A Nuclear Function of Hu Proteins as Neuron-specific Alternative RNA Processing Regulators

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Recent advances in genome-wide analysis of alternative splicing indicate that extensive alternative RNA processing is associated with many proteins that play important roles in the nervous system. Although differential splicing and polyadenylation make significant contributions to the complexity of the nervous system, our understanding of the regulatory mechanisms underlying the neuron-specific pathways is very limited. Mammalian neuron-specific embryonic lethal abnormal visual-like Hu proteins (HuB, HuC, and HuD) are a family of RNA-binding proteins implicated in neuronal differentiation and maintenance. It has been established that Hu proteins increase expression of proteins associated with neuronal function by up-regulating mRNA stability and/or translation in the cytoplasm. We report here a novel function of these proteins as RNA processing regulators in the nucleus. We further elucidate the underlying mechanism of this regulation. We show that in neuron-like cells, Hu proteins block the activity of TIA-1/TIAR, two previously identified, ubiquitously expressed proteins that promote the nonneuronal pathway of calcitonin/calcitonin gene-related peptide (CGRP) pre-mRNA processing. These studies define not only the first neuron-specific regulator of the calcitonin/CGRP system but also the first nuclear function of Hu proteins.

INTRODUCTION

Alternative RNA processing is one of the major mechanisms that expand the functional proteome from a limited genome size. Recent genome-wide bioinformatic analyses have demonstrated that the majority of human pre-mRNAs undergo alternative RNA processing (Modrek and Lee, 2002). A global survey of alternative splicing by using exon–junction microarrays indicated that at least 74% of multi-exonic human pre-mRNAs are alternatively spliced (Johnson *et al.*, 2003). The most extensive alternative splicing occurs in brain tissues (Xu *et al.*, 2002). It is not uncommon for hundreds of different isoforms to be generated from a single gene through alternative splicing in the mammalian nervous system. The genes that undergo extensive alternative splicing include those involved in cell-cell interactions, such as cell surface receptor molecules and various ion channels (Grabowski and Black, 2001; Black and Grabowski, 2003; Lipscombe, 2005). Significant advances have been made in dissecting the functional differences between different isoforms. As a result, it was postulated that alternative splicing plays a key role in supporting the complex functions of the nervous system (Grabowski and Black, 2001; Black and Grabowski, 2003; Lipscombe, 2005).

Although recent studies have provided exciting insight into the distinct functions played by individual alternatively spliced protein isoforms during neuronal development, un-

derstanding of the regulatory mechanisms that control neuron-specific alternative splicing remains very limited. Despite the extensive nature of alternative splicing in the nervous system, only a small number of factors involved in regulation of alternative splicing in the nervous system of mammals have been identified and even fewer characterized (Black and Grabowski, 2003). These factors include classical RRM-containing proteins such as nPTB/brPTB, NAPOR/CUGBP2, and Fox-1/Fox-2; KH-type proteins such as Nova; and the STAR/GRG family proteins such as QK1 (Ashiya and Grabowski, 1997; Zhang *et al.*, 1999; Jensen *et al.*, 2000; Markovtsov *et al.*, 2000; Polydorides *et al.*, 2000; Wu *et al.*, 2002; Zhang *et al.*, 2002; Dredge and Darnell, 2003; Ule *et al.*, 2003; Underwood *et al.*, 2005). All of these factors function by binding to their cognate target sequences on the pre-mRNA molecules and by modulating splicing of neuron-specifically processed exons either positively or negatively. In most cases, it is not clear how these factors interact with the basic splicing machinery to modulate splicing; however, one recent report demonstrated elegantly that the PTB protein blocks entry of U2AF into the presplicesomal E complex, thereby suppressing inclusion of the neuron-specific c-src N1 exon in nonneuronal cells; suppression is relieved in neuronal cells by the replacement of the ubiquitous form of PTB with a different form of PTB, nPTB/brPTB (Sharma *et al.*, 2005). A clue to how alternative splicing regulates brain-specific functions came from a study by Ule and colleagues, in which they provided compelling evidence to support the existence of a multi-tiered network regulated by Nova. They demonstrated that, in a coordinated manner, Nova regulates the exon content of RNAs encoding a large number of proteins that interact in the synapse, which accounts for 7% of the brain-specific alternative splicing in the neocortex (Ule *et al.*, 2005).

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Abbreviations used: CGRP, calcitonin gene-related peptide.

Mammalian Hu proteins are a group of RNA-binding proteins consisting of four family members: HuA (HuR in human), HuB (HelN1 in human), HuC, and HuD. Similar to Nova1 and Nova2, which are autoimmune targets in paraneoplastic opsoclonus myoclonus ataxia (Buckanovich *et al.*, 1996), HuD was originally cloned by screening a cerebellar expression library by using antisera from patients with paraneoplastic encephalomyelitis, one form of paraneoplastic syndrome in which Hu proteins are the autoimmune antigens (Szabo *et al.*, 1991). All three neuron-specific members of the Hu family, HuB, HuC, and HuD, were shown to be autoimmune antigens of the Hu syndrome (Posner and Dalmau, 1997). Several *in vivo* and *in vitro* experiments indicate an important role of neuron-specific Hu proteins in neuronal differentiation (Wakamatsu and Weston, 1997; Akamatsu *et al.*, 1999, 2005; Anderson *et al.*, 2000). At the molecular level, all members of the Hu protein family, including HuA (HuR) have been shown to play important roles in the cytoplasm. They interact with AU-rich elements (AREs) in 3'-untranslated regions (UTRs) to regulate mRNA stability (Jain *et al.*, 1997; Myer *et al.*, 1997; Anderson *et al.*, 2000); the human HuB and HuD proteins have also been shown to modulate translation (Antic *et al.*, 1999; Kullmann *et al.*, 2002).

