Role of ELAV-like RNA-binding proteins HuD and HuR in the post-transcriptional regulation of acetylcholinesterase in neurons and skeletal muscle cells

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Abstract

Over the last few years, several laboratories have focused their attention on elucidating the molecular events that control the expression and localization of acetylcholinesterase (AChE) in neurons and skeletal muscle cells. In this context, results from a number of studies have clearly shown the important contribution of transcriptional events in regulating AChE expression. Specifically, these studies have highlighted the roles of several cis- and trans-acting factors that control transcription of the AChE gene in these excitable cells. However, it has also become apparent that changes in the transcriptional activity of the AChE gene cannot fully account for the alterations seen in the overall abundance of AChE transcripts in neurons and muscle cells placed under a variety of experimental conditions. This indicates, therefore, that post-transcriptional mechanisms also play a significant role in controlling AChE mRNA expression. With this in mind, we have recently begun to address this issue in greater detail. Here, we provide a summary of our most recent findings dealing with the post-transcriptional regulation of AChE. Together, our studies have shown so far the important contribution of an AU-rich element located in the 3′UTR of AChE transcripts and of the stabilizing RNA-binding proteins of the ELAV-like family in regulating AChE expression in differentiating neuronal and muscle cells.

Keywords: Neuromuscular junction; Synaptic proteins; Differentiation; mRNA stability; AU-rich element

1. Introduction

Cholinergic neurotransmission at synapses of the central and peripheral nervous systems (CNS and PNS) is rapidly terminated by the enzymatic activity of acetylcholinesterase (AChE) (see for review [1–4]). Although in the brain, AChE is predominantly expressed in regions receiving cholinergic input, it can also be found in regions that do not typically involve cholinergic neurotransmission [5–8]. Several lines of evidence indicate that AChE is expressed during neuronal development, prior to the establishment of cholinergic synapses, and that it assumes additional functional roles such as in cell adhesion, promotion of neurite outgrowth and synaptogenesis (see for review [3,9]). Appropriate expression of AChE is not only important in the brain but it is also critical for normal skeletal muscle activity as exemplified by the phenotype seen in patients suffering from a myasthenic syndrome linked to a reduction of AChE at the neuromuscular junction [10,11].
Expression of AChE is known to increase markedly during differentiation of neurons and skeletal muscle cells. Additionally, the levels and localization of AChE in excitable cells is exquisitely sensitive to alterations in the amount and pattern of synaptic activity and to the presence of specific nerve-derived trophic factors. In recent years, several studies have begun to examine the molecular mechanisms regulating expression of AChE during neuronal and myogenic differentiation as well as in mature cells subjected to changes in activity or modifications in the supply of trophic factors. For example, we have recently shown that an increase in the transcriptional activity of the AChE gene could partially account for the initial increase in AChE mRNA levels observed in differentiating muscle cells grown in culture [12]. In this study, however, we also noted a clear discrepancy between the observed increase in transcription and the overall induction in AChE transcripts, thereby suggesting that transcription alone could not fully account for the increase in AChE expression seen at later stages of differentiation [12]. In agreement with a previous report [13], our results indicated that post-transcriptional events also participate in regulating the abundance of AChE mRNAs in differentiating muscle cells (see also [14]). Similarly, based on the few available reports, it appears that both transcriptional and post-transcriptional mechanisms also contribute to the regulation of AChE during neuronal development [15,16].

Several cis- and trans-acting factors involved in the transcriptional regulation of the AChE gene during neuronal and myogenic differentiation have been identified in recent years. In particular, we have shown that E- and N-box motifs located within the first intronic region, along with their respective transcription factors, myogenin and GABPα and β, participate in controlling transcription of the AChE gene during myogenesis [12]. More recent studies performed by others have also highlighted the contribution of specific 5′-regulatory regions in the AChE gene in muscle cells, including Sp1, Egr-1, CRE and NFAF elements [17–19]. During neurogenesis, CRE elements and CREB as well as several other transcription factors including for example Runc1/Amd1, AP-2, NF-κB and Sp1, have been implicated in the transcriptional regulation of AChE [20,21]. By comparison, relatively little is known about the cis- and trans-acting factors that are involved in the post-transcriptional regulation of AChE during differentiation of neurons and skeletal muscle cells. Here, we highlight our recent findings that specifically address this issue.

