Mammalian ELAV-like neuronal RNA-binding proteins HuB and HuC promote neuronal development in both the central and the peripheral nervous systems

WADO AKAMATSU*‡, HIROTAKA J. OKANO§, NORIKO OSUMI‖, TAKAYOSHI INOUE†, SHUN NAKAMURA‡, SHIN-ICHI SAKAKIBARA*‡, MASAYUKI MIURA*‡, NOBUTAKE MATSUO‡, ROBERT B. DARNELL§, AND HIDEYUKI OKANO*‡***

*Department of Neuroanatomy, Biomedical Research Center, Osaka University Graduate School of Medicine, 2-2 Yamadaoka, Suita, Osaka 565-0871, Japan; †Department of Pediatrics, Keio University School of Medicine, 35 Shinanomachi, Shinjuku, Tokyo 160-8582, Japan; ‡Core Research for Evolutional Science and Technology (CREST), Japan Science and Technology Corporation, 2-6-15 Shibakoen, Minato-ku, Tokyo 105-0011, Japan; §Laboratory of Molecular Neuro-Oncology, The Rockefeller University, New York, NY 10021; and ‖Division of Biochemistry and Cell Biology, National Institute of Neuroscience, National Center of Neurology and Psychiatry, 4-1-1 Ogawahigashi, Kodaira, Tokyo 187-8502, Japan

Communicated by Joan A. Steitz, Yale University, New Haven, CT, June 21, 1999 (received for review February 15, 1999)

ABSTRACT Hu proteins are mammalian embryonic lethal abnormal visual system (ELAV)-like neuronal RNA-binding proteins that contain three RNA recognition motifs. Although Drosophila ELAV is required for the correct differentiation and survival of neurons, the roles played by the Hu genes in the mammalian nervous system remain largely unknown. To explore the in vivo functions of mouse Hu proteins, we overexpressed them in rat pheochromocytoma PC12 cells, where they induced neuronal phenotype in the absence of nerve growth factor. We have characterized the functions of various forms of mHuB and mHuC bearing point mutations or deletions. Mutants of mHuC that had amino acid exchanges in the RNP1 domain of the first or second RNA recognition motifs (RRMs) lost biologic activity as well as RNA-binding activity. In addition, the mutants containing only the third RRM failed to induce the neuronal phenotype in PC12 cells and inhibited the biologic activity of cotransfected wild-type mHuB and mHuC, thus acting as a dominant-negative form. However, these mutants could not suppress the nerve growth factor-induced differentiation of PC12 cells. Further, we misexpressed wild-type and dominant-negative Hu in E9.5 mouse embryos, by using electroporation into the neural tube at the level of the rhombencephalon. mHuB and mHuC induced the ectopic expression of neuronal markers, whereas the dominant-negative forms of mHuB and mHuC suppressed the differentiation of central nervous system motor neurons. From these results, we suggest that Hu proteins are required for neuronal differentiation in the mammalian nervous system.

Neurons use a variety of means to regulate posttranscriptional gene expression, including alternative splicing, RNA transport, local translation, and RNA editing. Neural RNA-binding proteins are likely to play essential roles in mediating this regulation (1–4). Drosophila ELAV is a neuronal RNA-binding protein, and may be involved in their nuclear export (18, 30). Misexpression of Drosophila ELAV induces neuronal differentiation (32). HuB (Hel-N1) proteins are up-regulated during neuronal differentiation of embryonic carcinoma P19 cells (33), and its misexpression can induce formation of neurites in human tetracarcinoma cells (hNT2) (29). However, it remains to be studied whether the Hu genes are authentically required for the differentiation of neurons in vivo, especially those in the central nervous system (CNS).

Abbreviations: NGF, nerve growth factor; RRM, RNA recognition motif; ARE, AU-rich element; CNS, central nervous system; PNS, peripheral nervous system; RHP, ribohomopolymer; GFP, green fluorescent protein; ERK, extracellular signal-regulated kinase; ELAV, embryonic lethal abnormal visual system.