Despite evidence of nuclear-cytoplasmic shuttling (Fan and Steitz, 1998; Burry and Smith, 2006) and the predominant nuclear localization (Okano and Darnell, 1997), none of the Hu proteins have been reported to have a nuclear function. However, in *Drosophila*, the Hu protein homologue, the embryonic lethal abnormal visual (ELAV) protein, has been shown to have a very important function in the nucleus. ELAV regulates alternative pre-mRNA processing in neurons (Lisbin *et al.*, 2001; Soller and White, 2003, 2005). Although the idea that Hu proteins may regulate alternative splicing in mammalian neurons seemed extremely promising, it remains a mystery whether Hu proteins can function as splicing regulators, because no target sequence of Hu proteins as alternative splicing regulators has been identified. In this report, we describe our finding of the first such target of Hu proteins, the human calcitonin/calcitonin gene-related peptide (CGRP) pre-mRNA.

Calcitonin/CGRP pre-mRNA represents one of many RNA transcripts that undergo neuron-specific regulation of alternative RNA processing. In a subset of neurons, the six exon-containing calcitonin/CGRP pre-mRNA is processed to skip the alternative 3'-terminal exon 4, leading to the production of the neurotransmitter CGRP, in contrast to thyroid C cells where this exon is included and exons 5 and 6 are skipped leading to the production of the peptide hormone calcitonin (Figure 1A) (Rosenfeld *et al.*, 1982). Previous studies demonstrated that the rate-limiting step for processing the calcitonin/CGRP pre-mRNA is the decision about whether to include or exclude the nonneuronal exon 4, which contains suboptimal RNA processing signals (Bovenberg *et al.*, 1986; Adema *et al.*, 1990). An enhancer element located downstream of exon 4 was demonstrated to promote inclusion of the exon partly through enhancing polyadenylation (Lou *et al.*, 1996). Although it was speculated that neuron-specific factors exist to regulate the CGRP-specific pathway (Leff *et al.*, 1987; Roesser *et al.*, 1993), previous studies have only identified a number of factors that affect the nonneuronal pathway (Lou and Gagel, 1999; Tran *et al.*, 2003; Tran and Roesser, 2003; Zhu *et al.*, 2003; Roesser, 2004).

Here, we report the identification of the Hu proteins as pre-mRNA processing regulators promoting the CGRP-specific pathway. Previously, we demonstrated that the ubiqui-

Figure 1. Alternative pre-mRNA processing of the calcitonin/CGRP gene. (A) Schematic diagram of the calcitonin/CGRP gene and its alternative RNA processing in thyroid and neuronal cells. (B) Diagram showing the calcitonin/CGRP reporter gene and its cell-specific RNA processing patterns. (C) Left, RT-PCR assay of total RNA from HeLa or CA77 (rat medullary thyroid carcinoma) cells transfected with the diagrammed calcitonin/CGRP reporter gene. Amplification bands resulting from exon 4 inclusion or exclusion are indicated. Right, a picture of CA77 cells.

tously expressed TIA-1/TIAR proteins promote the calcitonin-specific pathway through interacting with a U-rich sequence located in the intronic element downstream of the calcitonin exon (Zhu *et al.*, 2003). In this report, we show that Hu proteins interact with the same U-rich sequence and strongly compete with TIA-1/TIAR for binding in neuron-like cells. We provide evidence that the major function of Hu proteins is to block the activity of TIA-1/TIAR. Hu proteins represent the first neuron-specific regulator of the calcitonin/CGRP system. Our studies also define the first and long speculated nuclear function of Hu proteins as RNA processing factors (Szabo *et al.*, 1991).

MATERIALS AND METHODS

Plasmids

The human calcitonin/CGRP reporter constructs used in transfection experiments consist of calcitonin/CGRP exons 4–6 fused to a heterologous first exon from adenovirus (Lou *et al.*, 1995). The reporter construct that contains mutations at the U-tract sequences (UUUUUUUUUUU to GUGUU-GAUGGU) and plasmids used to generate *in vitro* transcribed RNA substrates for UV cross-linking and gel-shift assays were described previously (Zhu *et al.*, 2003). The calcitonin/CGRP reporter construct containing a Rev-responsive element (RRE) sequence was generated by insertion of an XhoI restriction enzyme recognition sequence upstream of the U-tract through polymerase chain reaction (PCR)-directed mutagenesis followed by ligation of two oligonucleotides complementary to each other at the XhoI site (sense oligonucleotide, 5'-TCGAGTGGGCGCAGCGTCAATGACGCTGACGGTACAC; and antisense oligonucleotide, 5'-TCGAGTGTACCGTCAGCGTCATTGACGCTGCC-CCCAC).

