Involvement of Hu and Heterogeneous Nuclear Ribonucleoprotein K in Neuronal Differentiation through p21 mRNA Post-transcriptional Regulation*

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The Hu family is a group of neuronal RNA-binding proteins required for neuronal differentiation in the developing nervous system. Previously, Hu proteins have been shown to enhance the stabilization and/or translation of target mRNAs, such as p21 (CIP1), by binding to AU-rich elements in untranslated regions (UTRs). In this study, we show that Hu induces p21 expression, cell cycle arrest, and neuronal differentiation in mouse neuroblastoma N1E-115 cells. p21 expression is also up-regulated during Me2SO-induced differentiation in N1E-115 cells and is controlled by post-transcriptional mechanisms through its 3'-UTR. To investigate the molecular mechanisms of Hu functions, we used a proteomics strategy to isolate Hu-interacting proteins and identified heterogeneous nuclear ribonucleoprotein (hnRNP) K. hnRNP K also specifically binds to AU-rich sequences in p21 mRNA 3'-UTR and represses its translation in both nonneuronal and neuronal cells. Further, using RNA interference experiments, we show that the Hu-p21 pathway contributes to the regulation of neurite outgrowth and proliferation in N1E-115 cells, and this pathway is antagonized by hnRNP K. Our results suggest a model in which the mutually antagonistic action of two RNA-binding proteins, Hu and hnRNP K, control the timing of the switch from proliferation to neuronal differentiation through the post-transcriptional regulation of p21 mRNA.

Cell differentiation and development are achieved by differential gene expression, which is regulated by various mechanisms such as protein degradation, modification, transcription, and post-transcriptional regulation. Although post-translational protein modification and transcription have been thought to play central roles in gene regulation during the process of development, recent studies have revealed the important role of the post-transcriptional regulation of messenger RNA (1–4). Control at post-transcriptional levels has advantages over transcriptional gene regulation. It can rapidly switch gene expression according to incoming signals, without the need for de novo gene transcription (5). Therefore, post-transcriptional regulation enables a sharp timing control in rapidly progressing biological events like development. For example, the translational control of cyclin B mRNA is critical for meiotic cell division in Xenopus embryos (6). In meiosis, the level of expression of cyclin B and other genes, such as mos, cyclin A1, and cdk1, is controlled at the level of translation, through regulation of poly(A) length in the 3'-untranslated regions (UTRs)1 of mRNA (7–9). The mechanisms determining mRNA post-transcriptional regulation involve RNA-binding proteins that specifically recognize cis-elements in mRNA sequences (5).

Hu proteins have been identified as target antigens of autoantibodies appearing in the serum of patients with paraneoplastic encephalomyelitis (10, 11). Four members of the Hu family, HuC (Ple-21), HuD, HuB (Hel-N1), and HuA (HuR), have been cloned (10, 12–14) and identified as RNA-binding proteins similar to Drosophila Elav protein, which is required for the differentiation and survival of neurons (15, 16). Mammalian Hu proteins are expressed in both early postmitotic and mature neurons, with the exception of HuA (HuR), which is expressed ubiquitously (14, 17). Neuronal Hu proteins, HuB, HuC, and HuD, share highly homologous sequences, and all three Hu proteins have been shown to play an important role for neuronal differentiation in the mammalian nervous system. We and another group reported that the overexpression of neuronal Hu proteins induce neurite outgrowth in PC12 cells in the absence of nerve growth factor (18, 19). In vivo, the misexpression of Hu in E9.5 mouse embryos via electroporation in the neural tube induced the ectopic expression of neuronal markers (18). Both Hu proteins and Elav contain three well characterized RNA recognition motifs (RRMs) that mediate specific and high affinity binding to the AU-rich elements of target mRNAs (20). Importantly, the RNA binding ability of the RRM1 and RRM2 domains is required for the function of Hu to induce neurite extension in PC12 cells (18). Hu proteins have been shown to regulate the stability and/or translation of several putative target mRNAs, including p21, p27 (KIP1), GAP-43, tau, and neurofilament M (21–27). Interestingly, most of

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† The abbreviations used are: UTR, untranslated region; siRNA, short interfering RNA; RRM, RNA recognition motif; hnRNP, heterogeneous nuclear ribonucleoprotein; GST, glutathione S-transferase; PBS, phosphate-buffered saline; RT, reverse transcription; mRFP, monomeric red fluorescent protein; BedUrd, bromo-2-deoxyuridine; GFP, green fluorescent protein; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; DICE, differentiation control element; LOX, 15-lipoxygenase.
the target mRNAs encode proteins that play important roles in the regulation of differentiation and proliferation. These studies suggest that the simultaneous regulation of the expression of these mRNAs by neuronal Hu may play an important role in the timing control of growth/differentiation switching.

Key events in terminal differentiation, such as neurite outgrowth, are always accompanied by withdrawal from the cell cycle. An important regulator of this event is cyclin-dependent kinase inhibitor (e.g. p21 and p27 associate with several cyclin-dependent kinase-cyclin complexes to suppress cell cycle-promoting activity). Several papers have reported that p21 gene expression is up-regulated during neuronal differentiation. In PC12 cells, for instance, nerve growth factor induces p21 expression, accompanied by neurite extension (28, 29). However, p21 expression is regulated not only at the transcriptional level, but also at the post-transcriptional level (30). The stability of p21 mRNA is controlled by Hu proteins that specifically bind to AU-rich element in its 3′-UTR (21, 30).

To reveal the molecular mechanisms of the timing control of neuronal differentiation by Hu, we isolated and identified Hu-associated proteins. Here, we show that the RNA-binding protein hnRNP K directly interacts with Hu, represses the translation of p21 mRNA by binding to its 3′-UTR sequence, and antagonizes the action of the Hu protein, inducing cell cycle arrest and neurite outgrowth in neuroblastoma N1E-115 cells. Our findings suggest that the activity balance of these two functionally antagonizing RNA-binding proteins involved in p21 mRNA post-transcriptional regulation is important to switch from proliferation to differentiation.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—N1E-115, 293T, and NIH3T3 cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum, l-glutamine, penicillin, and streptomycin and grown in 5% CO2 at 37 °C.

