BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **244,** 226–229 (1998)
ARTICLE NO. RC988247

Expression of HuD Protein Is Essential for Initial Phase of Neuronal Differentiation in Rat Pheochromocytoma PC12 Cells

Yoh Dobashi, Mitsuhiko Shoji, Yoko Wakata, and Toru Kameya
Department of Pathology, Kitasato University School of Medicine, Sagamihara, Kanagawa, Japan

Received February 5, 1998

HuD is a neuronal-specific, RNA-binding protein. Here we examined the change in the expression of HuD protein during nerve growth factor-mediated differentiation of PC12 cells. As cells differentiated and extended neurites, expression of HuD gradually increased up to 1.5-fold. When HuD expression was counteracted by antisense oligonucleotide, neurite extension was completely inhibited, yet the morphology of differentiated cells remained unchanged even after that treatment. Furthermore, this morphological change correlated well with the downregulation of cyclin-dependent kinase 2 activity. These results suggest that the HuD is critically involved in the initial phase of neuronal differentiation. © 1998 Academic Press

The HuD gene is localized to the 1p34 region in human chromosomes and encodes a nuclear protein of 35-40 kD (1), depending on the particular pattern of alternative splicing (2-4). HuD is one of a distinct family of proteins, including HuC and Hel-N1, which are human homologues of Elav, a Drosophila protein required for neuronal development and maintenance (2,5). HuD is highly expressed in neuronal cells of the central and peripheral nervous system (4,6). Although HuD has been studied as a potential molecular target of autoantibodies found in patients with antibody-associated paraneoplastic encephalomyelitis (PEM), the exact function of this neuronal RNA binding protein is unclear (3,7). Here, we examined the change in HuD expression in rat pheochromocytoma PC12 cells during differentiation induced by nerve growth factor (NGF). We also investigated the essential role of HuD in differentiation using antisense oligonucleotide (OLN) to abrogate its expression and monitoring downregulation of cdk2 activity, a parameter previously shown to be a reliable indicator and a critical determinant of PC12 cell differentiation (8).

MATERIALS AND METHODS

Cell culture. PC12 cells were cultured in the absence or presence of NGF (50 ng/ml, Wako Ltd.) as previously described (8). After exposure to NGF for 0, 1, 3, 7 and 10 days, cells were harvested and used for further experiments.

Immunoblotting. Immunoblotting analysis using 50 μg of protein was performed as previously described (8) except that immunoblots were probed with serum from a patient who had been diagnosed with HuD-associated PEM. This serum has been demonstrated to be applicable for immunoblotting analysis to detect HuD protein (9). Each experiment was performed in triplicate.

Densitometric analysis. Densitometric quantification of the data obtained by immunoblotting analysis was done using a GT6500ARTS scanner (Epson Co., Tokyo). All scans were kept as 16-bit PICT files and incorporated into NIH images (version 1.56) for densitometric analysis (10).

Antisense oligonucleotide experiment. All the OLNs used in this experiments were synthesized and modified with phosphorothioate as previously described (11). The sense and the antisense 22-mer OLNs sequences were chosen around the initiation codon in the rat HuD cDNA sequence (4) as follows; sense (5′-AATTAGCACCATGG-AGCCTCAG-3′), antisense (5′-CTGAGGCTCCATGGTGCTAATT-3′). The cells were incubated with phosphate-buffered-saline (PBS), sense or antisense OLN (30 μM) for 96 hr. with three additions every 24 hr. On the 2nd day during this incubation period, NGF was added to the culture medium. Morphological change was observed at the end of the 4th day, after which cells were harvested for immunoblotting analysis. Alternatively, cells were pretreated with NGF for 48 hr. to induce differentiation ("primed")(12) and further incubated with PBS, sense or antisense OLN (30 μM) for 96 hr. with three additions every 24 hr. in the presence of NGF. Each experiment was performed in duplicate.

In vitro kinase reaction. Cell lysates obtained (200 μg) were used for cdk2 kinase assay as described previously (8).