To generate cDNA sequences of the mouse HuB and HuC, reverse transcription (RT)-PCR was carried out using RNA isolated from the mouse F9 cells and mHuB- or mHuC-specific oligonucleotides (5'-GCGGATCCAATG-GAAACACAACCTGTCTAA and 5'-GCGAATCTTAGGCTTTGTGCGTITTT-GTT for mHuB and 5'-GCGGATCCAATGGTCACTCAGATACTGGG and 5'-GCGAATCTCAGGCCTTGTGCTGCTTGC for mHuC). The PCR products were digested with BamHI and EcoRI and cloned into the pcDNA3.1HisB (Invitrogen, Carlsbad, CA) vector for glutathione S-transferase (GST) recombinant proteins production or the BamHI-EcoRI sites in the pcDNA3.1HisB (Invitrogen, Carlsbad, CA) vector for mammalian cell transfection. Truncated mHuC constructs were generated by similar PCR-directed cloning. mHuC RRM12 and mHuC RRM3 were created using oligonucleotide pairs 5'-GCG-

GATCCAATGGAAACACAACACTGTCTAA and 5'-GAATTCGTTTGGCAA-CTTGACCGT, and 5'-GCGGATCCAAAGACAGGGCAGGCCCTGCT and 5'-GCGAATTCTCAGGCCTTGCTGCTGCTGC, respectively. The TIA-1-Rev and HuC-Rev plasmids were constructed by PCR amplifying TIA-1 or HuC and inserting the PCR product into the SphI and BglII sites of the PC12-Rev vector (a gift from Dr. Christine Herrmann, Baylor College of Medicine, Houston, TX). All of these plasmids were verified by DNA sequencing.

Cell Culture, P19 Differentiation, and Cell Transfection

HeLa, Chinese hamster ovary (CHO), F9, PC12 TT, SK-N-SH, and P19 cell lines were maintained according to instructions from American Type Culture Collection (Manassas, VA). CA77 cells, a cell line derived from rat medullary thyroid carcinoma (a gift from Drs. Alison Hall at Case Western Reserve University, Cleveland, OH, and Andrew Russo, University of Iowa, Iowa City, IA) were cultured in DMEM/F-12 (Invitrogen) supplemented with 10% fetal bovine serum (Invitrogen) and 1% Pen/Strep (Invitrogen). For differentiation of P19 cells, cells were aggregated in bacterial culture dishes and either untreated or treated with 0.3 μ M all-*trans* retinoic acid (Sigma-Aldrich, St. Louis, MO) or 1% dimethyl sulfoxide (DMSO) for 4 d and then plated in tissue culture dishes. Transfection of differentiated P19 cells was carried out 48 h after the cells were transferred to tissue culture dishes by using Lipofectamine (Invitrogen), and cells were collected 4 d after transfection for analysis. HeLa cells were transfected as described previously (Zhu *et al.*, 2003). Transfection of CA77 cells was carried out essentially the same as HeLa cells except that Lipofectamine 2000 (Invitrogen) was used, and cells were grown for 72 h before and after transfection. Cotransfections used 1 μ g of the calcitonin/CGRP reporter plasmid and either 0.2–0.4 μ g of TIA-1, Rev, TIA-1-Rev or HuC-Rev plasmids, or 1–2 μ g of mHuC RRM12 or mHuC RRM3 plasmids.

RNA and Protein Analysis

Procedures for total RNA and protein isolation and RT-PCR analysis were described previously (Zhu *et al.*, 2003). Use of low cycle (18–22) PCR permitted determination of the relative abundance of individual RNA species. Quantification of exon inclusion was determined using a PhosphorImager (GE Healthcare). The results shown are representative of at least three independent transfections for each experiment. The effect of TIA-1, Rev, TIA-1-Rev, HuC-Rev, mHuC RRM12, or mHuC RRM3 on RNA processing of the reporter pre-mRNA was calculated as percentage of the calcitonin exon inclusion [calcitonin exon inclusion/(calcitonin exon inclusion + CGRP exon inclusion)]. Western blot analysis using the proteins isolated from the untransfected or transfected cells was carried out with anti-Xpress antibody (Invitrogen) or anti-TIA-1/TIAR antibody (3E6) (Zhu *et al.*, 2003) and anti-Hu sera (a gift from Dr. Jerome Posner, Sloan Kettering Cancer Center, New York, NY). Because it is difficult to detect overexpressed proteins in CA77 cells due to the extremely low transfection efficiency, protein expression of Rev, TIA-1-Rev, mHuC-Rev, mHuC RRM12, and mHuC RRM3 was verified using HeLa cells.

In Vitro Assays

UV cross-linking reactions were carried out as described previously (Zhu *et al.*, 2003). Cross-linked polypeptides were immunoprecipitated using monoclonal antibodies against TIA-1 and TIAR (3E6) or anti-Hu sera. Gel-shift assays were performed using recombinant GST-TIAR or His-HuD prepared from bacteria and in vitro-transcribed RNA substrates. The reactions were described previously (Zhu *et al.*, 2003).

