Full Paper

RNA Editing and Short Variant of Serotonin 2C Receptor mRNA in Neuronally Differentiated NG108-15 Cells

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Abstract. Two types of serotonin 2C subtype receptor mRNA, receptor-type and short variant, has been reported. The expression of the receptor-type mRNA could be detected as well as the short variant in NG108-15 cells by using a high temperature stable reversetranscriptase and the expression of the receptor-type mRNA was enhanced in drug-induced neuronal differentiated cells. The deleted sequence of the short variant include the RNA editing site by adenosine deaminase. Analysis of the sequence at the editing site revealed that the mRNA of undifferentiated cells was highly edited at sites A and B and that cytosine deaminase activity may also be involved in neuronal differentiation.

Keywords: serotonin, RNA editing, short variant, deaminase, neuronal differentiation

Introduction

Previous studies of the serotonin 2C subtype receptor (5-HT₂CR), which is functionally linked to the phosphoinositide hydrolysis pathway, have yielded many insights into this molecule: cloning by using the *Xenopus* oocyte functional expression system (1), changing the name from 1C to 2C, almost no reports demonstrating the endogenous expression in cloned cell lines even rich in the brain (2), and others. Recent interesting findings about 5-HT₂CR include RNA editing (3, 4) and a short variant of the mRNA (5). A 5-HT₂CR mRNA short variant with a 95-nt deletion in the region encoding the region from the second intracellular loop site to the fourth transmembrane site has been reported (5). Since the deletion induces a frame-shift, the translated protein has a different structure from the normal protein. Canton et al. (5) first isolated the short variant mRNA and showed that the predicted truncated protein is produced and has a different sequence of the 19 carboxyl terminal amino acids than the normal 5-HT₂CR. The functions and physiological roles of this variant are still unknown. The RNA editing of 5-HT₂CR is also of interest. It has been reported that the editing affects the affinity of G protein coupling

(3). Since the RNA editing site is included in the 95-nt deleted sequence of the short variant, it should be of interest to determine the relationship between editing and this deleted sequence. We previously reported that NG108-15 cells abundantly express the short variant mRNA, but show almost no expression of 5-HT₂CR-type mRNA in the normal condition (6). To study the relationship and the cellular function of short variant, drug induced neuronal differentiated NG108-15 cells were used for detection of 5-HT₂CR mRNA and the base sequences at the editing site compared with non-differentiated cells.

Materials and Methods

Cell culture

NG108-15 cells were cultured in Dulbecco's modified Eagle medium (DMEM) containing HAT (100 μ M hypoxanthine, 1 μ M aminopterin, and 16 μ M thymidine) and 1% fetal bovine serum at 37°C in a humidified atmosphere of 90% air, 10% CO₂. The culture dishes (35 mm) were coated with 0.1 mg/ml polyornithine (polymerization about 50) in 150 mM borate buffer (pH 8.4) to prevent detachment of the cells from the dishes. To induce neuronal differentiation of cells, 100 nM 12-*O*-tetradecanoylphorbol 13-acetate (TPA) and 1 mM dibutyryl cAMP were added to the medium

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simultaneously at the start of culture. The concentrations of drug stock solutions were 100 μ M TPA in dimethyl sulfoxide and 100 mM dibutyryl cAMP in water. The final concentration of dimethyl sulfoxide, 0.1%, did not affect the experimental results.

Isolation of RNA

Total RNA from the cells was isolated from 5 individual dishes by the method of Chomczynski and Sacchi (7). The medium was removed from a 35-mm dish. The cells were washed once with phosphate-buffered saline (PBS) and then 1 ml of Isogen[®] (Nippon Gene, Toyama) was added per dish. The cell lysate was transferred to a microtube, supplemented with 0.2 ml of CHCl₃, and centrifuged. RNA in the resultant aqueous phase was precipitated with 0.5 ml of isopropanol. The pellet was used as total RNA after washing with 70% ethanol.

Reverse transcription to make complementary DNA

The same amounts of total RNA (20 μ g/sample) were mix together and 2 μ g from the mixture was incubated with 5 μ M of the specific antisense nucleotide of 5-HT2CR (5'-CACAAAGAAT ACAATGCCAAGG-3', corresponding to nucleotides 1650 to 1629 in accession No. M21410) and Thermoscript[®] reverse transcriptase (RT) (Invitrogen, Carlsbad, CA, USA) for 60 min at 57°C in a 20- μ l reaction mixture. Each of the 1- μ l products was used as a template for the PCR reaction.