*Present address: Department of Developmental Neurobiology, Tohoku University Graduate School of Medicine, 2-1, Seiryo-machi, Aoba-ku, Sendai, Miyagi 980-8575, Japan.
‖To whom reprint requests should be addressed. E-mail: okano@nana.med.osaka-u.ac.jp.
To address these issues, in the present study we designed point and deletion mutants of Hu proteins to perform detailed structure-function analyses to identify dominant-negative forms of the proteins. The in vivo functions of the Hu proteins in the peripheral nervous systems (PNS) and CNS were examined by overexpressing the full-length Hu (mHuB and mHuC) proteins and their dominant-negative forms in the neural tube of developing mouse embryo. On the basis of the data, we conclude that mHuB and mHuC are required for and capable of inducing neuronal phenotype in both CNS and PNS.

**MATERIALS AND METHODS**

**Construction of Hu Expression Vectors.** The cDNA clones of mouse HuB and HuC were isolated by reverse transcription–PCR by using primers whose design was based on previous reports (34, 35), and their DNA sequences were confirmed. Subsequently, FLAG tags were added to the N termini of the coding regions of mHuB and mHuC by PCR. A series of deletion mutants (R1–2, lacking aa 205–360 of mHuB and 205–367 of mHuC and R3, lacking aa 1–277 of mHuB and 1–284 of mHuC) were constructed by PCR. Amino acid substitution mutations (RRM1 mt, in which Tyr-82, Phe-84, and Tyr-87 were replaced by Asp) or a 1:100 dilution of anti-Myc mAb was used as the primary antibody. For double staining of the FLAG and Myc proteins, they were first overexpressed in rat pheochromocytoma PC12 cells. The efficiency of transfection was approximately 10% based on immunostaining to the tag. However, we could drastically enrich the transfected cells (1:1,000 of anti-FLAG (Kodak), and horseradish peroxidase-conjugated anti-rabbit IgG (Chemicon) were used as secondary antibodies. For the enhanced immunostainings, TSA-direct kit was used (NEN Life Science). Immunofluorescent images were visualized by using an Axiosplan2 microscope equipped for fluorescence imaging (Zeiss).

**Ribohomopolymer (RHP)-Binding Assays.** T7 epitope-tagged and histidine-tagged full-length and mutant (R1–2, R3, RRM1mt, and RRM2mt) mHuC bacterial fusion proteins and a control (human cdr2) fusion protein were produced after cloning into pET21b (Novagen). Ten picomol of each fusion protein was incubated with RHP beads (Sigma) in RHPA buffer (10 mM Tris-HCl, pH 7.4/2.5 mM MgCl2/0.5% Triton X-100) with the indicated NaCl concentration and 1 mg/ml heparin in a volume of 500 μl. Binding was carried out at 4°C for 15 min. The beads were pelleted and washed five times in RHPA buffer with the indicated NaCl concentration, boiled in SDS buffer, and run on Western blots. Filters were probed with T7-tag monoclonal antibody (Novagen), and the relative amount of protein bound to RNA was visualized with ECL (Amersham Pharmacia) and quantitated by using National Institutes of Health IMAGE 1.61. These assays were able to detect binding of as little as 2–5% of the input protein.

**Electroporation of Embryos, Whole-Mount Embryo Culture, and Immunostaining.** Embryos were obtained from ICR mice at 9.5 d postcoitum (dpc). Whole embryos were cultured in vitro according to the method described previously (42). Each embryo was cultured in rat serum with 2 mg/ml glucose. After 2 hr of preculture, a DNA solution containing 5 μg/μl of the expression plasmid (pCXN2-FLAG-HuB, FLAG-HuC, HuB-R3, HuC-R3, or GFP-Myc) and 5 μg/μl of the marker plasmids pCAX-AFP (a mutant form of the GFP-expressing plasmid with the β-actin promoter, a gift from K. Umesono, Kyoto University, Japan) (43) mixed 1:1 with 0.01% fast green dye (Sigma) was injected into the neural tubes of embryos at the level of the rhombencephalon. DNA was injected by using micropipets made from capillary tubes pulled with a vertical microelectrode puller (Narushima, Tokyo) to a 10-μm internal diameter. Immediately after the injection of DNA, embryos were placed in Tyrode’s solution and electroporated by using a T820 electroporator and a BTX500 (BTX, San Diego) optimized (pulse 50 μs, 90 V × 5). After electroporation, embryos were cultured to 10.5 dpc and fixed in 4% paraformaldehyde and 1 × PBS. Frozen sections (12 microns thick) were cut on a cryostat and immunostaining was performed essentially as described (44) by using 2H3 monoclonal anti-165-kDa neurofilament antibody, anti-Issel1 antibody (both hybridoma supernatant, obtained from the Developmental Studies Hybridoma Bank, University of Iowa), or anti TuJ-1 antibody (Sigma). Detection of immunoreactivity was performed by using an ABC kit (Vector Laboratories) and a metal-enhanced DAB kit (Pierce).