Immunoprecipitation of CA77 Nuclear Extract and RT-PCR Analysis

Eight hundred micrograms of CA77 cell nuclear extract proteins was immunoprecipitated with anti-Hu sera in NET supplemented with RNase Out (Invitrogen). The pellet was washed five times in NET (150 mM NaCl, 50 mM Tris, pH 7.5, and 0.05% NP-40), treated with proteinase K, and extracted with phenol/chloroform, followed by RNA precipitation. RT-PCR was carried out as described previously (Zhu *et al.*, 2003). Oligonucleotides used for RT-PCR were TTAGCCCCGGAGGTTAAGCA and TTGATGCCACCTCTGAGCCT for calcitonin/CGRP (both in intron 4) and ACCTCCAACTGAAGAGC and TGGCAGGTTTCTCCAGCGGC for rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (in intron 3 and exon 5, respectively).

Nuclear Extract Preparation from HeLa and CA77 Cells

HeLa cell nuclear extracts were prepared using S3 suspension culture and standard techniques (Zhu *et al.*, 2003). To make nuclear extracts from CA77 cells, 100 100-mm dishes of monolayer of CA77 cells were collected and used following a standard procedure (Zhu *et al.*, 2003).

RESULTS

Correlation between Hu Protein Expression and CGRP-specific Processing

In mammals, Hu proteins comprise a family of RNA-binding proteins that contains four closely related members:

HuA (HuR in human), HuB (HelN1 in human), HuC, and HuD. Three of these proteins, HuB, HuC, and HuD, are normally exclusively expressed in all neurons of the central and peripheral nervous systems with the exception of HuB, which is also expressed in germ cells (Okano and Darnell, 1997). Although distinct combinations of the individual Hu proteins do exist for individual neurons, every neuron has at least one Hu protein (Okano and Darnell, 1997). CGRP is naturally produced in a subset of neurons, including hippocampal neurons, trigeminal ganglion, dorsal root ganglion, and spinal cord (Rosenfeld *et al.*, 1992). Thus, a correlation between Hu protein expression and CGRP production is obvious *in vivo*.

Several cell lines have been used in previous studies that recapitulate either the calcitonin- or CGRP-specific RNA processing pathway. In our studies, we use HeLa cells to mimic the calcitonin pathway, and CA77 cells derived from rat medullary thyroid carcinoma (MTC) to mimic the CGRP pathway. HeLa cells do not express the calcitonin/CGRP gene endogenously. However, when transfected with a calcitonin/CGRP reporter construct that contains a fragment of the human calcitonin/CGRP gene extending from the middle of intron 3 to the 3' end of the gene fused with a first exon and half of an intron from the major late transcription unit of adenovirus, these cells process the reporter pre-mRNA in the calcitonin-specific pathway (Figure 1, B and C, lane 1). To examine CGRP-specific processing, we chose CA77 cells in our current study. These cells provide an optimal system for our purposes because although the line is derived from a rat MTC, medullary thyroid carcinoma, the CA77 cells have a neuronal phenotype characterized by neurites and neuronal antigens (Russo *et al.*, 1992) (Figure 1C, right). Most relevant to us is the endogenous expression of the calcitonin/CGRP gene and the switch from 95% calcitonin to as high as 80–90% CGRP mRNA production (Russo *et al.*, 1992). When CA77 cells are transfected with the reporter construct shown in Figure 1B, processing of the reporter pre-mRNA closely mimics that of the endogenous calcitonin/CGRP, with CGRP-specific processing as the predominant pathway (Figure 1C, lane 2) (Russo *et al.*, 1992).

To examine the expression level of Hu family members, proteins in the whole cell lysate isolated from eight cell lines were separated on SDS-PAGE and probed with anti-Hu sera derived from patients who suffer from a paraneoplastic syndrome in which Hu proteins are autoimmune antigens. The anti-Hu sera can detect all three neuron-specific Hu proteins (HuB, HuC, and HuD) but not the ubiquitously expressed HuA (HuR) in a Western blot analysis. Strong signals were observed in F9, PC12, TT, CA77, mouse embryonic stem (ES) cells, and SK-N-SH cells, where the CGRP pathway is preferred based on published and unpublished work (Bennett and Amara, 1992; Russo *et al.*, 1992; our unpublished data). In contrast, no Hu signals were detected in the HeLa and CHO cells, where the calcitonin pathway is preferred (Figure 2A) (Lou *et al.*, 1996, 1998). Expression of a group of generally expressed splicing regulators, TIA-1 and TIAR, did not show significant differences among these cell lines (Figure 2A). In an attempt to further correlate Hu protein expression level and CGRP production, we isolated total RNA from four cell lines that endogenously express both Hu proteins and the calcitonin/CGRP gene and carried out RT-PCR analysis. It is very clear that the highest CGRP expression was observed in the two cell lines that express very high levels of Hu proteins, F9 and mouse ES cells. Note that only one-sixth of the total protein lysate was loaded for these two cell lines in the blot probed with anti-Hu sera. In CA77 and TT cells, where the Hu protein level is much