2. Post-transcriptional regulation of AChE in differentiating neurons

Several years ago, Coleman and Taylor (15) first suggested, using several complementary approaches, that the large increase in the overall abundance of AChE transcripts observed during retinoic acid-induced differentiation of P19 embryonic carcinoma cells involved changes in the stability of pre-synthesized transcripts as opposed to an induction in the transcriptional activity of the AChE gene. In agreement with these initial findings, others have also implicated the contribution of greater mRNA stability to the increase in AChE mRNA levels seen during thyroid hormone-induced neuronal differentiation of Neuro-2a neuroblastoma cells expressing the β1 thyroid receptor [22,23]. In a recent study, we examined the molecular mechanisms regulating AChE expression during neuronal differentiation with a clear focus on post-transcriptional mechanisms [24]. To this end, we made use of PC12 cells induced to differentiate via NGF treatment. As expected, we observed a pronounced increase in AChE transcripts in differentiating PC12 cells. Using several approaches, we determined that during the initial stages of differentiation, the increase in AChE transcripts appeared to involve a moderate increase in the transcriptional activity of the AChE gene (see also [16]).

The modest increase in transcription observed in differentiating PC12 cells was transient, however, and could not therefore account for the much larger rise in AChE mRNA levels thereby indicating that post-transcriptional events are also involved and likely to predominate. Close examination of the AChE 3′UTR revealed the presence of an AU-rich element (ARE). AREs consist of the AUUUA general sequence expressed as overlapping nonamers or dispersed throughout the 3′UTR. U-rich sequences and CU-rich motifs [25,26]. Several studies have shown that this element can either stabilize or destabilize transcripts depending on the number of sequential repeats, variations of the basic sequence and identity of the trans-acting factor that binds (see for review [27,28]). Amongst the increasing number of RNA-binding proteins known to bind AREs is the AUFI family of destabilizing proteins as well as ELAV (embryonic lethal abnormal vision)-like family of RNA-binding proteins that includes the neuron-specific HuB (HelN1), HuC, HuD proteins as well as the ubiquitously expressed HuR [27–29]. A major function of this later family of RNA-binding proteins is in stabilizing transcripts by inhibiting the de-adenylation and targeting of the transcripts to the exosome, or by blocking the activity of specific endonucleases that recognize the ARE [30–32].
Fig. 1. Schematic representation of domains in HuD and HuR. (A) Shows the various forms of HuD that arise from alternative splicing in the hinge domain (RRM; RNA-recognition motif). (B) Depicts HuR, which shares a high degree of similarity with HuD except in the hinge domain and N- and C-termini. The sequence of the hinge domain and the methylated arginines are indicated (underlined).

The presence of an ARE in the ACHe 3′ UTR and the known role of ELAV-like proteins led us to hypothesize that HuD is involved in regulating the stability of ACHe transcripts in differentiating neurons. HuD was the first of the Hu-proteins identified as the cause of paraneoplastic syndromes in small cell lung carcinoma patients [33]. The ELAV-like proteins consist of three RNA-recognition motifs (RRM), two located at the N-terminal and the third in the C-terminal separated from the second RRM by the hinge region (see Fig. 1A). The mammalian HuD gene is alternatively spliced within the hinge region linking the second and third RRM resulting in three different transcripts and molecular isoforms, termed HuDpro, HuD and HuDmex [34–37] (Fig. 1A). With the exception of the N-terminal domain and the hinge region, the protein sequences between the different ELAV-like proteins are very similar (70–85% similarity, see [35]). In vitro binding studies performed with HuD demonstrated that the RNA is bound primarily by the first RRM while the second RRM, which also binds the RNA, functions mostly to stabilize the complex [38,39]. The third RRM, in addition to participating in maintaining complex stability, binds to poly(A) tails [40,41]. The length of the poly(A) tail correlates with the overall binding activity of ELAV-like proteins. Thus, the longer the chain of adenines the more efficiently Hu proteins can bind their targets.

To examine the possibility of HuD being involved in ACHe mRNA expression, we capitalized on the use of PC12 cells engineered to over-express HuD or an antisense to HuD (as controls). In agreement with our working hypothesis, the abundance of ACHe transcripts in these cells correlates with the levels of HuD [24]. Furthermore, transfection of a reporter construct containing the ACHe 3′ UTR showed that this 3′ UTR can increase expression of the luciferase reporter in cells expressing HuD but not in cells expressing the antisense. Although RNA gel-shift assays and Northern blots revealed an increase in the binding activity of several protein complexes in differentiated neurons, immunoprecipitation experiments using differentiated cells demonstrated that HuD can indeed bind directly to ACHe transcripts. Taken together, these results show the importance of post-transcriptional mechanisms in regulating ACHe mRNA expression in differentiating neurons and implicate the ARE and HuD as key factors in these events. The fact that we observed the presence of multiple protein complexes that interact with the ACHe 3′ UTR further suggests that additional cis- and trans-acting factors are also likely involved in controlling post-transcriptionally the overall levels of ACHe mRNA in differentiating neurons. Finally, it is important to note that our results are in excellent agreement with those of another study that recently also showed the role of HuD in regulating ACHe mRNA expression [42].