**Purification of Hu-associated Proteins**—FLAG-tagged mouse HuB or GFP was overexpressed in 293T cells using recombinant adenovirus preparation as described previously (31). After 2 days, whole cell lysates were prepared in lysis buffer containing 10 mM Tris-HCl (pH 7.6), 50 mM NaCl, 1 mM sodium pyrophosphate, 50 mM β-glycerophosphate, 1% Triton X-100, and a protease inhibitor mixture (Complete; Roche Applied Science). Hu-associated proteins were immunopurified using an anti-FLAG M2 affinity beads (Sigma). After washing four times, the associated proteins were eluted by treatment with 200 μg/ml FLAG peptide (Sigma). The eluted products were separated with a 12% SDS-PAGE, electroblotted on a nitrocellulose membrane (Millipore Corp.). The blots were probed with anti-Myc monoclonal (1:1000 dilution; Sigma) and anti-FLAG monoclonal antibodies (1:500; Sigma) or anti-hnRNP K polyclonal antibodies (1:1500). Rabbit anti-hnRNP K polyclonal antibody was made by immunoizing animals with a full-length GST-hnRNP K bacterial fusion protein.

**Immunoprecipitation and RT-PCR**—Six-well plates of NIH3T3 cells were infected with recombinant retroviral expression vectors, pMY including FLAG alone, FLAG-HuB or FLAG-hnRNP K cDNA, and IRES GFP (a gift from Dr. T. Kitamura) (32). Forty-eight hours post-infection, the infected cells were lysed, and immunoprecipitation assays were prepared as described using a cytosol extraction buffer (25 mM Hepes, KOH (pH 7.9), 150 mM KCl, 2 mM EDTA, 20 mM NaF, 0.1% Nonidet P-40, Complete protease inhibitor mixture, and RNasin (Promega)). The amount of cell lysates was normalized according to the total protein. RT-PCR was performed using oligonucleotide primers specific for p21 (5′-GTTGAGAACGAAAACTCGGA-3′, 5′-GTC- TAGATGAGTTTGAACGTTG-3′) or GAPDH (5′-ACCAAGTTCGAT- TAGCAGTC-3′, 5′-TCTCACGGATGAGTCTA-3′) mRNA. The products of RT-PCR were resolved by 8% PAGE and visualized by staining with ethidium bromide. Real-time RT-PCR was performed using a MX3000P (Stratagene) with SYBR green (TaKaRa). PCR primer was 5′-GGGATCATTTCGACATTCTTGGTA-3′, 5′-GGGAT- CACAACAGATGAGGGCTA-3′ for p21 or as above for GAPDH.

**Luciferase Reporter Assay**—293T or N1E-115 cells were cultured in 12-well plates and transfected with 1 μg of pCXN2-Myc-GFP, carrying a CAG promoter (33), as a marker of transfection. Fusion proteins at final concentrations of 0, 10, 50, or 100 nmol in 20 μl of binding buffer (10 mM Tris-HCl (pH 7.4), 150 mM KCl, 1.5 mM MgCl2, and 0.5 mM dithiothreitol) at room temperature for 30 min. RNA-protein complexes were applied to 8% nondenaturing polyacrylamide gels in 0.5× TBE at room temperature. Bands were visualized by autoradiography.

**Luciferase Activity Measurement**—Luciferase activity was measured according to the manufacturer’s procedure (Promega). A Berthold Lumat LB9507 luminometer was used for detection.

**Cell Growth and Neurite Outgrowth Assay**—N1E-115 cells were culture in 6-well plates and co-transfected with 1 μg of pCXN2-Myc-GFP, -FLAG-HuB, -Myc-hnRNP K, or -Myc-p21 together with 0.2 μg of pCXN2-mRFP, carrying a CAG promoter (33), as a marker of transfected cells. The cells were cultured for 4 days after transfection. The number of cells with long neurites in monomeric red fluorescent protein (mRFP)-positive cells was then counted (neurite growth assay). The relative cell number represents the ratio of cells of day 2 to those on day 1 (cell growth).

**BrdUrd Incorporation**—48 h after transfection, N1E-115 cells were incubated with 10 μg/ml BrdUrd (Sigma) for 4 h. Cells attached to polyornithine-coated coverslips were washed once in PBS and fixed with 4% paraformaldehyde/PBS. The cells were rinsed with PBS two times and incubated in 0.3% Triton X-100 in PBS for 5 min at room temperature. After rinsing in PBS, the cells were incubated with 1% H2O2 in PBS followed by two rinses in PBS and then incubated at 4 °C overnight with anti-FLAG (M2) or anti-Myc (9E10) monoclonal antibody to a dilution of 1:5000 (Sigma) in tyramide signal amplification (TSA) buffer. After rinsing in PBS for one time, the cells were incubated with secondary antibodies conjugated with horseradish peroxidase, followed by observation using a tyramide signal amplification fluorescence system (PerkinElmer Life Sciences). After treatment with 1 mM HCl at 50 °C for 20 min, the samples were washed twice in PBS and incubated with anti-BrdUrd antibody at 1:200 (Fitzgerald) overnight. For the secondary antibody, Alexa 568 (A-21099) was used. After washing three times in PBS, the samples were mounted on slides and

**Anti-hnRNP K K/D antibody 3C2 (1:500; ImmunoQuest). Anti-Hu serum is reactive with HuB, C, and D.**

**Co-immunoprecipitation and Western Blotting**—293T cells were co-transfected with Myc-hnRNP K and FLAG-HuB using the FuGENE 6 transfection reagent (Roche Applied Science). 48 h after transfection, the cells were harvested with lysis buffer (10 mM Tris-HCl, pH 7.6, 100 mM NaCl, 1 mM EDTA, 1% Triton X-100, and protease inhibitor mixture) and centrifuged at 15,000 × g for 15 min. The supernatants, which included the FLAG fusion proteins, were immunoprecipitated using an anti-FLAG M2 antibody (Sigma) pretreated with or without 100 μg/ml FLAG peptide (Sigma) at 4 °C for 1 h. After washing three times with lysis buffer, the proteins were eluted by boiling in loading buffer, separated by SDS-PAGE, and transferred to an Immobilon P membrane (Millipore Corp.). The blots were probed with anti-Myc monoclonal (1:1000 dilution; Sigma) and anti-FLAG monoclonal antibodies (1:500; Sigma) or anti-hnRNP K polyclonal antibodies (1:500). Rabbit anti-HuB K polyclonal antibody was made by immunoizing animals with a full-length GST-hnRNP K bacterial fusion protein.