Figure 4. Hu proteins compete with TIAR for binding at the U-tract sequence. (A) Gel mobility shift assay. The 32 P-labeled in vitro transcribed RNAs indicated in Figure 3A were incubated with GST-TIAR and His-HuD. Lane 1, no protein added; lanes 2–5, 0.1 μ g of His-HuD and 0, 0.02, 0.2, and 2 μ g of GST-TIAR were added; and lanes 6–9, 0.1 μ g of GST-TIAR and 0, 0.02, 0.2, and 2 μ g of His-HuD were added. (B) UV cross-linking/IP assay. Top, 32 P-labeled in vitro-transcribed RNAs were UV cross-linked in CA77 cell nuclear extract in the absence (lane 1) or presence of 0.2, 1, and 5 μ g of GST-TIAR (lanes 2–4) and immunoprecipitated with antibodies specific to Hu proteins (anti-Hu sera) and TIA-1/TIAR (3E6). Bottom, 32 P-labeled in vitro-transcribed RNAs were UV cross-linked in HeLa cell nuclear extract in the absence (lane 1) or presence of 0.1, 0.5, and 2.5 μ g of GST-mHuB (lanes 2–3) and immunoprecipitated with antibodies specific to Hu proteins (anti-Hu sera) and TIA-1/TIAR (3E6). (C) Reduced binding of TIA-1/TIAR to the U-tract sequence in CA77 cells. Left, Western blot analysis using same amounts of nuclear proteins from HeLa and CA77 cells and anti-TIA-1/TIAR antibody. Right, UV cross-linking/IP assay using 32 P-labeled RNA substrate indicated in Figure 3A, same nuclear protein amounts of HeLa and CA77 cell nuclear extracts, and anti-TIA-1/TIAR antibody.

mobility shift assay using the same wild-type and mutant RNA substrates showed a result consistent with the UV cross-linking/IP experiment (Figure 3C).

To test whether Hu proteins and TIA-1/TIAR can compete with each other for binding at the U-tract sequence, we carried out two experiments: gel mobility shift and UV cross-linking/IP assays. In the gel mobility shift assay, we incubated both GST-TIAR and His-HuD with the RNA substrate shown in Figure 3A. When 0.1 μ g of either His-HuD or GST-TIAR was added, the majority of the RNA substrate moved slower to a position indicative of a complex between the RNA and His-HuD or GST-TIAR (Figure 4A, compare lanes 2 and 6 with lane 1). When the same, constant amount of His-HuD and increasing amounts of GST-TIAR were added, the complex formed between the RNA and His-HuD was gradually replaced by a new complex formed between the RNA and GST-TIAR, which moved more slowly on the gel due to the size difference between the two complexes (lanes 3–5). Likewise, when GST-TIAR was held constant and His-HuD added in increasing amounts, the larger complex was shifted to a smaller complex (lanes 7–9). To determine whether a similar competition between Hu proteins and TIA-1/TIAR exists in the nucleus of the CA77 cells, we also carried out UV cross-linking assay. In this assay, increasing amounts of recombinant GST-TIAR protein were added to the CA77 nuclear extract and binding of both proteins to the RNA substrate was examined. Figure 4B shows that Hu binding decreases when TIAR binding increases. The reciprocal experiment—adding recombinant HuD protein to HeLa cell nuclear extract—showed a similar result (Figure 4B). These experiments establish that Hu proteins do compete with TIA proteins for the calcitonin/CGRP regulatory RNA sequence and in cells.

We next compared binding of TIA-1/TIAR to the same sequence in HeLa and CA77 cells. Although the protein level of TIA-1/TIAR is the same in nuclear extracts isolated from HeLa and CA77 cells, cross-linking of these proteins to the U-tract sequence is significantly reduced in CA77 nuclear extract (Figure 4C). These results are consistent with the possibility that reduced binding of TIA-1/TIAR to the U-tract is the underlying cause of the neuron-specific exon 4 skipping phenotype in CA77 cells. Furthermore, they sug-

gest that competition between Hu proteins and TIA-1/TIAR may be responsible for the reduced binding of TIA-1/TIAR to the U-tract sequence in the calcitonin/CGRP pre-mRNA in these cells.

Tethering of TIA-1 Protein in the Vicinity of the U-Tract in CA77 Cells Promotes Exon 4 Inclusion

Our previous experiments using HeLa cells show that TIA-1/TIAR proteins are required for exon 4 inclusion (Zhu *et al.*, 2003). Because interaction of TIA-1/TIAR with the U-tract is dramatically reduced in CA77 cells, we reasoned that if we could increase binding of this protein, we might be able to increase exon 4 inclusion in these cells. We first tried to overexpress TIA-1 or TIAR in CA77 cells, but we observed no increase in exon 4 inclusion (our unpublished data). This result was not unexpected because binding of TIA-1/TIAR to the U-tract may remain low in CA77 cells even when the overall protein level is higher due to the presence of endogenous Hu proteins. Next, we took advantage of a well-established fusion protein approach to target TIA-1 to the vicinity of the U-tract sequence. This approach has been successfully used to bring protein factors to RNA targets (Tiley *et al.*, 1992) and is similar to the MS2-MS2 coat protein fusion approach. We introduced a human immunodeficiency virus RRE immediately upstream of the U-tract of the calcitonin/CGRP reporter gene (Figure 5A) and brought TIA-1 to the sequence through a TIA-1-Rev fusion protein. The introduced RRE sequence contains a minimal, 29-nucleotide single stem-loop structure shown to have strong binding for Rev (Tiley *et al.*, 1992). The resulting reporter construct behaved like the parental construct in that its pre-mRNA was processed to predominantly skip exon 4 (Figure 5B, lane 1, compare to lane 2 in Figure 1C). However, when a TIA-1-Rev fusion protein construct was cotransfected with the RRE-containing reporter gene, exon 4 inclusion was increased by 2- to 2.5-fold, whereas cotransfection of Rev alone construct did not significantly affect RNA processing of this reporter gene (Figure 5B, lanes 2–5). As expected, cotransfection of TIA-1 with the RRE-containing reporter did not change the splicing phenotype (our unpublished data). These experiments establish that reduced binding of TIA-1/