RESULTS AND DISCUSSION

Expression of the endogenous HuD protein during neuronal differentiation of PC12 cells. Cells were cultured in medium containing NGF. At days 0, 1, 3, 7 and 10, cells were lysed and the expression of HuD was evaluated by immunoblotting analysis. As shown in

0006-291X/98 $25.00

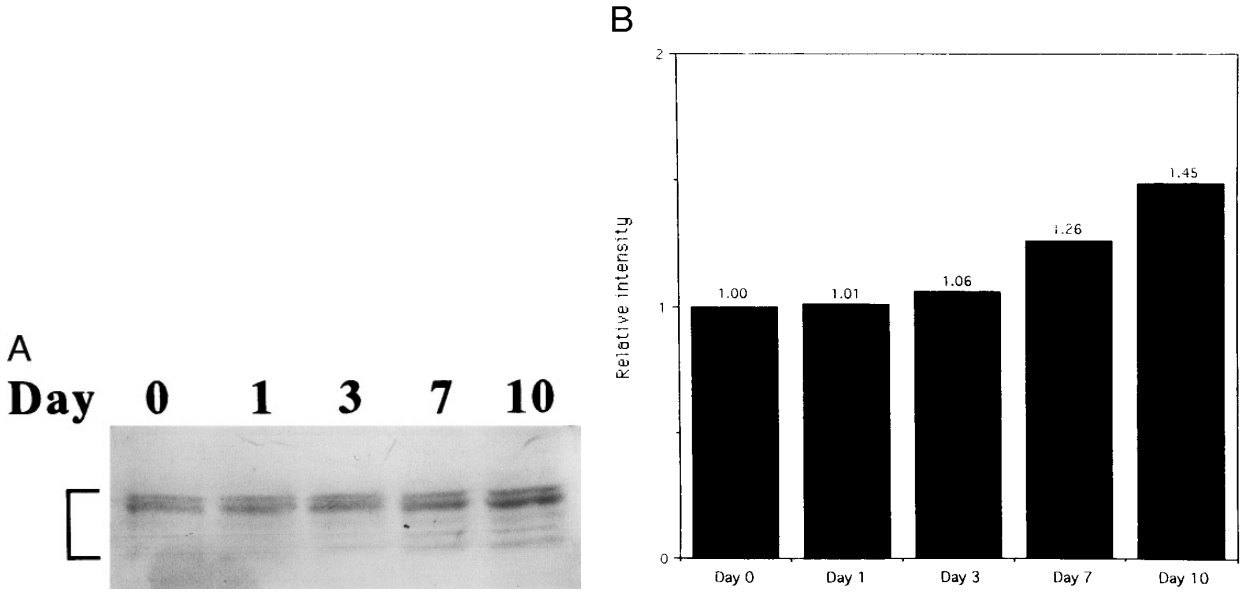

FIG. 1. Level of HuD protein expression during NGF-induced neuronal differentiation of PC12 cells. (a) Lysates were prepared from cells that had been cultured in the presence of NGF, harvested at the indicated times, and subjected to immunoblotting analysis. (b) Histograms were generated by quantitating the intensity of the bands from the nitrocellulose filters shown in panel (a). All values are expressed as ratios relative to those obtained on day 0.

Fig. 1, expression of HuD showed a gradual but significant increase, reaching 1.4 to 1.5-fold higher levels by day 10 (Fig. 1a, b).

Inhibition of differentiation in PC12 cells with antisense OLN. Control PC12 cells exhibited basal expression of HuD protein and showed prominent neurite

