The Elav-like Proteins Bind to a Conserved Regulatory Element in the 3'-Untranslated Region of GAP-43 mRNA*

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Previous studies have identified three brain proteins (40, 65 and 95 kDa, respectively) that specifically bind to the 3'-untranslated region of GAP-43 mRNA. In this study, using a specific monoclonal antibody, we now show that the 40-kDa proteins are members of the Elav-like protein family. This family of specific RNA-binding proteins comprise three neural specific members called HuD, HuC, and Hel-N1. We have shown that purified recombinant HuD can bind with high affinity to GAP-43 mRNA. In addition, we have mapped the binding site to a highly conserved 26-nucleotide sequence within the regulatory element. The binding of HuD to this site is readily displaced by RNA oligonucleotides encoding other HuD binding sites. We also show that only the first and second RNA binding domains of HuD are required for selective binding to GAP-43 mRNA.

The Elav-like genes encode a family of RNA-binding proteins that were first described in Drosophila (1–3). Elav, the founder member of this family, is expressed immediately after neuroblastic differentiation into neurons (4). The Elav gene product is necessary for neuronal differentiation and maintenance. In mutant flies, neuroblasts fail to differentiate and continue to proliferate (1). Interest in these proteins was stimulated by the discovery that their human homologues are tumor antigens (5). Subsequent cloning studies have shown that there are four members of the human Elav-like family, namely, HuD, HuC, Hel-N1, and HuR (5–8). HuD, HuC, and Hel-N1 are expressed in postmitotic neurons and in neuroendocrine tumors (6, 9–12). Recent studies indicate that they are also required for neuronal differentiation (13). HuR, the fourth and most recently discovered member, is expressed in all cells and is overexpressed in many human tumors (7). Its normal cellular function has yet to be clarified.

All four Elav-like proteins contain three RNA recognition motifs (RRM)1 of the RNP2/RNP1 type (14). The first and second of these RRMs are in tandem and are separated from the third by a segment rich in basic amino acids. A significant insight into the mechanism of action of these proteins was provided by the observation that they specifically bind to U-rich elements in the 3'-UTR of mRNAs that regulate cell growth and differentiation (7, 12, 15–17). These elements were first characterized by Shaw and Kamen (19), who showed that the U-rich element in the 3'-UTR of granulocyte macrophage-colony-stimulating factor mRNA regulates expression at the post-transcriptional level. Recent data have shown that transfection of the Elav-like genes into cells results in the increase in expression of mRNAs that contain such U-rich elements (20).

Thus, through binding to a common element in many mRNAs, the Elav-like proteins are important components of coordinate gene expression mechanisms.

One important question is the relationship between the HuR, which is ubiquitously expressed, and the neural specific members HuD, HuC, and Hel-N1. It is reasonable to postulate that HuD, HuC, and Hel-N1 interact with brain-specific mRNAs involved in pathways critical to neuronal differentiation. In our previous studies we have investigated the interaction between the Elav-like proteins and mRNAs that are expressed in all cells (16–17, 21). We have now investigated whether there are neural specific mRNAs that may be specifically regulated by the Elav-like proteins. Recent studies have indicated that post-transcriptional mechanisms significantly contribute to the regulation of GAP-43 gene expression (22–24). GAP-43 is a neuron-specific phosphoprotein that is required for the regeneration and remodeling of neuronal connections (25–34). Precise control of GAP-43 expression is thus of critical importance during nervous system development.

Two independent cis-acting elements that regulate expression have been mapped to the 3'-UTR of GAP-43 mRNA. One maps to the region proximal to the termination codon (35). Insertion of this element into a reporter mRNA results in destabilization of the reporter message (36). This pathway is regulated by nerve growth factor (36). The other element is U-rich in sequence and found within a highly conserved region of the 3'-UTR (37). Insertion of this element also confers instability to reporter constructs, which is reversed by treatment with TPA (38). Thus GAP-43 expression is regulated by at least two different pathways utilizing at least two different cis-acting elements. Cross-linking studies have identified three brain proteins of 40, 65, and 95 kDa that specifically interact with the 3'UTR of GAP-43 mRNA (37). It was noted that the 40-kDa protein was similar in size to the neuronal specific Elav-like proteins. Thus, in the current study, we have investigated whether this 40-kDa protein corresponds to a neuronal specific member of the Elav-like protein family and have investigated its interaction with GAP-43 mRNA in a purified system.