Figure 5. Targeted binding of TIA-1 to the U-tract region increased exon 4 inclusion. (A) Diagram showing the location of the RRE stem-loop sequence. (B) RT-PCR analysis using RNAs isolated from transfected CA77 cells that preferentially exclude exon 4. CA77 cells were transfected with a RRE-containing reporter (lanes 1–5) in the absence of any protein plasmids (lanes 1) or the presence of 1 and 2 μ g of Rev (lanes 2 and 3) or TIA-1-Rev (lanes 4 and 5). (C) RT-PCR analysis using RNAs isolated from CA77 cells transfected with the RRE-containing reporter in the absence of any proteins (lane 1) or the presence of 0.4 μ g of Rev (lane 2) or TIA-1-Rev (lanes 3–9) together with 0.2–0.4 μ g of HuC (lanes 4 and 5), Rev (lanes 6 and 7) or HuC-Rev (lanes 8 and 9). The bottom panel in B and C shows a bar graph indicating the level of the calcitonin exon inclusion upon overexpression of the transfected proteins.

TIAR at the U-tract sequence is responsible for the low level of exon 4 inclusion in CA77 cells.

Hu Proteins Are Functionally Involved in Regulating Exon 4 Skipping

To further investigate the role of Hu proteins in regulating CGRP processing, it is necessary to disrupt the function of the endogenous Hu protein in CA77 cells and to study its effect on alternative RNA processing of calcitonin/CGRP. Although the commonly used RNA interference approach is very attractive, it is very difficult to knockdown all three neuron-specific Hu proteins to sufficiently low levels to force a change in RNA processing. All of the experiments we have carried out to date suggest that individual Hu proteins have redundant functions in the calcitonin/CGRP system (our unpublished data). We therefore turned to the dominant-negative mutant protein approach. It was previously shown that either HuB or HuC RRM3 can function dominant negatively to interfere with the function of both HuB and HuC in a neuronal differentiation experiment (Akamatsu *et al.*, 1999). Thus, it is possible that a dominant-negative protein of one Hu protein will interfere with the activity of all of the Hu proteins. Different domains of Hu proteins have been shown to have distinct functions. In the study by Akamatsu *et al.* (1999), it was shown that the RRM1 and RRM2 of mouse HuB or HuC protein have strong binding affinity for poly(U)-Sephadex, whereas RRM3 binds to RNA only weakly. Other studies indicate that the RRM1 and RRM2 domains of the HuD protein have high RNA-binding activity and contribute to the RNA-binding properties of the HuD protein to the c-fos ARE (Chung *et al.*, 1996), whereas the RRM3 domain of human HuB (HelN1) binds to the c-myc ARE (Levine *et al.*, 1993; Gao and Keene, 1996). Moreover, the RRM3 of human HuB was shown to interfere with multimerization of the full-length HuB (Gao and Keene, 1996), whereas the hinge and RRM3 domains of the human HuA (HuR) was demonstrated to be necessary and sufficient for export of HuR through the CRM1 pathway (Gallouzi *et al.*, 2001).

When we cotransfected a construct containing the mouse HuC RRM3 together with the hinge domain with the calcitonin/CGRP reporter gene into CA77 cells, the production of CGRP was compromised and inclusion of the calcitonin exon was increased by approximately twofold compared

with cells transfected without any Hu proteins (Figure 6, A and B, compare lane 1 with lanes 4 and 5. Transfection of the CA77 cells with the mHuC RRM12 construct did not signif-

Figure 6. Neuronal exclusion of exon 4 in CA77 cells is compromised by a truncated mHuC protein containing RRM3 domain with the hinge domain. (A) Diagram of the two truncated forms of mHuC is shown in the top panel. (B) CA77 cells were cotransfected with a calcitonin/CGRP reporter and increasing amounts (2 and 4 μ g) of either mHuC RRM12 or mHuC RRM3 plasmid. The top panel shows an RT-PCR assay of total RNA from transfections of CA77 cells with the wild-type calcitonin/CGRP reporter gene and no mHuC (lane 1), increasing amount of mHuC RRM12 (lanes 2 and 3), or mHuC RRM3 (lanes 4 and 5). The middle panel shows Western blot analysis by using anti-Xpress antibody to indicate the level of the overexpressed mHuC proteins in HeLa cells. The bottom panel shows a bar graph indicating the level of the calcitonin exon inclusion upon overexpression of the transfected proteins.

Figure 7. Association of Hu proteins and the calcitonin/CGRP pre-mRNA in CA77 cells. The nuclear extract prepared from CA77 cells was immunoprecipitated with anti-Hu sera. RNA was isolated from either total nuclear extract or immunoprecipitated pellet and analyzed by RT-PCR. Minus RT lanes are included as controls. The oligonucleotides used to amplify the calcitonin/CGRP pre-mRNA anneal to sequences in intron 4, and the resulting PCR product is 225 nucleotides, whereas the oligonucleotides used to amplify the GAPDH pre-mRNA anneal to sequences in intron 3 and exon 5, and the resulting PCR product is 585 nucleotides.

icantly affect inclusion of the calcitonin exon (Figure 6, A and B, compare lane 1 with lanes 2 and 3).