PCR

PCR was carried out in a 10- μ l reaction mixture containing 1 μ l of template single strand cDNA (ss-cDNA), 1 μ M each of the forward and reverse 20 mer primer with 50% GC content (T_m = 55.4°C), deoxynucleosides triphosphate mixture (dNTPs) (2.5 mM), and 2 units of Taq DNA polymerase (Promega, Madison, WI, USA) in reaction buffer containing 2.5 mM MgCl₂. Primers for the region around the second intracellular loop site, including the deleted region for the short variant of 5-HT2CR, were 5'-TGGCAGTAAG CATGGAGAAG-3' (forward: nucleotides 917 to 936) and 5'-GACCCGATGA GAACGAAGTT-3' (backward: nucleotides 1346 to 1327) to produce a product of about 430 base pairs for the 5-HT2CR long variant sequence or 335 base pair product of short variant. The PCR cycling was performed as follows: 1) 94°C for 10 min; 2) the indicated number of cycles of 94°C for 1 min, 56°C for 2 min, and 72°C for 2 min; and then 3) 72°C for 10 min, followed by cooling to 4°C. The products were resolved by electrophoresis on 6% polyacrylamide gels and stained with SYBR Gold[®] (Molecular Probes Inc., Eugene, OR, USA). The bands were visualized under UV light and quantified with a

densitometer using Densitograph[®] 4.0 software (ATTO, Tokyo).

Determination of the bases in the position of RNA editing

To determine the bases in the position of RNA editing, PCR was performed under the same conditions described above. The template ss-cDNA was made at high temperature using the high-temperature-resistant reverse transcriptase. Primers for this experiment were the same forward primer described above and 5'-CGAATTGAAC CGGCTATGCT-3' (backward: nucleotides 1191 to 1172) which produced a product including the editing area. The estimated product size was 275 base pairs. The products were resolved by electrophoresis on 6% polyacrylamide gels and stained with SYBR Gold[®]. The cDNA bands amplified by PCR were cut out from the gels under UV transillumination. Each gel slice was heated to 90°C in 100 μ l of water for 12 min. Using the supernatant as template, reamplifying PCR was carried out with the same pair of primers as used in the initial reaction. The amplified DNA fragments were ligated into the EcoRV site of pBlue-script II KS(-) vector after T4 DNA polymerase treatment. The resultant vector was transfected into competent DH5 α cells (Toyobo, Tsuruga) to isolate and to amplify the clone. Finally, the isolated clone was sequenced using a Hitachi-5500 sequencer (Hitachi, Tokyo).

Results

Figure 1 shows that two major PCR fragments were made from the RT product of the high-temperature-stable enzyme. Sequence analysis revealed that the low molecular weight band corresponded to the same short variant 5-HT2CR mRNA as we previously reported (6). The high molecular band was shown to have the sequence of intact 5-HT2CR mRNA. These results suggest that the short variant and intact 5-HT2CR mRNAs may be the same except for a structural difference in the region from the second intracellular loop to the fourth transmembrane domain.

To examine the cellular function of the short variant 5-HT2CR, changes of the expression of the short variant and intact 5-HT2CR mRNAs were examined in neuronal differentiated and undifferentiated NG108-15 cells. We previously reported that simultaneous application of dibutyryl cAMP and phorbol 12-myristate 13-acetate, an activator of protein kinase C, induced stronger neuronal differentiation than cAMP alone (8). Treatment with these drugs induces strong neurite outgrowth and enhancement of the expression of GAP-43 (9).

Fig. 1. PCR detection of 5-HT2CR mRNA in NG108-15 cells. RNAs were isolated from rat brain (R) and NG108-15 cells (NG) by acidic phenol extraction. Reverse transcription was performed at 57°C using 5-HT2CR-specific primer. Then PCR was performed using a primer set that detected a region around the second intracellular loop domain of 5-HT2CR, including the deletion site in the short variant. The products were resolved by electrophoresis on 6% polyacrylamide gels and stained. The structure of the 5-HT2CR mRNA sequence, region detected by PCR, and the region deleted in the short variant are shown at the top of the figure. M, molecular markers: Msp I-digested pBR322 plasmid with bands of 622, 527, 404, 307, 242, 238 nt, and other smaller bands.