3. Post-transcriptional regulation of ACHe in differentiating skeletal muscle cells

Prior to differentiation, C2C12 myoblasts express very little ACHe transcript and protein [12,13,43,44]. As differentiation proceeds and myoblasts fuse to form multinucleated myotubes, expression of ACHe sharply increases [12,13,43]. As mentioned above, increases in ACHe transcript levels during myogenic differentiation appear to involve transcriptional regulatory events, with some having been described in detail, as well as post-transcriptional mechanisms whose specific nature remains elusive. Given our recent data showing that HuD and the ARE regulate post-transcriptionally ACHe expression during neuronal differentiation, we recently investigated whether similar mechanisms operate in muscle cells to control the levels of ACHe mRNA during their differentiation.

To this end, we specifically examined the role of the short 3′ UTR since we already established that it contains elements important for its regulation [24,42] and since previous studies have demonstrated that the first polyadenylation signal is used preferentially over the second signal [13,45]. Using an engineered reporter construct containing the short ACHe 3′ UTR, we observed a significant increase in reporter expression in differentiating myotubes thereby indicating that the 3′ UTR is involved in regulating ACHe transcript levels during myogenic differentiation [46]. In addition, RNA gel-shift assays...
showed that the binding intensity of protein complexes interacting with the ARE increased as a result of myogenic differentiation.

Since in our earlier work dealing with PC12 cells [24], we identified HuD as one of the RNA-binding proteins interacting with the ARE located in the AChE 3′UTR, we chose to examine whether the more ubiquitously expressed member of the Hu-family of proteins, namely HuR, could bind to AChE transcripts in muscle cells. HuR shares a high degree of similarity with HuD but differs in the C-terminal domain and hinge region that separates the second and third RRM ([35,47]; see Fig. 1B). Because of its ubiquitous pattern of expression, HuR has been studied more extensively than its neuronal counterparts. Similar to HuD, HuR is characterized as an mRNA stabilizing protein. However, HuR differs significantly from HuD with regards to its sub-cellular location, which is predominantly nuclear in non-stimulated or non-stressed cells [47]. Recent studies found that HuR can interact with several transcripts in skeletal muscle including VEGF [48]. Notably, HuR has been identified as one of the principal regulators of myogenic differentiation through its specific binding to transcripts encoding the myogenic factors MyoD, myogenin and p21 [49,50].

Using several complementary approaches, including supershift assays, mRNA-binding protein pull-down assays and immunoprecipitation followed by RT-PCR, we recently showed that HuR from differentiated muscle cells specifically binds to AChE transcripts via direct interaction with the ARE [46]. Functionally, over-expression of HuR in C2C12 cells induced an increase in the endogenous levels of AChE transcripts as well as an increase in the expression of the reporter construct containing the AChE 3′UTR. Similarly, siRNA experiments aimed at decreasing the levels of HuR in differentiating muscle cells, led to a reduction in AChE mRNA levels. In vitro stability assays showed indeed, that HuR mediates these effects by decreasing the rate of decay of AChE transcripts. Together, these results show that during myogenic differentiation HuR interacts directly with the 3′UTR of AChE transcripts and regulates their stability. Accordingly, these findings show that HuR is an important regulator of AChE expression in differentiating muscle cells.

4. Discussion

Our recent efforts have focused on trying to elucidate some of the post-transcriptional mechanisms that appear so critical in regulating expression of AChE in both neuronal and muscle cells. Our findings to date have shown that the ARE located within the AChE 3′UTR, interacts with members of the ELAV-like RNA-binding protein family leading to an increase in the stability of AChE transcripts. Taken together, results from our studies emphasize the role of Hu proteins in regulating AChE expression during differentiation of excitable cells.

The ELAV-like RNA-binding proteins are also well known for stabilizing a number of additional transcripts encoding proteins with distinct and essential functions in differentiating cells (see for review [29,47,51]). Notably, HuD is recognized as an essential regulator of morphological differentiation in neurons [29,52–55]. In this case, there is now compelling evidence indicating that HuD binds and stabilizes a number of transcripts known to encode proteins implicated in neurite elongation, such as tau, GAP-43 and AChE [24,52,53,56]. In addition, an increased level of HuD in neural crest cells precipitates neurotrophic dependence thereby implicating HuD in cell survival as evidenced by the development of para-neoplastic encephalomyelitis and sensory neuropathy in patients harboring anti-Hu antibodies in their serum [55,57]. These results taken into consideration with the preferential expression of HuD in projection neurons, strongly suggests that HuD, through its stabilizing effect on transcripts, is an essential regulator of the development and maintenance of neuronal processes.