**Biotin Pull-down Assay**—Recombinant GST- or His-tagged mouse hnRNP K/βIVBP was incubated with 0.1 mM FLAG peptide (Sigma) at 4 °C for 1 h. After washing three times with washing buffer, the proteins were eluted by boiling in loading buffer, separated by SDS-PAGE, and transferred to an Immobilon P membrane. After blocks with 5% BSA, the blots were probed with anti-FLAG monoclonal (1:1000 dilution; Sigma) and anti-FLAG monoclonal antibodies (1:500; Sigma) or anti-hnRNP K polyclonal antibodies (1:500). Rabbit anti-HuB K polyclonal antibody was made by immunoizing animals with a full-length GST-hnRNP K bacterial fusion protein.

**MALDI-TOF mass analysis** was performed by APRO Science (Tokushima, Japan).

**Recombinant Proteins, in Vitro Translation, and GST Pull-down Assays**—Recombinant GST- or His-tagged mouse hnRNP K, HuB, and HuC fusion proteins were produced by transforming BL21 competent cells, followed by isopropyl 1-thio-β-D-galactopyranoside induction and purification by nickel chelation or glutathione chromatography. Full-length and mutant hnRNP K RNAs were transcribed and translated in vitro using PURESYSTEM classic (Post Genome Institute Co., Ltd.) with L-[35S]methionine (Amersham Biosciences). Ten micrograms of GST-fused Hu or GST protein was incubated with 32S-labeled, in vitro translated, hnRNP K deletion mutant proteins or the indicated cell lysates in the binding buffer (PBS with 0.1% Nonidet P-40 and protease inhibitor mixture) for 2 h at 4 °C. Glutathione-Sepharose beads were added, and the mixture was rotated at 4 °C for 1 h; the beads were then washed three times with binding buffer and resolved using 10–15% SDS-PAGE. Bound proteins were analyzed by autoradiography or Western blotting.

**Biotin Pull-down Assay**—40 μg of 293T or PC12 cell lysate or 1 μg of GST-hnRNP K or HuB protein was incubated with 0.1 μg of biotinylated in vitro transcribed p21 3′-UTR RNA, together with 0–0.2 μg of nonbiotinylated p21 3′-UTR RNA, for 2 h at 4 °C. RNA-protein complexes were isolated with streptavidin beads. After washing three times, the samples were subjected to Western blot analysis to detect Hu or hnRNP K protein using anti-Hu human serum (1:2000; a gift from Dr. Robert Darnell), anti-hnRNP K polyclonal antibody (1:500), or anti-
FIG. 1. A, isolation of Hu-associated proteins. Cell extracts prepared from 293T cells infected with FLAG-HuB or GFP adenovirus were bound to FLAG beads and eluted with FLAG peptide. Eluted products were resolved on 10% SDS-PAGE, and three bands were excised and analyzed by MALDI-TOF. B, His-HuC bacterial fusion protein was subjected to a pull-down assay with GST or GST-hnRNP K. Binding was detected by Western blotting using an anti-His tag antibody. To confirm the activity of RNase A (2 μg/ml), reactions were mixed with yeast tRNA (60 μg/sample), and aliquots were run on an agarose gel and stained with ethidium bromide. A similar result was obtained by using HuB fusion protein (data not shown). C, Myc-tagged or FLAG-tagged GFP, HuB, and hnRNP K were co-expressed in 293T cells and then immunoprecipitated with anti-FLAG antibody beads or peptide-blocked FLAG antibody (+Flag peptide) beads. The precipitates were analyzed by Western blotting using anti-FLAG, Myc, or hnRNP K antibodies.

examine using a universal fluorescence microscope (Axiophot; Carl Zeiss, Oberkochen, Germany).

RNA Interference—Short interfering RNA (siRNA) duplexes for p21 and the recommended negative control were obtained from Ambion in annealed and purified forms. Transfection of the siRNA duplexes was carried out using Lipofectamine 2000 (Invitrogen). Three microliter of Lipofectamine 2000 reagent with 100 pmol of siRNA duplexes, 0.5 μg of FLAG-HuB, and 0.1 μg of mRFP expression vectors were used per well (12-well plate). After transfection, the cells were subjected to a cell growth assay, a neurite outgrowth assay, or a Western blot analysis (34).

RESULTS

Isolation of Hu-associated Protein Complexes—As a first step in our study of the biological functions of neuronal Hu proteins in vivo, we tried to identify Hu-interacting proteins. For this purpose, we isolated protein complexes containing HuB from 293T whole cell extracts using FLAG tag affinity purification. The protein fractions from control GFP- or FLAG-tagged HuB (FLAG-HuB) that had been overexpressed by recombinant adenoviruses were separated using SDS-PAGE, followed by silver staining (Fig. 1A). Several bands were found specifically in the lane of the FLAG-HuB immune complexes, compared with the control lane. Three major bands were excised and analyzed by mass spectrometry (MALDI-TOF). A protein with a molecular mass of about 80 kDa appeared to be the RNA-binding protein hnRNP K, whereas the other two bands representing proteins with a molecular mass of about 70 and 45 kDa were the arginine methyltransferase skb-1 and nuclear protein NF45, respectively. We were particularly interested in hnRNP K, because this protein is a translational repressor for mRNA containing differentiation control elements (DICEs), such as 15-lipoxygenase (LOX) mRNA (4).

To demonstrate the direct interaction between hnRNP K and Hu proteins, we performed a GST pull-down assay and co-immunoprecipitation experiments. In vitro, purified HuC or HuB-bacterially prepared fusion protein was tested for binding to glutathione beads prebound with GST alone or GST-hnRNP K. The results indicated that HuC associated with hnRNP K, and the interaction between hnRNP K and HuC was also detected after the treatment with RNase A, although the interaction was partly affected by RNase A (Fig. 1B). A similar result was obtained by using HuB fusion protein (data not shown). Taken together, these data demonstrate that hnRNP K and two proteins of Hu family are capable of interacting directly in the absence of an RNA intermediate.