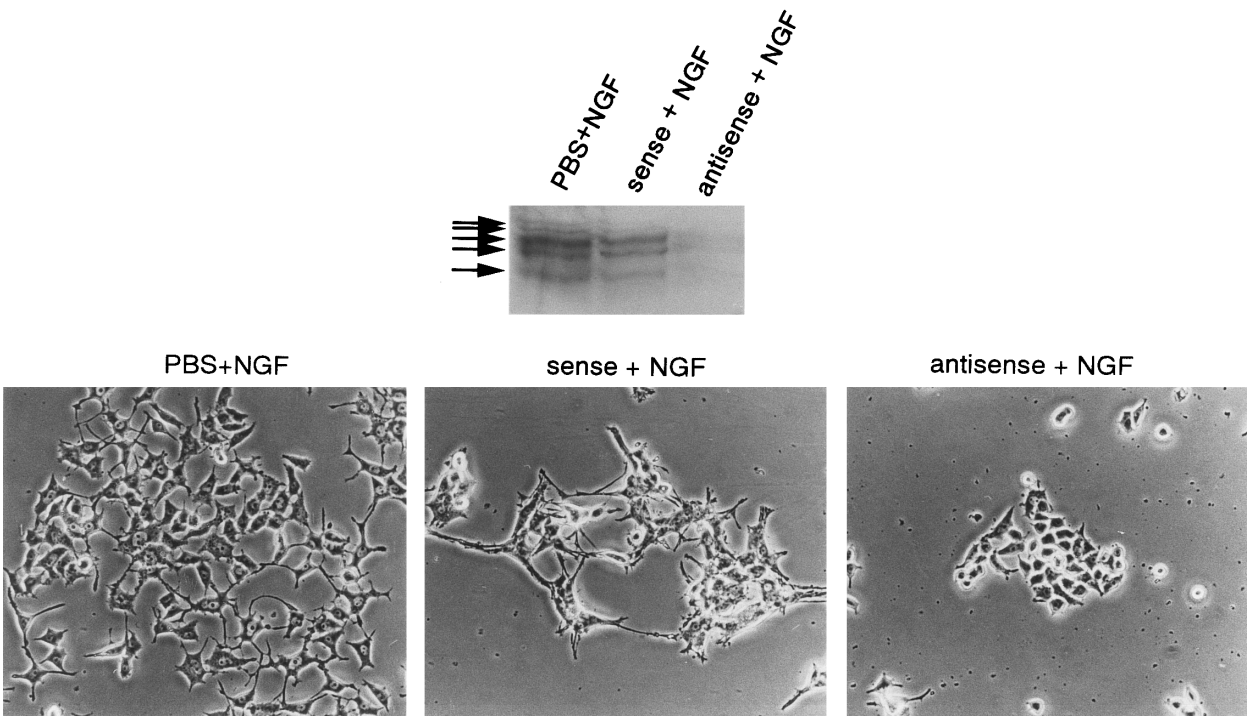

FIG. 2. Effect of treatment with oligonucleotides (OLNs) followed by NGF in PC12 cells. (upper panel) The changes in HuD protein expression after incubation with OLNs were confirmed. Lysates were prepared from cells that had been cultured in the presence of PBS, sense or antisense OLN for 4 days and were subjected to immunoblotting analysis to detect HuD protein. (lower panels) Morphological changes in PC12 cells by NGF treatment following exposure to PBS, sense or antisense OLNs.