HuR has also been implicated in the process of cellular differentiation. During myogenic differentiation for example, expression of several muscle-specific and synaptic genes is often induced transcriptionally by myogenic regulatory factors such as MyoD and myogenin [58]. Recently, two related studies demonstrated that the 3′UTR of these myogenic factors interact with HuR and regulate the stability of these transcripts [49,50]. The increase in the stability of these transcripts is important in dictating the overall levels of proteins encoded by these mRNAs during myogenic differentiation. Thus, transcription factors that are key to the process of myogenic differentiation, and to the initial increase in AChE gene transcription [12], are themselves regulated post-transcriptionally by HuR.

Given the significant roles of these two RNA-binding proteins in developing neurons and muscle cells, it becomes important to understand how expression and binding activity of these proteins are regulated. Due to its ubiquitous nature, a number of studies have attempted to decipher the molecular and cellular mechanisms that regulate the binding activity of HuR. One possible mechanism that could regulate HuR involves its sub-cellular localization. For example, previous studies have demonstrated that HuR is predominantly located in the nucleus of myoblasts and that during the initial phases of differentiation, it either shuttles out to the cytoplasm bound to
target transcripts, or interacts with and stabilizes specific transcripts once it appears in the cytoplasm [49, 50].

The regulation of HuR and HuD binding could also be mediated by post-translational modifications, such as phosphorylation or methylation. In this context, metabolic stress was recently shown to regulate the cytoplasmic localization of HuR and its binding through activation of the AMP-activated kinase [59]. Binding activity of HuD could also be the downstream target of phosphorylation, since protein kinase C has been implicated in regulating the actions of HuD during neuronal differentiation [56]. In addition, HuR has been shown recently to be methylated by the arginine methyltransferase CARM1 (co-activator associated arginine methyltransferase) in the hinge region adjacent to the novel nuclear localization signal, resulting in a nuclear-cytoplasmic shuttling ability and/or enhanced interactions with other proteins or transcripts [60, 61]. Consequently, several putative mechanisms may be regulating the ability of these proteins to bind their mRNA targets.

Among additional mechanisms that could regulate the ability of HuR and HuD to mediate their effects, it appears highly plausible that these ELAV-like proteins do not function alone as single entities. In this context, we observed in our recent studies the presence of several binding complexes and individual proteins that could interact with the AChE 3′ UTR. HuD and HuR have both been found in ribonucleoprotein complexes interacting with other RNA-binding proteins, scaffolding proteins and sub-cellular localization proteins, such as nuclear export proteins, poly(C)-binding proteins, IGF-II mRNA binding protein 1 (IMP-1, the orthologue of zipcode-binding protein), kinesin as well as several others [26, 62–65]. Accordingly, post-transcriptional regulation of AChE transcripts may involve several additional RNA-binding proteins as well as specific interactions between HuD/HuR and other factors. The ELAV-like proteins specifically recognize ARE, which are a common cis-acting element that can be recognized by a number of different stabilizing and destabilizing trans-acting factors, including AUFI, Tristetraprolin and many others [66, 67]. Consequently, the fate of the AChE transcript may depend in part on the relative abundance of these different binding factors, as it has been demonstrated for other transcripts [68, 69].

In recent years, several studies have focused on deciphering the molecular mechanisms regulating AChE expression either in different muscle types, such as fast and slow muscles, or following muscle denervation. In this regard, one of our previous studies examined the levels of AChE in fast EDL versus slow soleus muscles [70]. In this study, we observed that AChE mRNA levels were significantly greater in EDL muscles as compared to the soleus. Importantly, this difference could not be attributed to enhanced AChE gene transcription in fast muscle as determined by nuclear run-on assays, thereby indicating that mRNA stability is important in regulating the overall content of AChE transcripts in fast versus slow muscles. Similarly, post-transcriptional regulatory mechanisms were also implicated in the pronounced decrease in AChE transcript levels seen following denervation of adult skeletal muscle [14]. Based on our recent findings obtained with cultured muscle cells, it thus becomes important to determine whether these changes in AChE mRNA expression in vivo are caused by the ability of HuR to bind to the AChE 3′UTR and hence, modulate the stability of these transcripts in skeletal muscle fibers placed under variety of experimental conditions. Similarly, it appears timely to also investigate whether HuD modulates AChE mRNA expression in neurons in vivo, following for example, anatomy and treatment with neurotrophic factors (see [71]).

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