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1 The abbreviations used are: RRM, RNA recognition motif; UTR, untranslated region; TPA, 12-O-tetradecanoylphorbol-13-acetate; mAb, monoclonal antibody.

2 W.-J. Ma, S. Chung, and H. M. Furneaux, submitted for publication.
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**Materials and Methods**

Preparation of Labeled RNA Transcripts—Plasmid DNAs were digested with the appropriate restriction enzymes and transcribed with T3 RNA polymerase for GAP-43B, and GAP-43C or T7 RNA polymerase for GAP-43A in the presence of [32P]uridine triphosphate (Amersham Corp.) as described previously (39). GAP-43A was linearized with HinIII, yielding a transcript of 221 nucleotides and 114 nucleotides of the 3′-untranslated region. pGAP43B and pGAP43C were linearized with EcoRI, yielding 221 nucleotides and 114 nucleotides of the 3′-untranslated region, respectively.

Immunoprecipitation of UV-cross-linked Complexes between the GAP-43 3′-UTR and Cytosolic Proteins from Neonatal Rat Brains—S100 extracts were prepared from freshly dissected brains from postnatal day 4 rats as described by Dignam et al. (40). Extracts containing 50 μg of protein were incubated with 0.5 μg of 32P-labeled RNA (1.5 × 106 cpm) and 10 units of RNasin (Promega) in a buffer containing 10 mM HepES (pH 7.6), 3 mM MgCl2, 40 mM KCl, 5% (v/v) glycerol, and 1 mM dithiothreitol for 10 min at 4°C (41). Following digestion with RNase T1 (1 unit/μl, Calbiochem), RNA-protein complexes were exposed to UV irradiation for 30 min at 4°C using a germicidal lamp (Sylvania G50Tb), and samples were then subjected to an additional digestion with RNase A (1 μg/ml) for 15 min at 37°C. Immunoprecipitation assays were performed using 16A11 antibody (10) or an anti-tubulin MAb as a negative control. We used the protocol developed by De Graan et al. (42) with the following modifications. UV-cross-linked complexes were incubated in 200 μl of Nonidet P-40 buffer (10 mM Tris-HCl [pH 7.5], 1% Nonidet P-40, 1% bovine serum albumin, 150 mM NaCl, and 2 mM EDTA) containing the indicated MAb (at 1:100 dilution) for 1 h at 25°C. Next, 20 μl of a 50% slurry of protein G-Sepharose (Sigma) in Nonidet P-40 buffer was added. Samples were further incubated for 4 h at 4°C. Beads containing the immunoprecipitates were then separated by centrifugation and proteins analyzed by 10% SDS-polyacrylamide gel electrophoresis. Gels were dried and the radioactivity associated with each band was analyzed using a PhosphorImager (Molecular Dynamics).

Purification of GST-HuD Proteins—An overnight culture of Escherichia coli BL 21, transformed with each HuD construct (17), was diluted in 1:50 LB media. At an A600 of 0.4, the culture was induced with isopropyl-β-D-thiogalactopyranoside (0.1 mM). After 4 h of further growth, the cells were spun down and resuspended in 10 ml of buffer A (50 mM Tris (pH 8.0), 200 mM NaCl, 1 mM EDTA). The cells were lysed by adding lysozyme (0.2 mg/ml) and Triton (1%). The lysate was centrifuged at 12,000 × g for 30 min. The resultant supernatant was loaded onto a glutathione-agarose affinity column (13 mg of protein/ml of resin). After washing the column with buffer B (50 mM Tris (pH 8.0), 200 mM NaCl, 1 mM EDTA, 1% Triton), GST-HuD was eluted with (50 mM Tris (pH 8.0), 5 mM glutathione). Active protein was determined by RNA-complex formation, pooled, and stored at −70°C.