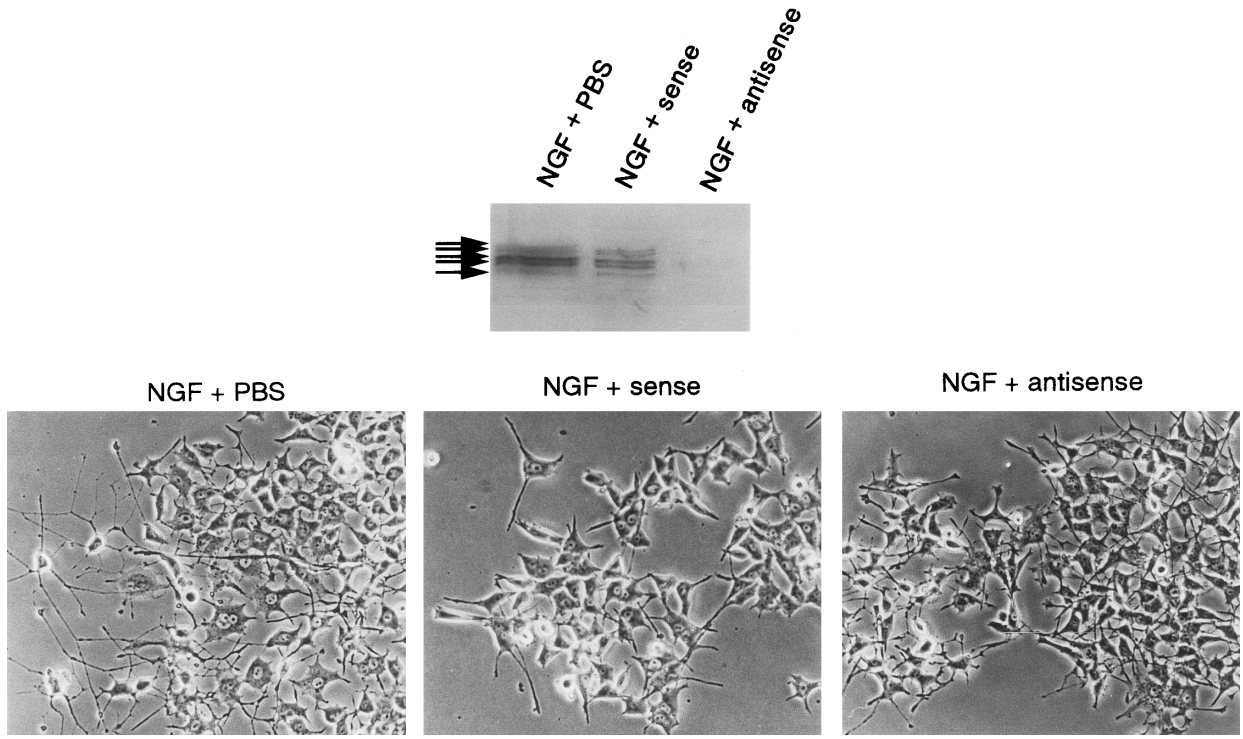

FIG. 3. Effect of treatment with OLNs observed in primed PC12 cells. (upper panel) The changes in HuD protein expression after treatment with OLNs were confirmed. Lysates were prepared from primed cells that had been further cultured in the presence of PBS, sense or antisense OLNs for 96 hr. These were subjected to immunoblotting analysis to detect HuD. (lower panels) Morphological changes in primed PC12 cells by treatment with PBS alone, sense or antisense OLNs.

extension following exposure to NGF for 2 days (Fig. 2). Although 20 to 30 % of the cells treated with sense OLN died, probably due to the cytotoxicity of nucleotide solution, expression of the HuD protein remained unchanged for 96 hr. Surviving cells also exhibited neurite extension comparable to that observed in control cells treated with PBS alone (Fig. 2). However, treatment with the antisense OLN resulted in the marked downregulation of HuD protein expression and complete inhibition of NGF-induced neurite extension during the 96-hr. trial (Fig. 2).

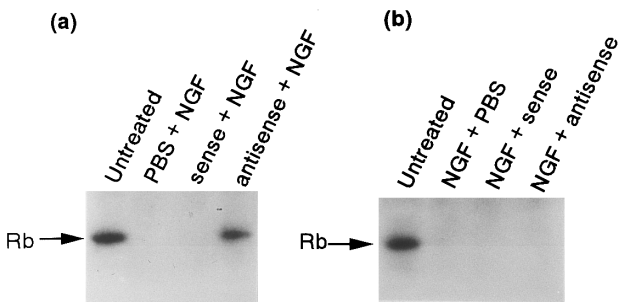

FIG. 4. Changes in cdk2 activity by treatment with NGF and OLNs in PC12 cells. (a) The changes in cdk2 activity by incubation with PBS or OLNs followed by NGF are shown. The kinase activity of untreated PC12 cells is shown as a control. (b) The changes in cdk2 activity by incubation with PBS or OLNs in primed PC12 cells are shown. The kinase activity of untreated cells is shown as a control.

Antisense OLN does not reverse neurite extension in primed cells. We next asked whether persistent expression of HuD is necessary for neurite extension in PC12 cells. Cells pretreated with NGF for 48 hr. ("primed") were cultured in the absence or presence of OLNs for an additional 96 hr. and the expression of HuD protein was examined. Although HuD expression was maintained in cells treated with PBS or sense OLN, it was markedly downregulated in cells treated with antisense OLN (Fig. 3). However, neither sense- or antisense-treated cells showed any reversal of neurite extension (Fig. 3).

Correlation of cdk2 activity with morphological change. We have previously shown that morphological change occurring during NGF-mediated differentiation in PC12 cells correlated well with downregulation of cdk2 activity (8) and that constitutive overexpression of cdk2 inhibits NGF-mediated neuronal differentiation. In order to confirm that the morphological change we observed is genuine differentiation, and to examine the effect of HuD expression on cdk2 activity, lysates from cells treated with OLNs were assayed for *in vitro*

cdk2 kinase activity. In cells treated with sense HuD OLN, NGF stimulation resulted in a decline in cdk2 activity similar to that seen in control cells (Fig. 4a). In contrast, in cells treated with antisense HuD OLN, cdk2 kinase activity did not decline following NGF stimulation, but rather remained at a level similar to that observed in control cells not stimulated with NGF (Fig. 4a). However, the low cdk2 activity caused by priming cells with NGF was not reversed by subsequent treatment with any of OLNs (Fig. 4b).

The HuD protein has been reported to be abundantly expressed in the neurons of the central and peripheral nervous system, chromaffin cells of the adrenal medulla and in small cell carcinoma of the lung (1,2,13). During chicken and quail embryonic development, Hu gene products are localized in the nuclei of proliferating neural crest-derived cells and the sensory and sympathetic ganglia (6). These results suggested that up-regulation of Hu gene product expression coincides with the generation of neurogenic cells and that this expression is maintained as the cells withdraw from the mitotic cycle. This notion is basically consistent with our current results showing that HuD protein expression is upregulated during NGF-mediated neuronal differentiation of PC12 cells. Furthermore, our antisense OLN experiments demonstrated that HuD protein is essential only for the initial phase of neuronal differentiation. This is in contrast to DCC (deleted in colorectal cancer) which was shown to be essential for both neuronal differentiation and maintenance of the differentiated phenotype in primed cells (14).