**RESULTS**

**Neuronal Phenotypes Are Induced by Overexpression of Hu Proteins in PC12 Cells.** To elucidate the function of Hu proteins, they were first overexpressed in rat pheochromocytoma PC12 cells, which have been widely used for molecular analyses of the signaling pathways that lead to differentiation of PNS neurons. We transiently transfected pCXN2-FLAG-HuB or -FLAG-HuC into PC12 cells. The efficiency of transient transfection was approximately 10% based on immunostainings to the tag. However, we could drastically enrich the cells expressing exogenous Hu through G418 selection (more than 90% of the survived cells after the selections over 9 d)
FIG. 1. HuC-induced neuronal phenotype of PC12 cells. (A) Morphology of pCXN2-FLAG-HuC-transfected PC12 cells (Nomarski differential interference contrast optics). Neuron-like morphology, similar to NGF-induced differentiation, appeared 9 d after transfection. (D–F) Negative control (pCXN2-GFP-Myc transfected PC12 cells) showed no morphological changes 9 d after transfection. Double staining for overexpressed FLAG-HuC (or GFP-Myc) (B and E) and neurofilament H (C and F). Antibodies for tagged sequence were used for the detection of transfected fusion protein. Overexpressed HuC protein localized mainly to the cytoplasm, and these cells showed increased expression of Neurofilament H (C), which is also known to increase in the NGF-induced differentiation of PC12 cells (53). (Bar = 15 μm.)

without increase of nontransfected cells. About 6 d after transfection, pCXN2-FLAG-HuC-transfected cells began to extend neurites. About 9 d after transfection, most of the FLAG-HuC (Fig. 1 A–C) or FLAG-HuB (data not shown) transfected cells were neuron-like in appearance and stopped proliferating (data not shown), a phenotype associated with neuronal differentiation. In contrast, control cells (transfected with pCXN2-GFP-Myc) (Fig. 1 D–F) showed no morphological changes.

Transfection of HuB or HuC without the FLAG tag showed similar morphological changes in PC12 cells as with the FLAG tag (data not shown). Expression of the neuronal marker neurofilament H, which is not known to be a direct target of Hu protein, was increased in PC12 cells expressing FLAG-HuC (Fig. 1 B and C), in contrast to the control cells (Fig. 1 E and F).

Mutation Analysis of Hu Proteins and Design of Dominant-Negative Forms. To further examine these findings, the functional domains of Hu proteins essential for the induction of neuronal phenotype were identified by mutation analysis. Hu proteins have three RRMs (RRM 1–3) (8, 9). We constructed deletion mutants lacking one or two of the RRMs from full-length mHuB and mHuC (Fig. 2A): mutants lacking the third RRM (termed “R1–2”) or the first and second RRMs (termed “R3”). Furthermore, to examine the role of the RNA-binding activity of HuC protein, amino acid residues that are essential for RNA binding were mutated in the RRM1 (termed “RRM1mt”) or RRM2 (termed “RRM2mt”) of HuC.

To assess the ability of each of these mutants to bind RNA, binding to RHPs was assessed under varying conditions of stringency (Fig. 2B). Full-length HuC and HuC-R1–2 bound RHP avidly with a preference for ribouridine (rU) under high stringency (e.g., 54% and 39% of input protein, respectively, bound in 0.5 M NaCl). RRM3 bound RHP only weakly, with a sequence preference for rU (e.g., 5% of input protein in 0.5 M NaCl). Similar results were found for full-length and R3
constructs of HuD tested, whereas constructs harboring the hinge region between RRM2 and RRM3 together with RRM3 bound no better than R3 alone (data not shown). In addition, no significant RNA binding was detected with the RRM1mt and RRM2mt constructs, consistent with prior structure-function studies of the RRM (45). Moreover, RNA binding of Hu constructs was tested against specific RNA ligands (fos ARE and additional RNA ligands identified by RNA selection, H.J.O. and R.B.D., unpublished data) by filter-binding assay. Full length HuC and HuD constructs showed high-affinity binding to these ligands (K\textsubscript{d} values \(\approx 7-12\), R1–2 constructs bound with \(\approx 5-10\) fold lower affinity, consistent with prior gel shift studies on similar R1–2 constructs (27, 34, 46), and R1 mt, R2 mt, and R3 Hu constructs showed no measurable RNA binding.