There are two possibilities for how Hu proteins contribute to the CGRP-specific processing. Hu proteins may simply block the activity of TIA-1/TIAR proteins, or they have a secondary function upon binding to the U-tract sequence. We carried out a simple experiment to address this question, again, by using the Rev-RRE approach. As shown in Figure 5B, TIA-1-Rev increased the calcitonin exon inclusion of the transcribed RRE-containing reporter pre-mRNA. This activity can be competed away by cotransfection of the Rev alone construct in increasing amounts together with TIA-1-Rev (Figure 5C, lanes 3, 6, and 7). If Hu proteins do not have any additional function other than blocking TIA-1/TIAR activity, cotransfection of a HuC-Rev fusion construct would decrease the calcitonin exon inclusion to the similar extent as Rev alone, which is exactly what we observed (Figure 5C, compare lanes 6 and 7 and 8 and 9). Cotransfection of HuC without the Rev fusion and TIA-1-Rev did not have a different effect from TIA-1-Rev (compare lane 3 with lanes 4 and 5). This result implies that the major function of the Hu proteins upon binding to the intronic U-tract sequence is to block the activity of TIA-1/TIAR.

Hu Proteins Interact with Endogenously Expressed Calcitonin/CGRP pre-mRNA

We took advantage of the fact that Hu proteins and calcitonin/CGRP are both endogenously expressed in CA77 cells and tested whether they interact in the natural environment of these cells. We immunoprecipitated the Hu protein-containing complex from a CA77 cell nuclear extract by using anti-Hu sera and examined the RNA in the complex by RT-PCR analysis. As shown in Figure 7, significant association of calcitonin/CGRP pre-mRNA was present in the immunoprecipitate, whereas no GAPDH pre-mRNA was brought down. PhosphorImager measurement of radioactivity in the RT-PCR products indicates that 55% of the input calcitonin/CGRP pre-mRNA was pulled down by the anti-Hu sera. The strong association of Hu proteins and calcitonin/CGRP pre-mRNA supports the role of Hu proteins in regulating the neuron-specific RNA processing of this pre-mRNA.

DISCUSSION

In this report, we demonstrate a novel mechanism of neuron-specific regulation of alternative RNA processing. We argue that the mammalian Hu proteins regulate neuron-specific alternative RNA processing of the calcitonin/CGRP

pre-mRNA by blocking the activity of ubiquitously expressed TIA-1/TIAR, which were previously shown to play an important role in inclusion of the nonneuronal 3'-terminal exon, exon 4 (Zhu *et al.*, 2003). The two groups of proteins compete for binding to the same U-tract sequence located in an intronic element of the calcitonin/CGRP pre-mRNA. It is interesting that even though these two groups of proteins have a similar affinity to the U-tract-containing RNA target in a gel mobility shift assay, Hu proteins seem to cross-link RNA more strongly in the neuron-like CA77 cells (Figure 4C). This result, coupled with the lack of effect of overexpression of TIA-1 on exon 4 inclusion in CA77 cells, implies that other factors in these cells may contribute to the apparently stronger interaction of Hu proteins with the target in these cells. We demonstrated previously that TIA-1/TIAR binding at this particular U-tract sequence depends on the interaction between U1 Small nuclear ribonucleoprotein (snRNP) and a pseudo-5' splice site sequence located immediately upstream of the U-tract (Zhu *et al.*, 2003). The reported direct interaction between U1C and TIA-1 is a likely liaison between U1 snRNP and TIA-1/TIAR in the calcitonin/CGRP system (Forch *et al.*, 2002). In CA77 cells, this interaction may be blocked by Hu proteins, thereby leading to reduced binding of TIA-1/TIAR to the U-tract, indicated by the cross-linking/IP analysis. It is also possible that other neuron-specific factors exist to help Hu proteins outcompete TIA-1/TIAR proteins.

It seems that the major function of Hu proteins upon binding to the intronic U-tract sequence in neuronal or neuron-like cells, such as CA77 cells, is to block the activity of TIA-1/TIAR proteins, which would otherwise promote the inclusion of the calcitonin exon 4 (Figure 5C). Of particular note is the fact that in the experiment where TIA-1-Rev fusion protein is cotransfected with the RRE-containing reporter construct into CA77 cells, Hu proteins are presumably still able to bind the U-tract immediately downstream of the RRE stem-loop structure. However, such binding does not impact the inclusion of the calcitonin exon if TIA-1-Rev is present. Likewise, increasing amounts of HuC protein did not change the outcome either if TIA-1-Rev is present (Figure 5C).