Furthermore, we also cloned a related intrinsic factor, sproutin (TA20) (10). We used a semi-quantitative RT/PCR analysis method to compare the expression levels of short variant and intact 5-HT2CR mRNAs in drugs-induced-differentiated and undifferentiated NG108-15 cells.

Regardless of the number of cycles of PCR, we observed that the level of expression of the short variant mRNA was almost equal to that of the intact 5-HT2CR mRNA in undifferentiated cells (Fig. 2B), when the expression was measured using the high-temperature condition (57°C) at the RT step. In contrast, in differentiated cells, higher levels of the PCR product corresponding to the intact type of 5-HT2CR mRNA were detected regardless of the number of cycles of PCR (Fig. 2A). The levels of expression were quantitated by densitometry. The results showed that the intact (receptor) type of 5-HT2CR mRNA was more highly expressed in differentiated cells. On the other hand, the short variant was more highly expressed in undifferentiated cells (Fig. 2). These results suggest that some modification(s) of 5-HT2CR mRNA may occur during the neuronal differentiation step. One possible type of modification is RNA editing, since the deleted sequence of the short variant includes all five of the RNA editing positions in the mRNA. To examine this possibility, we designed a PCR primer set that allowed us to amplify

the sequence in the editing site in differentiated and undifferentiated cells and to read this sequence. The results are shown in Fig. 3. At editing site A, all of the samples from undifferentiated cells were edited from adenosine to guanosine. Site B was also highly edited (7 out of 9) in undifferentiated cells. In contrast, in differentiated cells, only 3 samples were edited at site A and none at site B. No editing was observed at sites C', C, or D in either differentiated or undifferentiated cells.

Interestingly, another editing position was found in this area. The nucleotide immediately downstream of position B was cytosine in samples from undifferentiated cells but was thymidine in samples from differentiated cells, except in one sample. This result indicates that cytosine deaminase activity may also be involved in neuronal differentiation.

Discussion

The present results may demonstrate the following interesting hypothesis: 1) The short variant mRNA may be derived from the same mRNA which produces 5-HT2CR by posttranscriptional modification of mRNA. 2) RNA editing may be directly involved in the generation of the short variant. 3) RNA editing may have some role in regulating the neuronal differentiation/proliferation.

A) differentiated NG108-15 **B) undifferentiated**

Fig. 2. Quantitative detection of 5-HT_{2C} mRNA variant expression in neuronally differentiated and undifferentiated NG108-15 cells using the RT/PCR method. Neuronal differentiation of NG108-15 cells was induced by treatment with 100 nM phorbol 12-myristate 13-acetate and 1 mM dibutyryl cAMP. RNAs were isolated by acidic phenol extraction. Reverse transcription was performed at 57°C using a 5-HT_{2C}-specific primer. Then PCR was performed for the number of cycles indicated below the photographs of the gels. The products were resolved by electrophoresis on 6% polyacrylamide gels and stained. The upper panels show the gel images and cell morphology obtained from differentiated (A) and undifferentiated (B) cells. M, molecular markers: Msp I-digested pBR322 plasmid with bands of 622, 527, 404, and 307 nt. The lower panels show the intensity of each band measured using “Densitograph” Ver. 4.0 (ATTO).

Although Wang et al. (11) reported that the short variant is generated by alternative splicing, this report demonstrates the possibility that the mRNA of 5-

HT_{2C} is modified after transcription from the DNA to make the short variant. Wang et al. also thought that the RNA editing must occur before splicing, since the

Fig. 3. Identification of bases in positions of RNA editing in differentiated and undifferentiated NG108-15 cells. Neuronal differentiation of NG108-15 cells was induced by treatment with 100 nM phorbol 12-myristate 13-acetate and 1 mM dibutyryl cAMP. RNAs were isolated from 5 individual dishes by acidic phenol extraction. The same amounts of total RNA (20 mg/sample) were mix together and 2 mg from the mixture was used for reverse transcription at 57°C using a 5-HT2CR-specific primer. Then PCR was performed using a specific primer set that amplified the editing region. The PCR products, with an estimated size of 275 base pairs, were purified by electrophoresis on a 6% polyacrylamide gel. After the products were reamplified, and they were ligated into the pBluescript II KS(-) vector. The resultant plasmids were transfected into competent DH5 α cells (Toyobo, Tsuruga) to isolate amplify clones. Finally, the isolated clones were sequenced using a Hitachi-5500 sequencer (Hitachi, Tokyo). The sequence results of this editing parts are shown in left side. The right side bar graph shows % of editing at the site of A, B, and the site for cytidine deaminase as shown in the top.

