have not fully clarified the action of V-1 in the cells, we showed that ATF-2 in the V-1 cells were activated and that activation of ATF-2 enhances TH gene transcription. We are now investigating the general role of ATF-2 in transcription of the TH gene as well as that of other catecholamine-synthesizing genes.

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Research report

Localization of sepiapterin reductase in the human brain

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Abstract

Sepiapterin reductase (SPR) is the enzyme that catalyzes the final step of the synthesis of tetrahydrobiopterin (BH4), the cofactor for phenylalanine hydroxylase, tyrosine hydroxylase (TH), tryptophan hydroxylase, and nitric oxide synthase (NOS). Although SPR is essential for synthesizing BH4, the distribution of SPR in the human brain has not yet been clarified. In the present study, we purified recombinant human SPR from cDNA, raised an antibody against human SPR (hSPR), and examined the localization of SPR protein and SPR activity. Human brain homogenates from the substantia nigra (SN), caudate nucleus (CN), gray and white matters of the cerebral cortex (CTX), and dorsal and ventral parts of the medulla oblongata (MO) were subjected to Western blot analysis with anti-hSPR antibody or with anti-TH antibody. Whereas TH protein showed a restricted localization, being mainly detected in the SN and CN, SPR protein was detected in all brain regions examined. SPR activity was relatively high compared with the activity of GTP cyclohydrolase I (GCH), the rate-limiting biosynthetic enzyme of BH4, and was more widely distributed than GCH activity. Immunohistochemistry revealed SPR immunoreactivity in pyramidal neurons in the cerebral CTX, in a small number of striatal neurons, and in neurons of the hypothalamic and brain stem monoaminergic fields and olivary nucleus. Double-staining immunohistochemistry showed that TH and SPR were colocalized in the SN dopamine neurons. Localization of SPR immunoreactive neurons corresponded to monoamine or NOS neuronal fields, and also to the areas where no monoamine or NOS neurons were located. The results indicate that there might be a BH4 biosynthetic pathway where GCH is not involved and that SPR might have some yet unidentified function(s) in addition to BH4 biosynthesis.

Theme: Neurotransmitters, modulators, transporters, and receptors

Topic: Other neurotransmitters

Keywords: Sepiapterin reductase; Human; Immunohistochemistry; Tyrosine hydroxylase; Tetrahydrobiopterin; Monoamine

1. Introduction

Sepiapterin reductase (SPR), a polypeptide of 30 kDa generally existing in a homodimeric form [53], is the terminal-step enzyme for the synthesis of tetrahydrobiopterin (BH4) [27,28,34,52], which is the reduced pteridine cofactor for pteridine-requiring enzymes such as phenylalanine hydroxylase, tyrosine hydroxylase (TH), tryptophan hydroxylase (TPH) [37], and nitric oxide synthase (NOS) [15,50]. The genomic organization and chromosomal localization of SPR in rats [47], mice [29], and humans [45,56] have already been determined. It has been established that the first-step and rate-limiting BH4-synthesizing enzyme is guanosine triphosphate (GTP) cyclohydrolase I (GCH), and the second-step one, pyruvoyltetrahydropterin synthase (PTPS) [1,16,36].

Catecholamines (dopamine, noradrenaline, and adrenaline) are synthesized from tyrosine starting with TH: tyrosine→dopa→dopamine→noradrenaline→adrenaline: and indoleamines (serotonin and melatonin) from tryptophan, starting with TPH: tryptophan→5-hydroxytryptophan → serotonin → *N*-acetylserotonin → melatonin.

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Catecholamines and serotonin are known as monoamines. Nitric oxide (NO) is synthesized by NOS from arginine: arginine→NO+citrulline.

BH4 also plays important roles in immune or cytokine systems [26,39,51,58,59,62] and in cell proliferation [55]. In humans, the lack of BH4 due to mutation of GCH gene is a cause of dopa-responsive dystonia (Segawa's disease) [20,24].

Although previous studies examining human and rodent brain tissues indicated that GCH immunoreactivity or GCH mRNA expression was localized in monoamine neurons, neither could be detected in pteridine-requiring NOS neuronal fields [33,40–42]. The significance of such a discrepancy is unclear, and the question arises as to whether a BH4 biosynthetic pathway where GCH is not involved might exist.