If the major function of Hu proteins is to block TIA-1 and TIAR proteins, why does the RRM3 domain, but not the RRM1 and RRM2 domains together, have a dominant-negative effect? The lack of a dominant-negative effect by RRM1 and RRM2 can be explained by the fact that this protein, although binding to RNA specifically, has lowered binding affinity as judged by RNA gel-shift analysis (our unpublished data). Consistent with our results, the study by Chung *et al.* (1996) demonstrated that the RNA-binding affinity of the HuD full-length protein is almost 8 times greater than that of the HuDRRM1+2 protein. It is unclear at present how the RRM3 and hinge domains of Hu proteins functions as a dominant-negative protein. However, a hint came from the following studies. In one study, RRM3 of the human HuB protein interferes with the multimerization of the full-length proteins (Gao and Keene, 1996). Two other studies indicated that HuD as well as ELAV, the *Drosophila* homologue of Hu proteins, exist as multimers (Kasashima *et al.*, 2002; Solter and White, 2005). The study on ELAV further demonstrated that binding of the multimerized ELAV on *ewg* RNA is functionally important (Solter and White, 2005). Thus, it is very tempting to suggest that multimerization of Hu proteins is important for their functions as RNA processing factors, and overexpression of the RRM3 and hinge domains interferes with multimerization of the full-length proteins, which causes its dominant-negative effect.

Although in RNA gel mobility shift assay, recombinant proteins of the three neuron-specific Hu proteins show strong affinity for the calcitonin/CGRP intronic element containing the U-rich sequence (our unpublished data), our cell transfection experiments did not distinguish effect by individual Hu proteins (Figures 5 and 6). In CGRP-producing neurons, including hippocampus, dorsal root ganglia, and spinal cord, all three of the neuron-specific Hu proteins, HuB, HuC, and HuD, are present in adult mice (Okano and Darnell, 1997). A recent study using HuD-deficient mice demonstrated that HuD is involved at multiple stages during neuronal development. However, no difference of expression of several Hu protein targets was detected, suggesting at least a partial functional redundancy of Hu family proteins (Akamatsu *et al.*, 2005).

The precise role of TIA-1/TIAR in promoting the nonneuronal inclusion of calcitonin/CGRP exon 4 is not clear. We propose that TIA-1/TIAR may play one of the two roles: inhibit recognition of the 3' splice site of exon 5, or enhance recognition of the 3' splice site of exon 4 (Zhu *et al.*, 2003). In addition to the function mediated by intronic element, TIA-1/TIAR have been shown to promote authentic 5' splice site recognition by U1 snRNP (Del Gatto-Konczak *et al.*, 2000; Forch *et al.*, 2000; Le Guiner *et al.*, 2001). In those examples, the TIA-1/TIAR binding sites are located immediately downstream of suboptimal 5' splice sites. Recent studies demonstrate an emerging theme of regulated alternative splicing by competing activities of TIA-1 and PTB. In one example, the two proteins bind to two different RNA sequences on the Fas pre-mRNA and have opposing activities in regulating the fate of Fas exon 6 (Izquierdo *et al.*, 2005). In the other two examples, TIA-1 and PTB compete for binding at the same intronic sequences following the 5' splice sites (Zuccato *et al.*, 2004; Shukla *et al.*, 2005). The competing nature of binding of Hu and TIA-1/TIAR proteins at similar target sequence may be a wide spread phenomenon of neuron-specific alternatively spliced exons. Conceptually, it is likely that a subset of alternatively spliced exons is controlled by the competing activity of these two groups of proteins. However, at this point, it remains possible that other AU-rich sequence-binding proteins that we did not examine may also be involved in regulated RNA processing.

Experiments reported in this communication reveal a functional role of Hu proteins in regulating the neuron-specific alternative RNA processing of the calcitonin/CGRP pre-mRNA. However, although Hu proteins are necessary for the regulation, they are not sufficient. In HeLa cells, overexpression of Hu proteins, alone or in combination, did not switch the RNA processing phenotype (our unpublished data). This result suggests that other proteins may also be required for regulation in neurons. There is precedent that more than one neuron-specific protein is needed to include an alternative exon. At least two neuron-enriched proteins, nPTB and Fox-1, are required for inclusion of the c-src N1 exon in neurons (Markovtsov *et al.*, 2000; Underwood *et al.*, 2005).

Experiments using cell lines, embryos, and knockout mice demonstrated that Hu proteins are involved in early neuronal differentiation (Wakamatsu and Weston, 1997; Akamatsu *et al.*, 1999, 2005; Anderson *et al.*, 2000). Because some of the neuron-specific members of the Hu protein family have been shown to regulate mRNA stability and translation, it was postulated that the function of Hu proteins during neuron differentiation is to stabilize or promote translation of those mRNAs involved in neuronal function, such as the growth-associated protein-43 and neurofilament M, through binding at the AU-rich element located in 3'

UTR (Antic *et al.*, 1999; Anderson *et al.*, 2000). Our results define the first nuclear function of Hu proteins and therefore suggest a novel mechanism by which Hu proteins regulate neuronal differentiation. It is entirely possible that Hu proteins regulate expression of genes associated with neuronal differentiation or maintenance by affecting alternative splicing of their pre-mRNAs. It is therefore of great importance to identify additional targets of Hu proteins and study the role of Hu proteins in regulating alternative splicing of these Hu target-containing pre-mRNAs. Our initial search of the alternative splicing database identified several neuron-specific alternatively spliced exons that are surrounded by U-rich sequences. Experiments are under way to investigate the role of Hu proteins in regulating these alternative splicing events.

Our studies reported here add Hu proteins to the currently very short list of tissue-specific RNA processing regulators. Additional studies of these proteins will provide significant insight into the regulatory mechanisms of neuron-specific alternative RNA processing.

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