To confirm the interaction between hnRNP K and Hu proteins in vivo, co-immunoprecipitation experiments were performed. FLAG-GFP or FLAG-HuB with Myc-tagged hnRNP K (Myc-hnRNP K) were overexpressed in 293T cells, and whole lysates were immunoprecipitated with anti-FLAG antibody-beads, followed by Western blot analysis with an antibody against Myc tag or hnRNP K. Myc-hnRNP K and endogenous hnRNP K, expressed in 293T cells, were both co-precipitated equally well with transfected FLAG-HuB protein but not with the GFP control protein (Fig. 1C). The specificity of the immunoprecipitation study was further confirmed using anti-FLAG antibody-beads preincubated with FLAG peptide. These results indicated that hnRNP K interacts directly with Hu proteins both in vivo and in vitro.

We further examined the interaction between hnRNP K and HuB using truncated constructs in a pull-down assay. The truncated versions of hnRNP K, as shown in Fig. 2A, were translated in vitro, subjected to a GST pull-down assay, and visualized by autoradiography. As shown in Fig. 2A, the spacer domain of hnRNP K containing an RGG box (Kd5) was sufficient to produce as robust an interaction with HuB as was full-length hnRNP K. We also demonstrated that both HuBM and HuBS, splicing isoforms of HuB, bound to hnRNP K. In contrast, no single hnRNP K deletion construct without an RGG box was able to mediate a strong interaction with HuB. Next, by using two in vitro translated C-terminal domains with or without the RGG box (Kd2 and Kd3, respectively), we mapped the interaction regions in HuB that bind to hnRNP K. The resulting data indicated that the Kd2 interacted with all HuB truncated mutants except the RR1 domain alone (Fig. 2B).
hnRNP K Repressed p21 mRNA Translation—hnRNP K has been shown to bind to CU-rich DICE in the 3'-UTR of Lox mRNA and silence its translation (4). On the other hand, neuronal Hu as well as HuB have been shown to bind to an AU-rich element in the 3'-UTR of p21 mRNA. Interestingly, three potential consensus binding sites (CU-rich regions) for hnRNP K exist downstream of the Hu-binding element and the CU-rich regions and have been shown not to bind to Hu (21). We examined whether hnRNP K might also bind to these elements in p21 mRNA 3'-UTR (Fig. 3A). To study the interaction between hnRNP K and p21 mRNA 3'-UTR in vitro, we performed a combined immunoprecipitation/RT-PCR assay. NIH3T3 cells were infected with retrovirus expressing FLAG alone, FLAG-HuB, or FLAG-hnRNP K, and each FLAG-tagged protein was immunoprecipitated with anti-FLAG antibody-beads, followed by RT-PCR with p21- or GAPDH-specific primers. PCR products were resolved by 8% PAGE or 2% agarose gel electrophoresis (Fig. 3B). Specific 180-bp bands for p21 mRNA were detected in the FLAG-HuB and FLAG-hnRNP K lanes but not in the control or RT (-) lanes. In contrast, GAPDH mRNA was not detected in any of the immunoprecipitants. To identify hnRNP K binding sequence in the p21 mRNA 3'-UTR, a gel shift assay was performed using four in vitro transcribed 20–40-kb RNA fragments (Hu binding site, CU-rich1, CU-rich2, and CU-rich3 regions). hnRNP K interacted with CU-rich2 with a high affinity and with CU-rich3, whereas no detectable binding to the Hu binding site or CU-rich1 was detected (Fig. 3C). To assess whether endogenously expressed Hu proteins or hnRNP K were also able to interact with the p21 mRNA 3'-UTR, we performed a biotin pull-down assay with extracts from 293T or PC12 cells using synthesized biotin-labeled RNA (Fig. 3D). In the 293T cells, which endogenously express Hu proteins but not neuronal Hu proteins, a robust association of hnRNP K with biotinylated p21 mRNA 3'-UTR was detected, and this association decreased upon competition with increased amounts of nonbiotinylated transcripts. The same assay using extracts from PC12 cells, which express both Hu proteins and hnRNP K, showed that both endogenous Hu proteins and hnRNP K bound specifically with p21 mRNA 3'-UTR (Fig. 3D and data not shown). These results indicated that hnRNP K binds directly to specific sites in p21 mRNA 3'-UTR in vitro and in vivo. Next, to assess whether HuB and hnRNP K compete at the level of RNA binding, we carried out a biotin pull-down assay using AU element of the Hu binding site or AU-CU element, containing both Hu binding and hnRNP K binding (CU-rich1 and CU-rich2) sites of p21 mRNA 3'-UTR. These results showed that GST-hnRNP K interacted with AU-CU element but not with AU element and control beads. Interestingly, GST-hnRNP K was pulled down with the AU element in the presence of GST-HuB, and furthermore equally strong interactions were detected between HuB and AU-CU RNA element in the presence or absence of hnRNP K (Fig. 3E). These data indicated that HuB-hnRNP K interaction did not interfere with the RNA-binding ability of HuB, and as a result, HuB, hnRNP K, and RNA formed an RNA-protein-protein triple complex.