Assay of GAP-43 HuD Complex Formation—Reaction mixtures (0.02 ml) contained 50 mM Tris (pH 7.0), 150 mM NaCl, 0.25 mg/ml tRNA, 0.25 mg/ml bovine serum albumin, 10 fmol of labeled RNA and protein as indicated. Mixtures were incubated at 37°C for 10 min. Following incubation, 4 μl of a dye mixture (50% glycerol, 0.1% bromphenol blue, 0.1% xylene cyanol) was added, and 20% of the reaction mixture was immediately loaded on a 1% agarose gel in TAE buffer (40 mM Tris acetate, 1 mM EDTA). The gel was then electrophoresed at 40 V for 2.5 h. The gel was dried on DE81 (Whatman) with a backing of gel drying paper (Hudson City Paper, West Caldwell, NJ) and exposed to XAR5 film (Eastman Kodak Co.) for 4–5 h at −70°C.

RNase T1 Selection Assay—Reaction mixtures (0.02 ml) contained 50 mM Tris (pH 7.0), 150 mM NaCl, 0.25 mg/ml bovine serum albumin, 0.25 mg/ml tRNA, 20 fmol of radiolabeled mRNA, purified HuD as indicated. After 10 min of incubation at 37°C, 0.5 unit of RNase T1 was added to each reaction and incubated at 37°C for 10 min further. The mixtures were diluted 1:6 with buffer F (20 mM Tris (pH 7.0), 150 mM NaCl, 0.05 mg/ml tRNA) and filtered through nitrocellulose (BA 85, Schleicher & Schuell). After washing the nitrocellulose twice with buffer F, bound HuD-RNA complex was extracted with phenol/chloroform and concentrated by ethanol precipitation. The resultant RNA was dissolved in formamide buffer and denatured at 65°C for 2 min. Samples were analyzed by 12% polyacrylamide/urea gel electrophoresis. The gel was fixed with 1:1:8 acetic acid: methanol: water, dried, and exposed to XAR5 film at −70°C overnight.

Nitrocellulose Filter Binding Assay—Reaction mixtures (0.02 ml) contained 50 mM Tris (pH 7.0), 150 mM NaCl, 0.25 mg/ml bovine serum albumin, 0.25 mg/ml tRNA, 10 fmol of radiolabeled mRNA, and purified HuD as indicated. After 10 min of incubation at 37°C, the mixtures were diluted 1:6 with buffer F and filtered using nitrocellulose. After washing the filter twice with buffer F, bound radioactivity was determined by Cerenkov counting.

Results

The 40-kDa Protein Is Recognized by a Monoclonal Antibody Directed against the Neuronal Members of the Elav-like Protein Family—Fig. 1 shows the structure of the GAP-43 mRNA and the transcripts (encoded by plasmids GAP-43H, GAP-43A, GAP-43C, and GAP-43B) used in these studies. Kohn et al. (37) have previously described 40, 65, and 90-kDa brain proteins that cross-link to the transcript encoded by plasmid GAP-43H. To investigate whether the 40-kDa protein was related to the Elav-like proteins, the labeled cross-linked material was precipitated with a monoclonal antibody specific for the neuronal members of the Elav-like protein family (10). The anti-Elav-like monoclonal (16A11) specifically precipitated the 40-kDa species (Fig. 2, lanes 1–3). The 40-kDa protein was not detected.
The 40-kDa species is a member of the Elav-like RNA-binding protein family. 0.5 ng of $^{32}$P-labeled GAP-43 RNA (derived from plasmid GAP-43H) was incubated with S100 extract (50 µg of protein) and cross-linked by UV irradiation. The cross-linked material was then precipitated with mAb 16A11 or an anti-tubulin mAb and analyzed by SDS-gel electrophoresis (lanes 1–3). The nonprecipitated material is shown in lane 4.

Fig. 2. The 40-kDa species is a member of the Elav-like RNA-binding protein family.

FIG. 3. HuD binds to the GAP-43 RNA 3′-untranslated region. 10 fmol (50,000 cpmpmol UMP) of each $^{32}$P-labeled RNA was incubated without protein or with indicated concentrations of HuD protein. After 10 min of incubation at 37 °C, 20% of the reaction mixtures was resolved on a 1% agarose gel.

Fig. 3. HuD binds to the GAP-43 RNA 3′-untranslated region.

FIG. 4. The affinity of HuD for GAP-43 RNA. RNA-protein complex formation was assayed by nitrocellulose filtration. 10 fmol of each RNA (specific activity, 100,000 cpmpmol) was incubated with the indicated concentration of HuD for 10 min at 37 °C. A, plot of percentage of RNA bound versus log of HuD concentration. ◆, GAP-43A; ●, GAP-43B; □, GAP-43C. B, plot of log complex/free RNA versus log HuD concentration.