The complementary DNAs (cDNAs) of SPR have been cloned from rat [8], mouse [31,46], and human [19] sources: and the primary structures of their proteins were elucidated. Recently we produced for the first time a highly specific antibody against human SPR (hSPR) protein. Using it, we conducted the present study to determine the localization of SPR enzyme activity and SPR immunoreactivity in the human brain by Western blotting and immunohistochemistry. The coexistence of SPR and TH was also examined by a double-staining immunohistochemical method.

2. Materials and methods

2.1. Purification of recombinant hSPR for use as an immunogen

Human SPR cDNA was introduced into the *Nde*I and *Bam*HI sites of a pET16b vector (Novagen), which expresses a foreign gene as the fusion protein with a stretch of 10 histidine residues. *Escherichia coli* (*E. coli*) strain BL21 (DE3, pLysS) carrying the vector were cultured at 37 °C to a late-log phase, and the expression of the fusion protein was induced by the addition of isopropyl β-D-thiogalactopyranoside (IPTG). Protein concentrations were determined by the method of Bradford [7], with bovine γ-globulin used as a standard. Bacterial cells containing the fusion proteins were sonicated in ice-cold phosphate-buffered saline (PBS), and the lysate was centrifuged at 100 000×*g* for 30 min. The supernatant was filtered through a 0.22-μm filter (Millipore) and applied to a Ni^{2+}-chelating column (Amersham–Pharmacia Biotech) equilibrated with binding buffer (20 mM Tris–HCl (pH 8.0), 500 mM NaCl, and 5 mM imidazol) at 4 °C. The column was washed with 10 ml of binding buffer, then with 6 ml of washing buffer (20 mM Tris–HCl (pH 8.0), 500 mM NaCl, and 50 mM imidazol) and then again with 10 ml of binding buffer. Subsequently elution was achieved with 20 ml of stripping buffer (20 mM Tris–HCl (pH 8.0), 500 mM NaCl, and 100 mM EDTA). The eluate was dialyzed against PBS and stored at −80 °C until used.

2.2. Production of an anti-hSPR antibody

A rabbit polyclonal antibody against the purified recombinant SPR was raised. For immunization, a solution containing the purified recombinant protein (1 mg/ml) was emulsified with Freud's complete adjuvant having two times the volume of the antigen solution. A rabbit received a dose of 0.5 mg of the protein intradermally at multiple sites on its back. Doses of 0.5 mg of the protein in Freund's incomplete adjuvant were then given as booster injections at intervals of 2 weeks by subcutaneous injections. More than 10 booster injections were necessary to obtain a satisfactory antibody titer.

2.3. Immunoblot analysis

Western blot analysis of human and mouse liver lysates and of human brain homogenates from the substantia nigra (SN), caudate nucleus (CN), gray and white matters of the cerebral cortex (CTX) and dorsal and ventral parts of the medulla oblongata (MO) was performed. With the consent of the bereaved family, the human brain tissue was obtained from a pathological autopsy case (78-year-old female, with tissues taken 5 h postmortem) with no clinical or pathological signs of neurological or psychiatric diseases at Nagoya University Hospital. The tissues were homogenized in 20 mM Tris–HCl buffer (pH 7.5) containing 100 mM KCl, 1 mM EDTA, 1 mM DTT, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 1 mM PMSF. The homogenate was then centrifuged at 1500×*g* for 10 min, and the supernatant was stored at −80 °C until used. The extract or the purified protein was subjected to SDS–12% polyacrylamide gel electrophoresis (SDS–PAGE), and the proteins transferred to a PVDF membrane (Bio-Rad) at a constant voltage of 15 V for 1 h. The membrane, after having been blocked with 3% skim milk, was incubated with the antibodies (1:1000 dilution), and then incubated with anti-rabbit IgG conjugated to horseradish peroxidase. Following repeated washing of the membrane, the signals were visualized with ECL Plus (Amersham–Pharmacia Biotech).