To investigate whether hnRNP K mediates the translational silencing of p21 mRNA, we next used a chimeric reporter gene assay system containing Renilla luciferase cdna fused with the p21 mRNA 3'-UTR sequence. Three Renilla luciferase reporter constructs containing a 1.2-kb full-length (Luc-FL) sequence or a 0.3-kb fragment (Luc-3CU) with either the deletion of the CU-rich regions or Luc as a negative control were placed under control of the herpes simplex virus thymidine kinase promoter. A firefly luciferase expression vector under the control of an SV40 promoter was co-transfected as an internal control (Fig. 4A). As shown in Fig. 4B, the luciferase activity of Luc-FL was repressed by hnRNP K in a dose-dependent manner, whereas the luciferase activities of Luc-3CU and a construct with a reversed sequence of p21 mRNA 3'-UTR were not affected by hnRNP K (Fig. 4B; data not shown). Importantly, Renilla Luc mRNA levels remained unchanged under all con-
A condition when the total RNA was analyzed by Northern blotting (Fig. 4C). Taken together, we concluded that hnRNP K repressed \( p21 \) gene expression translationally through interaction with the cell lysates. After the RNA was isolated from the precipitates, RT-PCR was performed using \( p21 \) or GAPDH mRNA-specific primers. The PCR products were resolved by PAGE or agarose gel and visualized by staining with ethidium bromide. The sites of \( p21 \) primers are indicated in A (arrows). C, gel shift assay: \( ^3P \)-Labeled transcripts (Hu binding site and CU-rich1, -2, and -3 indicated in A) were incubated with 100 nM GST or with 0, 10, 50, or 100 nM GST-hnRNP K fusion protein. After incubation, the reaction mixture was resolved on 8% PAGE and autoradiographed. D, biotin pull-down assay. 293T or PC12 cell extracts were incubated with in vitro transcribed \( p21 \) 3'-UTR biotinylated RNA together with or without unbiotinylated competitor RNA. RNA-protein complexes were isolated with streptavidin beads, and the pulled-down proteins were detected by Western blot using anti-Hu or hnRNP K antibody. E, GST-HuB and/or GST-hnRNP K proteins were subjected to a biotin pull-down assay with the indicated biotinylated RNAs. AU RNA contains the Hu binding site alone, and AU-CU RNA contains the Hu binding site and the CU-rich1 and CU-rich2 sites shown in A. The pulled down proteins were detected by Western blotting using anti-Hu or -hnRNP K antibody (3C2).

**Fig. 3.** hnRNP K interacts with \( p21 \) mRNA 3'-UTR in vitro and in vivo. A, schematic and nucleotide sequences of Hu binding site and three candidate hnRNP K binding sites (CU-rich1, -2, and -3) in \( p21 \) mRNA 3'-UTR. B, immunoprecipitation/RT-PCR assay. NIH3T3 cells were infected with a retrovirus expressing FLAG alone, FLAG-HuB, or FLAG-hnRNP K, and the resulting FLAG proteins were immunoprecipitated from the cell lysates. The FLAG products were resolved by PAGE or agarose gel and visualized by staining with ethidium bromide. The sites of \( p21 \) primers are indicated in A (arrows). C, gel shift assay: \( ^3P \)-Labeled transcripts (Hu binding site and CU-rich1, -2, and -3 indicated in A) were incubated with 100 nM GST or with 0, 10, 50, or 100 nM GST-hnRNP K fusion protein. After incubation, the reaction mixture was resolved on 8% PAGE and autoradiographed. D, biotin pull-down assay. 293T or PC12 cell extracts were incubated with in vitro transcribed \( p21 \) 3'-UTR biotinylated RNA together with or without unbiotinylated competitor RNA. RNA-protein complexes were isolated with streptavidin beads, and the pulled-down proteins were detected by Western blot using anti-Hu or hnRNP K antibody. E, GST-HuB and/or GST-hnRNP K proteins were subjected to a biotin pull-down assay with the indicated biotinylated RNAs. AU RNA contains the Hu binding site alone, and AU-CU RNA contains the Hu binding site and the CU-rich1 and CU-rich2 sites shown in A. The pulled down proteins were detected by Western blotting using anti-Hu or -hnRNP K antibody (3C2).

**p21 Expression Is Post-transcriptionally Controlled during Neuronal Differentiation in N1E-115 Cells**—It has been previously reported that \( p21 \) not only inhibits the cell cycle but also contributes as a developmental regulator that induces axon and dendrite extension in newborn neurons (35). To investigate whether \( p21 \) expression is post-transcriptionally regulated during neuronal differentiation, we used mouse neuroblastoma N1E-115 cells, which were induced to extend their neurites by treatment with Me2SO (35). We transfected the \( Renilla \) luciferase expression vector with or without \( p21 \) mRNA 3'-UTR into N1E-115 cells, in which a /H9252-galactosidase reporter plasmid was co-transfected as an internal control. The cells were then either induced to differentiate with Me2SO or maintain in a proliferation condition without Me2SO for 48 h; the cell lysates were prepared and assayed for luciferase and \( /H9252 \)-galactosidase activities. The activity of luciferase with \( p21 \) mRNA 3'-UTR decreased to about half of that in the control, under proliferation condition, whereas its activity under differentiation condition was about 2-fold higher than that under proliferation condition. On the other hand, the control luciferase and \( /H9252 \)-galactosidase activities did not change under either condition (Fig. 5A). This result indicated that \( p21 \) mRNA 3'-UTR contains a translation-inhibitory element that suppresses protein synthesis in immature cells, whereas suppression is released in differentiated cells, and the post-transcriptional regulatory mechanism contributes to the up-regulation of \( p21 \) expression during neuronal differentiation. Further, the overexpression of hnRNP K dramatically repressed luciferase activity with \( p21 \) mRNA 3'-UTR under both conditions, whereas control luciferase expression was not significantly affected by hnRNP K (Fig. 5B). We next examined the endogenous expression of the \( p21 \) gene at mRNA and protein levels during neuronal differentiation in N1E-115 cells. Western blotting and real time RT-PCR analysis revealed that the \( p21 \) protein level was at an undetectable level, whereas \( p21 \) mRNA was produced under proliferation condition. By Me2SO treatment, \( p21 \) protein production was dramatically increased in consistent with a previous report (35), and its mRNA level increased slightly by 1.3-fold (n = 3).
for up to 96 h (Fig. 5, C and D). These results strongly suggested that p21 protein production is regulated post-transcriptionally and is positively switched on during the transition from proliferation to neuronal differentiation. It is important to note that this post-transcriptional regulation mechanism of p21 expression depends on the presence of the 3'/H11032-UTR sequence. We next performed Western blot analysis to detect endogenous p21 protein expression levels using lysates from N1E-115 cells transfected with HuB and hnRNP K. In HuB-transfected cells, the p21 protein level markedly increased, compared with those with GFP control. Furthermore, we found that the induction of p21 protein by HuB transfection was canceled by co-transfection with hnRNP K, suggesting that hnRNP K has an antagonistic effect on HuB in induction of p21 protein production (Fig. 5E).