site for editing is removed by splicing. This report, however, suggests the possibility that the RNA editing leads to formation of the short variant. When the high-temperature-resistant RT enzyme was used at the RT step with a primer specific for 5-HT2CR, approximately equal amounts of both the long and short types of mRNAs were detected in undifferentiated cells. In contrast, in neuronally differentiated cells, a much larger amount of the long type was detected the short type, suggesting that the RNA in itself may be modified during the neuronal differentiation step, resulting in greater formation of the long-type product. Since the eliminated site in the short variant includes the RNA editing area, the sequence of the RNA editing site was compared between undifferentiated and differentiated cells. The sequencing results showed the possibility that the RNA editing catalyzed by adenosine deaminase was inhibited in differentiated cells. Although we have no direct evidence for it, the fact that both inhibition of RNA editing and high expression of the long type of mRNA are observed in differentiated cells might indicate that RNA editing plays some role in neuronal differentiation/proliferation. It has been reported that adenosine deaminase type 2 (AD-2) acts to convert adenosine to guanosine in its own mRNA (12). This editing results in the addition of 47 nucleotides in the

coding region of AD-2 which induces a frameshift, suggesting that this self-editing can control the production of the enzyme activity through alternative splicing. In the case of 5-HT2CR mRNA, it has been reported that RNA editing of this mRNA results in a change of the affinity of 5-HT2CR for G protein, resulting in the inhibition of inositol phosphates (IPn) formation (11) and Ca²⁺ mobilization (13). Another report about the basal activity of 5-HT2CR also showed that the editing induced inhibition of IPn formation (14). Gurevich et al. (15, 16) reported that 5-HT2CR mRNA editing is stimulated at site C' (E) and is inhibited at site D in the dorsal prefrontal cortex of suicide victims compared with normal humans. The report by Gurevich et al. (15, 16) also demonstrated the possibility that fluoxetine could overcome the editing changes observed in suicide victims. These reports indicate that the editing of 5-HT2CR mRNA clearly changes the activity of the effector system. However, the detailed mechanism of these changes and the effects on cellular physiology are still unclear. The present report suggest that two kinds of protein, 5-HT2CR and the short variant, can be made from one mRNA and that the editing activity has an important role in the switching: the editing activity may produce the short variant protein. Although we did not try to detect the short variant protein in the present

experiments, Canton et al. showed the existence of this protein using an antibody (5).

The functional role(s) of the short variant protein are of interest. This short variant seems to be involved in regulating cell growth, as indicated by the following facts. When the 5-HT_{2C} cDNA was first cloned by Julius et al. (1) using the *Xenopus* oocytes functional expression system, it was found that 4 of the 5 editable-position bases were guanosine instead of adenosine residues, that means they were edited. When the cloned 5-HT_{2C} cDNA (edited type) was transfected into NIH3T3 fibroblast cells, foci were formed (17), suggesting that 5-HT_{2C} (edited type) is involved in regulating cell proliferation. Although a considerable level of 5-HT_{2C} has been reported to be widespread in the brain (2), there have been few reports about endogenous expression of the receptor type of 5-HT_{2C} in cloned cell lines. Cloned cell lines, including NG108-15, generally have high growth potential. It may be expected that the functional expression of receptor type of 5-HT_{2C} is inhibited in the cell growing step. In accord with the fact that neuronal differentiation inhibits cell growth, the proportion of mRNA for the short variant, which seems to play a role in cell growth, decreased with the diminution of RNA editing activity in differentiated NG108-15 cells. Cytosine deaminase also seems to be involved in neuronal differentiation. Interestingly, while adenosine deaminase activity is reduced, cytosine deaminase activity is elevated in differentiated cells. Recently, cytosine deaminase has received attention for its possible use in cancer treatment via gene therapy (18, 19). However, the physiological role of cytosine deaminase is still unclear. The present results may yield insight into the role of this enzyme in cell growth control and/or differentiation. Further studies will be necessary to clarify the cellular function of the short variant of 5-HT_{2C}, including its relationship to cell growth and RNA editing.

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