The distribution and the mode of HuD expression was initially elucidated by Steller et al. who demonstrated a strong induction of HuD mRNA in mouse embryonal carcinoma P19 cells after neuronal differentiation induced by retinoic acid. They also observed that HuD mRNA was unchanged during the NGF-mediated neuronal differentiation of PC12 cells. Our results contrast with this previous report. This discrepancy may be the result of differences in our experimental procedures, such as the use of RT-PCR versus immunoblotting analysis. Alternatively, the expression of HuD protein may be regulated at the level of protein degradation.

We have previously described that regulation of cdk2 activity occurred in strict correlation with the extent of neurite formation in the presence of NGF (8). *In vitro* kinase assays, in our present study, demonstrated that the morphological change induced by NGF treatment and inhibition by OLNs correlated well with the regulation of cdk2 kinase activity. Although it is still unclear whether this decline in cdk2 activity is the cause or result of differentiation, HuD definitely seems to exert its effect via the regulation of cdk2 activity. However, HuD does not affect cdk2 kinase activity once cells are primed and cdk2 kinase activity is downregulated.

Taken together, our results suggest that in cells of neuronal lineage the HuD protein may participate in complex neuronal cell functions: for example, basal expression of the HuD protein may be a prerequisite for proliferation and neuronal differentiation (15), yet not essential for maintenance of the established neuronal phenotype once cells have differentiated. Further elucidation of the physiological significance of HuD protein during neuronal differentiation, in particular, its interaction with cdk2, is underway in our laboratory.

REFERENCES

1. Steffen, C. B., Anderson, N. E., Rosenblum, M. K., and Posner, J. (1988) *Cancer Res.* **48,** 430–434.
2. Szabo, A., Dalmau, J., Manley, G., Rosenfeld, M., Wong, G., Henson, J., Posner, J. B., and Furneaux, H. M. (1991) *Cell* **67,** 25–333.
3. Sekido, Y., Bader, S. A., Carbone, D. P., Johnson, B. E., and Minna, J. D. (1994) *Cancer Res.* **54,** 4988–4992.
4. Steller, U., Kohls, S., Muller, B., Soeller, R., Muller, R., Schlender, J., and Blohm, D. H. (1996) *Mol. Brain Res.* **35,** 285–296.
5. Campos, A. R., Grossman, D., and White, K. (1985) *J. Neurogenet.* **2,** 197–218.
6. Marusich, M. F., and Weston, J. A. (1992) *Dev. Biol.* **149,** 295–306.
7. Altermatt, H. J., Rodriguez, M., Scheithauer, B. W., and Lennon, V. A. (1991) *Lab. Invest.* **65,** 412–420.
8. Dobashi, Y., Kudoh, T., Matsumine, A., Toyoshima, K., and Akiyama, T. (1995) *J. Biol. Chem.* **270,** 23031–23037.
9. Yagi, Y., Inuzuka, T., Takada, K., Nakano, R., Hozumi, I., Yoshimoto, H., Onodera, O., Tanaka, K., Sato, S., and Takahashi, M. (1997) *Eur. Neurol.* **37,** 122–123.
10. Masters, D. B., Griggs, C. T., and Berde, C. B. (1992) *Biotechniques* **12,** 902–906.
11. Matsukura, M., Shinozuka, K., Zon, G., Mitsuya, H., Reitz, M., Cohen, J. S., and Broder, S. (1987) *Proc. Natl. Acad. Sci. U.S.A.* **84,** 7706–7710.
12. Rukenstein, A., Rydel, R. E., and Greene, L. A. (1991) *J. Neurosci.* **11,** 2552–2563.
13. Dalmau, J., Furneaux, H. M., Carlos, C., and Posner, J. B. (1992) *Am. J. Pathol.* **141,** 881–886.
14. Lawlor, K. G., and Narayanan, R. (1992) *Cell Growth Diff.* **3,** 609–616.
15. Liu, J., Dalmau, J., Szabo, A., Rosenfeld, M., Huber, J., and Furneaux, H. (1995) *Neurology* **45,** 544–550.