Negative Regulation of the Proliferation of N1E-115 Cells by HuB Is Antagonized by hnRNP K—We next investigated the role of hnRNP K and HuB in relation to cell cycle regulation. N1E-115 cells were co-transfected with either hnRNP K, HuB, or p21 together with mRFP, and the relative increase in the number of mRFP-positive cells was counted 1 and 2 days after transfection. Although the overexpression of HuB or p21 re-
duced the proliferation of N1E-115 cells, hnRNP K and the GFP control did not, suggesting that HuB as well as p21 induced cell cycle arrest in N1E-115 cells (Fig. 6A). To investigate whether the increased production of endogenous p21 is necessary for HuB-induced cell cycle arrest, we performed an RNA interference experiment to reduce the amount of endogenous p21. As shown in Fig. 6B, the introduction of p21 siRNA significantly decreased the expression of p21 protein and partially reversed the effect of HuB, promoting cell cycle arrest. In contrast, siRNA had no effect on the expression level of HuB and p27 cyclin-dependent kinase inhibitor protein. These results indicated that HuB was able to induce cell cycle arrest, and this effect might be due, at least in part, to the induction of p21 expression by HuB. We next employed combined overexpression of HuB and hnRNP K in N1E-115 cells to determine whether the cell cycle-arresting function of HuB was antagonized by hnRNP K. Cells were transfected with either Myc-GFP or FLAG-HuB and increasing amounts of Myc-hnRNP K, cultured for 48 h after transfection, and fixed following 4 h of incubation with BrdUrd. The cells were subsequently immunostained with anti-BrdUrd antibody and either anti-FLAG or anti-Myc antibody, and then the ratio of BrdUrd/tag double-positive cells to the total number of tag-positive cells was quantified. In control GFP-transfected cells, the BrdUrd incorporation rate was about 90%, whereas the ratio of BrdUrd/tag double-positive cells in HuB-transfected cells decreased to 48%, consistent with the results shown in Fig. 6A. As shown in Fig. 6C, the overexpression of hnRNP K significantly restored the cell cycle-arresting activity of HuB and promoted cell division in a dose-dependent manner. Taken together, these results demonstrated that hnRNP K had an antagonistic function against the ability of HuB to induce p21 expression as well as the ability to suppress the cell cycle.

Neurite Outgrowth Induced by HuB Is Partially Antagonized by hnRNP K—It has been reported that the overexpression of HuB or HuC alone induced neuronal differentiation in PC12 cells in the absence of nerve growth factor (18). In this study, we examined whether the overexpression of HuB induces neurite outgrowth in N1E-115 cells under proliferation condition. N1E-115 cells were co-transfected with mRFP and with either HuB, hnRNP K, GFP, or p21 and cultured for 4 days without Me2SO, and the number of cells that had extended neurites was counted among the mRFP-positive cells. In HuB-transfected cells, 27.1% of the cells exhibited neurite outgrowth (Fig. 7A), with a phenotype similar to that of N1E-115 cells treated with Me2SO. In contrast, almost no neurite outgrowth was observed in GFP- or hnRNP K-transfected cells (2.9 and 2.1%, respectively). Although p21 alone induced neurite outgrowth as well, its activity was almost half of HuB (15.1%). To investigate whether p21 contributes as a downstream effector of HuB in neuronal differentiation, we performed an RNA interference experiment to knockdown p21 expression. The introduction of p21 siRNA significantly, but not completely, canceled the differentiation-promoting activity of HuB in N1E-115 cells, whereas the control siRNA had no effect. This finding suggested that the p21 pathway, as a downstream effector of HuB,
FIG. 7. Neurite outgrowth assay. A, N1E-115 cells were co-transfected with Myc-GFP, FLAG-HuB, Myc-hnRNP K, or Myc-p21 expression vectors together with an mRFP expression vector. The percentage of cells with long neurites (arrowheads) among the total transfected cells was quantified (bottom). B, N1E-115 cells were transfected with HuB together with p21 or control siRNA. After 4 days of culture without Me2SO (DMSO), the neurite outgrowth assay described in A was performed. C, N1E-115 cells were co-transfected with HuB (1 μg) and increasing amounts of hnRNP K (0-, 1-, or 2-μg) expression vectors. Four days after transfection, the percentage of cells with long neurites among the total transfected cells was quantified. Values are shown as the mean ± S.D. B, n = 6; *, p < 0.01; two-tailed t test. C, n = 3; *, p < 0.05; two-tailed t test.

corresponds to the neuronal differentiation of N1E-115 cells, at least in part (Fig. 7B). Given the significant antagonistic effect of hnRNP K against HuB in the cell cycle control, we next tested whether hnRNP K affects the ability of HuB to promote neuronal differentiation. N1E-115 cells were co-transfected with HuB together with increasing amounts of hnRNP K, and a neurite outgrowth assay was carried out. The overexpression of hnRNP K restored HuB-induced neurite outgrowth to almost half the level of cells transfected with HuB alone in a dose-dependent manner (Fig. 7C). Taken together, these experimental results indicated that hnRNP K has antagonistic effects on the function of HuB not only via cell cycle regulation but also in the promotion of neuronal differentiation.

DISCUSSION

We have identified hnRNP K, a protein that specifically interacts with the neuron-specific RNA-binding protein Hu. The present study extended our previous findings showing that Hu acts to promote neuronal differentiation in the mammalian nervous system. We and other groups have previously shown that Hu directly binds to several target RNAs and that the RNA-binding ability of Hu is necessary for its neuronal differentiation-promoting activity. The data presented here suggest that hnRNP K may antagonize the Hu-dependent induction of neuronal differentiation and cell cycle arrest.