Expression levels of transfected Hu proteins were examined by using immunoblotting (Fig. 2C). Quantification by densitometric scanning (Scanning Imager, Molecular Dynamics) demonstrated high levels of overexpression of both full-length and R3 constructs (approximately 10-fold) relative to endogenous Hu proteins.

The biologic activities of these mutants were then assayed in PC12 cells (Fig. 2D). The R1–2 mutants were still capable of inducing the neuronal phenotype (neurite extension) in PC12 cells, but their effects were weaker than those of the wild-type proteins (20 \(\approx 25\%\) of the wild-type control). The R3 mutants, however, essentially had no neuronal phenotype-inducing activity and exerted no significant effect on proliferating activity of transfected cells. RRM1mt and RRM2mt also showed no significant neuronal phenotype-inducing activity (Fig. 2D).

The R3 mutants, which essentially lack (or have greatly diminished) neuronal phenotype-inducing and RNA-binding activities, may inhibit the normal functions of HuC as dominant-negative forms. To examine this possibility, R3 mutants were coexpressed with wild-type HuB or HuC. PC12 cells expressing both the wild-type FLAG-HuC, and mutant HuC-R3 showed a significantly lower percentage of cells with neuronal phenotype than the cells expressing only FLAG-HuC (Fig. 2E). Interestingly, combinations of different Hu proteins (HuC/HuB-R3 and HuB/HuC-R3) showed essentially the same results (Fig. 2E), indicating that the R3 mutant of one type of Hu protein is capable of inhibiting other member(s) of the Hu family as a dominant-negative form.

**Actions of Hu in PC12 Cells: Relationship to Nerve Growth Factor (NGF)/Ras Signaling.** The molecular mechanisms underlying the neuronal phenotype-inducing activity of Hu proteins were examined in relation to NGF/Ras signals, which are also known to induce the neuronal differentiation of PC12 cells (47). First, we performed the in situ detection of activated ERK (the doubly phosphorylated form), which is known to be increased in the NGF/Ras-mediated differentiation of PC12 cells (48). PC12 cells that differentiated in response to NGF treatment showed strong expression of activated ERK, especially in their nuclei (Fig. 3E). In contrast, the PC12 cells, whose neuronal phenotype was induced by the misexpression of HuC, showed no detectable expression of activated ERK (Fig. 3C). Next, we examined whether the misexpression of the dominant-negative form of HuC (R3) could prevent the NGF-induced differentiation of PC12 cells. Although the forced expression of dominant-negative H-Ras (H-Ras\textsuperscript{N17}) (49) prevented NGF-induced PC12 cell differentiation, the misexpression of HuC-R3 did not, even at a lower dose of NGF (Fig. 3F). Furthermore, HuC-R3 could not suppress the differentiation of PC12 cells induced by the misexpression of active H-Ras (H-Ras\textsuperscript{V12}) (50) (Fig. 3G). However, wild-type HuC (FLAG-HuC) induced the neuronal phenotype of PC12 cells that otherwise should have been inhibited by H-Ras\textsuperscript{N17} (Fig. 3G). Taken together, these results indicate that Hu is likely to induce the neuronal phenotype of PC12 cells in a parallel and distinct pathway from NGF/Ras and its subse-
and F). Thus, overexpression of HuB or HuC appears to be sufficient to induce the ectopic neuronal development within embryonic CNS.

To address whether Hu is required for normal neuronal development in the embryonic CNS, dominant-negative Hu proteins (R3) were misexpressed in the CNS of mouse embryos. When HuC-R3 (Fig. 4 G and H) or HuB-R3 (data not shown) was transfected into E9.5 mouse embryos, expression of the early motor neuronal marker, Islet-1, was shown to be suppressed coinciding with transgene expression. At this stage, endogenous expression of Hu proteins was already detected in this region by immunohistochemistry (data not shown). These findings suggest that the Hu-R3 mutants acted as dominant-negative forms to inhibit the ability of endogenous Hu proteins to promote the differentiation of CNS neurons.