Fig. 4. The affinity of HuD for GAP-43 RNA. RNA-protein complex formation was assayed by nitrocellulose filtration. 10 fmol of each RNA (specific activity, 100,000 cpmpmol) was incubated with the indicated concentration of HuD for 10 min at 37 °C. A, plot of percentage of RNA bound versus log of HuD concentration. ◆, GAP-43A; ●, GAP-43B; □, GAP-43C. B, plot of log complex/free RNA versus log HuD concentration.
HuD Binds to a Conserved U-rich Segment of GAP-43 mRNA—We have further mapped the binding site using an RNaseT1 digestion technique. In this technique the HuD-RNA complex is digested with RNaseT1, and the protein-bound RNA fragments are isolated by absorption to nitrocellulose (17). Fig. 5A shows the HuD-dependent retention of a single fragment on incubation with GAP-43 mRNA. A, the indicated concentrations of HuD or glutathione S-transferase (GST) (lanes 1, 2, 4, 5, 7, and 8) were incubated with 32P-labeled Gap-43 RNAs (20 fmol, 50,000 cpm/pmol UTP) at 37 °C for 10 min. After treating the reaction mixture with T1 RNase (0.5 unit per reaction), the reaction mixtures were filtered through nitrocellulose. The bound RNA fragments were extracted and resolved on 12% denaturing polyacrylamide gel. Lane M, φX174 HindIII fragments; lane 3, RNase T1 digestion of GAP-43A RNA; lane 6, RNase T1 digestion of GAP-43B; lane 9, RNase T1 digestion of GAP-43C. B, the selected fragment was eluted from the preparative gel and digested by further digestion with RNase T1.

We have compared the sequence of the GAP-43 mRNA binding site with those we have found in c-Fos, interleukin-3, c-Myc, Tau, IVA2, and GAP-43 mRNAs (Fig. 6). The GAP-43 mRNA binding site is similar to the others in that it contains U-rich tracts. We have shown that these U-rich tracts are essential for the binding of HuD to the element in the 3’-UTR of c-Fos mRNA (17). Next, we tested whether the HuD-GAP-43 complex would be displaced by the 27-nucleotide c-Fos oligonucleotide but not by a mutant oligonucleotide (27–8) (17) which contains substitutions of the U-rich tracts (Fig. 7). Thus the GAP-43 binding site is functionally similar to those we have described in other mRNAs.

The First and Second RNA Binding Domains Are Essential for Binding to GAP-43 mRNA—HuD contains three RNA binding domains (5). The first and second are in tandem and are separated from the third by a basic segment, which we call the basic domain. Previous studies have shown that the first and second RNA binding domains are both essential for binding to
the AU-rich elements, whereas the third RNA binding domain binds to the poly(A) tail (17).

To determine the domains of HuD required for binding to GAP-43 mRNA, we utilized the deletion constructs shown in Fig. 8. Gel retardation analysis showed that the first and second RRMs were essential for binding to the GAP-43 site (Fig. 8). The third RRM did not display any detectable binding (Fig. 8, lanes 8 and 9). As noted previously in our studies on the c-Fos element, the first and second RRMs are required in tandem for complete binding activity (Fig. 8, lanes 4–6). The basic domain alone did not exhibit any binding activity.

**DISCUSSION**

The Elav-like RNA-binding proteins regulate gene expression via an interaction with U-rich elements in the 3'-UTR of specific mRNAs. The mechanism underlying the specificity of this regulation is poorly understood. There are four members of this family (HuD, HuC, Hel-N1, and HuR) that are homologues of Elav, a Drosophila gene required for neuronal differentiation. The existence of three family members in human neurons suggests that they specifically modulate mRNAs that regulate neuronal differentiation. The RNA binding specificity, however, of all four members is very similar (7, 12, 17). Thus, it is likely that HuD, HuC, and Hel-N1 are selectively expressed in neurons (5–8). The neuronal members are the homologues of Elav, a Drosophila gene required for neuronal differentiation. The three family members (HuD, HuC, and Hel-N1) are ubiquitously expressed, whereas HuR is specifically expressed in neurons (5–8). The neuronal members are the homologues of Elav, a Drosophila gene required for neuronal differentiation.

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