Isolation of Hu-associated Proteins—We isolated protein complexes containing Hu using biochemical methods and identified several components included in the complexes. Of interest, we identified three proteins, skb-1, NF45, and hnRNP K, all of which play a role in RNA metabolism. The skb homologue 1 is arginine methyltransferase, which contributes to the formation of RNP complexes by methylating arginine residues in RGG boxes, an arginine/glycine-rich motif shared by numerous RNA-binding proteins (36). NF45 contains a C2H2 zinc finger motif and forms a heterodimer with double-stranded RNA-binding protein NF90, which binds directly to and stabilizes IL-2 mRNA in activated T cells (37). hnRNP K is involved in multiple steps that compose gene expression, such as transcription, RNA splicing, mRNA stability, and translation (38). hnRNP K contains three KH-type RNA-binding motifs and RGG box. This protein also has multiple protein-interacting domains, and each domain is responsible for an interaction with specific binding partners, including YB-1, Src, and Lyn (38). In a co-expression study, we showed that two RNA-binding proteins, HuB and hnRNP K, directly interact and form a protein complex in vivo (Fig. 1C). We found that HuB interacts with the RGG box between the second and third KH domains of hnRNP K (Fig. 2A).

Post-transcriptional Regulation of p21 by hnRNP K and Hu—Although many studies have provided evidence of hnRNP K protein involvement in multiple processes of gene regulation, the mechanisms of hnRNP K action are best studied in its translational regulation. Consensus RNA sequences that hnRNP K binds have been identified as a cytosine-rich cluster using the SELEX method (39), and three C-rich patches have been identified using a yeast three-hybrid system (40). Notably, similar CU-rich repetitive stretches are found in the DICE of LOX mRNA 3’-UTR, and hnRNP K does indeed silence the translation of LOX mRNA as well as a reporter mRNA containing a DICE element (4). hnRNP K also specifically binds to and inhibits the translation of human papillomavirus type 16 (HPV-16) L2 mRNA in squamous epithelial cells when these cells are immature (41).

In this study, we demonstrated that hnRNP K binds specifically to two CU-rich regions in p21 mRNA 3’-UTR and represses the translation of reporter mRNA containing p21 mRNA 3’-UTR. Post-transcriptional regulation of p21 is particularly important in the initiation and maintenance of differentiation in mouse embryonic muscle cells, C2C12. In this differentiation state, a striking increase in the half-life of p21 mRNA was observed as a result of an increase in mRNA stability mediated by HuB, known to be a major protein binding to the AU-rich regions of p21 mRNA 3’-UTR (42, 43). Recent growing evidence suggests that the control systems that determine the functional integrity of mRNAs may be susceptible to
differentiation.

RNA-binding proteins, Hu and hnRNP K, during neuronal differentiation.

hnRNP K is regulated by extracellular signals. c-Src kinase reports indicate that the translation-silencing activity of hnRNP K is partially canceled by the overexpression of HuB, even in proliferating cells, and this HuB-induced derepression of translation is almost completely canceled by the overexpression of hnRNP K. Since hnRNP K directly binds to but does not interfere with the RNA binding ability of HuB (Fig. 3E), it is speculated that hnRNP K and HuB oppositely regulate recruitment of a regulatory machinery for mRNA translation/stability onto p21 mRNA. The simplest model consistent with our data is that hnRNP K binds the CU-rich elements adjacent to the Hu binding site in p21 mRNA 3′-UTR, mediating an inhibitory effect on Hu-dependent mRNA stabilization through protein-protein interactions, perhaps by preventing the assembly of a multiprotein complex necessary to stabilize mRNA. Another possibility is the regulation of polysome recruitment to the target mRNA. Translation silencing through the DICE element of hnRNP K occurs at blocking of the recruitment of the 60 S ribosomal subunit and the formation of translation component 80 S ribosome (46). On the other hand, NF-M mRNA, which is one of the target mRNAs of HuB, is recruited to heavy polysomes in hNT-2 cells transfected with HuB (26). These reports suggest that two RNA-binding proteins, hnRNP K and HuB, may regulate the recruitment of ribosomes onto p21 mRNA. Surprisingly, the amount of Hu and hnRNP K endogenous proteins did not change before and after Me2SO treatment (data not shown). These results strongly support the idea that the activity balance of these two RNA-binding proteins may be crucial for the regulation of p21 protein production during neurogenesis (Fig. 8). Although we do not yet understand the mechanisms underlying the regulation of neuronal Hu protein activities, several reports indicate that the translation-silencing activity of hnRNP K is regulated by extracellular signals. c-Src kinase mediates the tyrosine phosphorylation of hnRNP K, which inhibits the binding of hnRNP K to the DICE and releases the translational silencing of LOX mRNA (47, 48). In contrast, serine phosphorylation by extracellular signal-regulated kinase 1/2 drives the cytoplasmic accumulation of hnRNP K and represses DICE-mediated translation (49).

Regulation of Cell Cycle and Neuronal Differentiation through the Hu-p21 Pathway—Cyclin-dependent kinase inhibitor p21 is also known as a positive regulator of neuronal differentiation through induction of the cell cycle arrest of neuronal progenitor cells (28, 29). Although p21 localizes in the nucleus to bind and inhibit cyclin-dependent kinases in dividing cells, the cytoplasmic accumulation of p21 occurs during the course of differentiation in chick retinal precursor cells and N1E-115 cells (35). A recent paper showed that cytoplasmic p21 inhibits Rho-kinase activity and induces neurite outgrowth in culture cells as well as in primary hippocampal neurons (35). In the current study, we demonstrated that the overexpression of Hu alone is sufficient to induce neurite outgrowth and inhibits cell growth in N1E-115 cells; however, these effects are partially canceled by introduction of p21 siRNA. Furthermore, like siRNA, the overexpression of hnRNP K can also overcome the functions of Hu in a dose-dependent manner. As shown in this study, p21 protein production is regulated positively by neuronal Hu and negatively by hnRNP K at the post-transcriptional level. Together, these observations indicate that p21 is a direct downstream effector of Hu function in differentiation induction/cell cycle inhibition and that this Hu-p21 pathway is antagonized by hnRNP K in immature neuronal progenitor cells. Although the role of hnRNP K in the process of neuronal differentiation remains unclear, several lines of evidence indicate that hnRNP K may be a negative regulator of terminal differentiation in nonneuronal tissue through translational repression (4, 44, 45). The expression of hnRNP K mRNA is increased in actively proliferating hepatocytes and hepatoma cells, compared with quiescent cells (50). The mutant fly gene bancal, a Drosophila homologue of hnRNP K exhibits defects in cell proliferation in the imaginal disc, and this phenotype can be rescued by the expression of human hnRNP K (51). In the mammalian embryonic brain, by using newly generated anti-hnRNP K polyclonal antibody, we find that hnRNP K expression is relatively higher in the ventricular zone of the cortex, where Hu-negative immature neural stem/progenitor cells are distributed, than in the cortical plate, in which most of the cells are postmitotic neurons that express high levels of Hu (17). Consistent with these findings, p21 is also strongly expressed in postmitotic neurons in the developing brain (52). These reports and our results suggest that hnRNP K may promote cell proliferation and have a negative effect against the promotion of differentiation in vivo.