**DISCUSSION**

Here, we investigated the roles of Hu in the mammalian nervous system by misexpressing full-length and dominant-negative forms of HuB and HuC. The full-length HuB and HuC induced neuronal phenotypes of PC12 cells, resulting in neuron-like morphological changes, increased expression of a neuronal marker gene (Fig. 1 C and D), and mitotic arrest (data not shown). Overexpression of Hu in chicken neural crest-derived cells results in the premature appearance of neuronal markers (32) and the HuB protein family is upregulated during neuronal differentiation of embryonic carcinoma P19 cells (33). Taken together, these results indicate that Hu proteins are likely to act as positive regulators of neuronal development in a way that is similar to *Drosophila* ELAV. Furthermore, we showed that Hu proteins function not only in the PNS but also in the developing CNS. In the developing mouse embryo, we observed misexpression of HuB/C or their R3-mutant affected the expression of neuronal markers. In addition to the possible direct translational activation of neurofilament-M by HuB (29), it is notable that expression of other two neuronal markers (TuJ-1 and Islet-1) are also affected. Collectively, we can conclude that Hu proteins promote neuronal development.

We have shown that neuronal differentiation in PC12 cells and in vivo can be mediated by full-length HuB and HuC expression constructs and, albeit with less efficiency, in PC12 cells by constructs harboring only RRM 1–2. In contrast, full-length Hu constructs harboring point mutations in either RRM1 or RRM2 failed to promote neuronal phenotype. These biologic results correlate with the RNA-binding activities of each construct—full-length or R1–2 constructs bound RNA avidly and promote neuronal phenotype in PC12 cells, whereas point mutants showed no RNA-binding to RHPs and no biologic activity. Thus the biologic activities we observed are likely to depend on the RNA-binding activity of the Hu proteins. The Hu proteins stabilize target RNAs through sequence-specific binding to AREs (18, 22, 24, 25, 31). Interestingly, Steitz and colleagues have examined the ARE binding with RRM1–2 constructs (aa 1–185 of HuA) nearly identical to the R1–2 construct used here (aa 1–204 of HuC) and found that the RRM1–2 failed to stabilize ARE containing target RNAs in L929 cells (18). Although directly comparable stability assays using HuB/C constructs have not been reported, these observations suggest that the biologic activity of HuB/C-R1–2 constructs in neuronal cells may not act through stabilization of ARE containing RNAs. Thus, one possible explanation is that Hu proteins may have other biologically important RNA targets in neurons in addition to ARE elements.

Our results also point to the importance of the RRM3 of Hu in neuronal differentiation, because HuB/C-R3 appears to act in a dominant-negative manner to inhibit the biologic activity of Hu in PC12 cells and in vivo. Previous studies have demonstrated not only that HuA RRM3 is necessary for stabilization of ARE-containing target RNAs (18), but that RRM3 may bind polyA RNA in vitro (34, 52). We did not find evidence for significant RNA binding of RRM3 to poly rA or
other RHPs, this discrepancy may be accounted for by the different stringency of binding assays—we examined RNA binding under stringent conditions (Fig. 2B), whereas previous studies examined polyA binding under nonstringent binding conditions (34, 52). Nonetheless, our observations do not rule out the possibility that HuC/B-R3 acts as a dominant-negative domain by competing for binding of full-length protein to specific RNA target sequences. Another possibility is that HuC/B-R3 blocked Hu function by competing at the level of protein–protein interaction. The only candidate protein interaction domain identified in Hu proteins is a tightly defined NLS/NESS domain (amino acids 205–237 in HuA) (28), which is close to but distinct from RRM3. Notably, the analogous HuB/C sequence elements were absent from our dominant-negative constructs (HuC/B-R3), suggesting that the dominant-negative action may not result from direct interaction with nuclear-cytoplasmic shuttling activity. Finally, it is possible that the action of HuC-R3 involves inhibition of both RNA and protein binding.

Availability of a dominant-negative form of Hu is likely to provide a useful tool for exploring its in vivo functions. HuB/C and D proteins have more than 90% homology at the amino acid level. The expression pattern of each Hu gene is different, Neurology Neurology (1995) 17, 8300–8312.


This work was supported by grants to H.O. from the Japanese Ministry of Education, Science and Culture, CREST, Japan Science and Technology Corporation, and the Human Frontier Science Program Organization, and grants to R.B.D. from the National Institute of Neurological Disorders and Stroke (RO1 NS34389) and the Irma Hirsch Trust. H.J.O. was supported by National Research Service Award Postdoctoral Training Grant CA 09673-18.