run is crucial for splicing regulation, we mutated either or both of the G-runs in the minigene construct. RT-PCR analysis revealed that disruption of a single G-run marginally affected splicing, whereas disruption of both G-runs resulted in a splicing shift towards the $AChE_H$ isoform from the $AChE_T$ isoform (Figure 3D). We introduced the bacteriophage PP7 coat protein-binding hairpin-loop replacing the two G-runs in E5a-Δ4d. PP7 mediated artificial tethering of hnRNP H efficiently induced the selection of the distal 3' ss (Figure 3E).

We then identified a cryptic PAS with a weak polyadenylation signal sequence, UAUAAA, in exon 5a (pA-1 in Figure 4A,B). 3' RACE confirmed that SH-SY5Y cells use the cryptic PAS in exon 5a (pA-1) and the canonical PAS at the end of exon 5b (pA-2) (Figure 4B). Sequencing of the 3' RACE products revealed that both the cryptic and canonical PASs are indeed used (Figure 4C). The 3' RACE analysis in hnRNP H/F-knocked down SH-SY5Y cells revealed that downregulation of hnRNP H upregulated the selection of the cryptic PAS (higher pA-1/pA-2 ratio), in both the endogenous and minigene contexts; and these effects were rescued by overexpression of si-resistant hnRNP H (Figure 4D,E). An essential 3' processing factor, CstF64, binds to GU/U-rich regions located within ~40 nucleotides downstream of the cleavage site. Several UG-rich elements are present immediately downstream of the cleavage site, and are overlapping with the two essential G-runs in exon 5a, suggesting that hnRNP H and CstF64 may compete each other for RNA-binding (Figure 5A). We individually depleted hnRNP H/F and CstF64 from HeLa nuclear extracts (Figure 5B), and performed RNA affinity purification assay using a biotinylated RNA probe carrying the cryptic PAS and its flanking regions (Figure 5A). Depletion of CstF64 resulted in increased binding of hnRNP H to the RNA probe (lane 3 in Figure 5C), whereas depletion of hnRNP H/F resulted in increased binding of CstF64 to the RNA probe (lane 2 in Figure 5C).

To further examine the molecular mechanism of coupled regulation of AS and APA, we introduced mutations to disrupt the cryptic PAS (UAUAAA>UAUUGA) in the *ACHE* minigene, which resulted in exclusive selection of the canonical PAS (pA-2)

(Figure 5D). We then converted the weak cryptic PAS to an optimal one (UAUAAA>AAUAAA), or introduced three G>T mutations in exon 5a to disrupt binding of hnRNP H/F; both resulted in efficient selection of pA-1. RT-PCR of the alternative *ACHE* isoforms revealed that the selection of the cryptic PAS was accompanied by the expression of $AChE_H$ isoform arising from these constructs (Figure 5D). In order to examine whether hnRNP H-mediated antagonizing regulation of AS and APA is generally observed in humans, we analyzed RNA-seq of *HNRNPH*-knocked down HEK293T cells (GSE16642) and CLIP-seq of hnRNP H in HEK293T cells (GSE23694) in the GEO database. We detected 43,152 transcripts with different APA sites, and the splicing analysis tool MISO revealed that 635 APA sites are located between a constitutive 5' ss and an alternative distal 3' ss, as we observed in the human *ACHE* gene. To evaluate the effect of hnRNP H on the selection of an APA site and an alternative 3' ss, we divided the APA sites into five categories from the most activated APA sites to the most downregulated APA sites by hnRNP H. Similar to the human *ACHE* gene, APA sites that were suppressed by hnRNP H were associated with the selection of the distal alternative 3' ss's (Figure 6A).

Discussion

We have analyzed the molecular mechanisms underlying generation of human *ACHE* isoforms, and have shown that alternative splicing of *ACHE* is coupled to selection APA sites, which is coordinately regulated by hnRNP H and its antagonizing CstF64. In human *ACHE*, hnRNP H activates the selection of the distal 3' ss, which produces the muscle- and brain-specific $AChE_T$ isoform (Figure 6B). On the contrary, lack of hnRNP H allows CstF64 to bind downstream to the cryptic PAS (pA-1), which generates a truncated transcript lacking the distal 3' ss. Two alternative choices for this short transcript are either to select the proximal 3'ss ($AChE_H$) or to retain intron 4 ($AChE_R$) (Figure 6B). Similar effects of hnRNP H on both activation of the distal 3' ss and suppression of APA are observed in global analysis of CLIP-seq and RNA-seq (Figure 6A). We propose that hnRNP H is an essential factor that competitively regulates alternative splicing and alternative polyadenylation.