Based on our experimental results, the existence of additional downstream parallel pathways of Hu that share similar roles with p21 is strongly suspected, since p21 siRNA can only reverse 30–40% of the effects of Hu on the cell cycle and differentiation. One such candidate molecule is p27, another cyclin-dependent kinase inhibitor (Fig. 8). As with p21, p27 inhibits cell growth by binding cyclin-dependent kinases and also has additional roles in the regulation of cell differentiation. Interestingly, p27 also binds to RhoA and inhibits RhoA activation (53), and the overexpression of p27 induces neuronal differentiation in N1E-115 cells (34). Furthermore, p27 is known as a putative target gene of HuR and HuD (22), suggesting that the p27 pathway may also play an important role through its roles in the regulation of cell cycle and neuronal differentiation.

2 M. Yano, H. J. Okano, and H. Okano, unpublished results.
3 H. J. Okano, unpublished results.
Antagonistic Action of RNA-binding Proteins Hu and hnRNP K

The present study suggests that neuronal Hu and hnRNP K antagonized each other in the timing control of neuronal differentiation through the post-transcriptional regulation of p21. Activation of the neuronal Hu-p21 pathway and inactivation of differentiation through the post-transcriptional regulation of p21.

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REFERENCES

1. Okabe, M., Imai, T., Kurusu, M., Hiromi, Y., and Okano, H. (2001) Nature 411, 94–98.
2. Okano, H., Imai, T., and Okabe, M. (2002) J. Cell Sci. 115, 1355–1359.
3. Imai, T., Tokunaga, A., Yoshida, T., Hashimoto, M., Mikoshiba, K., Weinmaster, G., Nakafuku, M., and Okano, H. (2001) Mol. Cell. Biol. 21, 3888–3900.
4. Osterrech, D. H., Osterrech-Lederer, A., Wilm, M., Thiele, B. J., Mann, M., and Hentze, M. W. (1997) Cell 89, 597–606.
5. Darnell, R. B. (2002) Cell 110, 545–550.
6. Sheets, M. D., Fox, C. A., Hunt, T., and Wickens, M. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 1017–1022.
7. Stebbins-Boaz, B., Cao, Q., de Moor, C. H., Mendez, R., and Richter, J. D. (1996) Genes Dev. 10, 926–938.
8. Stebbins-Boaz, B., Hake, L. E., and Richter, J. D. (1998) EMBO J. 17, 2582–2592.
9. Mendez, R., and Richter, J. D. (2001) Nat. Rev. Mol. Cell. Biol. 2, 521–529.
10. Szabo, A., Dalmau, J., Manley, G., Rosenfeld, M., Henson, J., Posner, J. B., and Furneaux, H. M. (1991) Cell 67, 325–333.
11. Lange, R., and Darnell, R. B. (2003) Proc. Natl. Acad. Sci. U. S. A. 100, 1543–1554.
12. Sakai, K., Gofuku, M., Kitagawa, Y., Ogawa, T., Hirase, G., Yamazaki, M., Koh, C. S., Yangasigawa, N., and Steinman, L. (1994) Biochem. Biophys. Res. Commun. 199, 1200–1208.
13. Levine, T. D., Gao, F., King, P. H., Andrews, L. G., and Keene, J. D. (1993) Mol. Cell. Biol. 13, 3494–3504.
14. Imai, T., Tokunaga, A., Yoshida, T., Hashimoto, M., Mikoshiba, K., Weinmaster, G., Nakafuku, M., and Okano, H. (2001) Mol. Cell. Biol. 21, 3888–3900.
15. Imai, T., Tokunaga, A., Yoshida, T., Hashimoto, M., Mikoshiba, K., Weinmaster, G., Nakafuku, M., and Okano, H. (2001) Mol. Cell. Biol. 21, 3888–3900.
16. Sheets, M. D., Fox, C. A., Hunt, T., Vande Woude, G., and Wickens, M. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 1017–1022.
17. Stebbins-Boaz, B., Hake, L. E., and Richter, J. D. (1998) EMBO J. 17, 2582–2592.
18. Szabo, A., Dalmau, J., Manley, G., Rosenfeld, M., Henson, J., Posner, J. B., and Furneaux, H. M. (1991) Cell 67, 325–333.
19. Darnell, R. B. (2002) Nat. Rev. Mol. Cell. Biol. 3, 247–257.
20. Szabo, A., Dalmau, J., Manley, G., Rosenfeld, M., Henson, J., Posner, J. B., and Furneaux, H. M. (1991) Cell 67, 325–333.
21. Szabo, A., Dalmau, J., Manley, G., Rosenfeld, M., Henson, J., Posner, J. B., and Furneaux, H. M. (1991) Cell 67, 325–333.
22. Szabo, A., Dalmau, J., Manley, G., Rosenfeld, M., Henson, J., Posner, J. B., and Furneaux, H. M. (1991) Cell 67, 325–333.
23. Szabo, A., Dalmau, J., Manley, G., Rosenfeld, M., Henson, J., Posner, J. B., and Furneaux, H. M. (1991) Cell 67, 325–333.
24. Szabo, A., Dalmau, J., Manley, G., Rosenfeld, M., Henson, J., Posner, J. B., and Furneaux, H. M. (1991) Cell 67, 325–333.