2.4. Measurement of SPR and GCH activities

The enzyme activities were measured by using 10 μg of the brain homogenate, which was prepared as described above. SPR activity was assayed as described previously with a slight modification [11]. The incubation mixture (total volume of 50 μl) contained 100 mM potassium phosphate buffer (pH 6.4), 0.1 mM NADPH, and 0.15 mM sepiapterin as substrate. The reaction was carried out at 37 °C for 1 h and then terminated by the addition of 25 μl of iodine solution (1% iodine and 2% KI) and kept at room

temperature in the dark for oxidation of the product, 7,8-dihydrobiopterin, to biopterin. After centrifugation to remove precipitated proteins, the excess of iodine was reduced by adding 25 μl of 2% ascorbic acid. Biopterin was separated by high-performance liquid chromatography (HPLC) on a reverse-phase HPLC column and detected fluorometrically. GCH activity was assayed as described previously with a slight modification [54]. The incubation mixture (total volume of 100 μl) contained 100 mM Tris–HCl (pH 8.0), 300 mM KCl, 2.5 mM EDTA, 10% glycerol, and 1 mM GTP as substrate. The reaction was carried out at 37 °C for 1 h. The product, 7,8-dihydroneopterin triphosphate, was oxidized with iodine and dephosphorylated with alkaline phosphatase to neopterin. Neopterin was also separated and detected by the same method as used for biopterin.

2.5. Immunohistochemistry

2.5.1. Tissue processing

Human brains were obtained from five autopsy cases (27–72 years old, both sexes, postmortem interval: 4–5 h) at the Department of Legal Medicine, Shiga University of Medical Science, Japan, in compliance with the ethical code of the Ethical Committee of the Japanese Society of Legal Medicine. Each case had died from natural causes, and no signs of neurological or psychiatric diseases were detected clinically or pathologically. The brains were immediately sliced into 1-cm slabs and immersed in fresh fixative at 4 °C for 48–72 h. The slices were then transferred to phosphate buffer containing 15% sucrose and 0.1% sodium azide for storage at 4 °C. The brain sections were cut in coronal planes to a thickness of 50 μm and thereafter treated with 40% methanol and 1% H_2O_2 for 20 min to inhibit endogenous peroxidase [21].

2.5.2. Immunocytochemical methods

2.5.2.1. Single staining. The sections were incubated with antibodies against hSPR (diluted 1:1000–20 000) or TH (diluted 1:10 000) [38] in PBS containing 0.3% Triton X-100 (PBST) at 4 °C for 1 week. They were then rinsed in PBST (10 min×3), incubated in biotinylated rabbit IgG (Vector Laboratory, 1:1000) for 12 h at room temperature, washed again in PBST (10 min×3), and incubated with avidin–biotin–peroxidase complex (Vector Laboratory, 1:1000) for 1 h at room temperature. After final washes in PBST (10 min×3), the peroxidase activity was visualized with 50 mM Tris–HCl buffer (pH 7.6) containing 0.0003% H_2O_2, 0.01% 3,3 diaminobenzidine–4HCl (DAB), and 1% nickel ammonium sulfate. The sections were then rinsed in PBS, mounted on gelatin-coated glass slides, dehydrated, cleared in xylene, and coverslipped. Some sections were counterstained with neutral red. Atlases of Pearson et al. [49], Hokfelt et al. [17], Paxinos and Huang [48], Mai et al. [35], and Kitahama et al. [30] were used to determine the anatomical territories.

2.5.2.2. Double staining. For SPR/TH double-staining immunohistochemistry, sections were incubated in a mixture of mouse anti-TH antibody (LNCI; Incstar, Stillwater, MN; diluted 1:1000) and the rabbit anti-hSPR antibody (diluted 1:10 000) for 1 week at 4 °C. They were then rinsed in PBST (10 min×3), and were incubated with a mixture of secondary antibodies, i.e., Cy3-conjugated donkey anti-mouse IgG (AP192C; Chemicon, Temecula, CA; 1:200) for TH and fluorescein-conjugated donkey anti-rabbit IgG (AP182F; Chemicon, 1:200) for hSPR. Sections were rinsed with PBST (10 min×3) and subsequently mounted on glass slides and coverslipped with medium containing 70% glycerol and 0.1% *p*-phenylenediamine in PBS (Vector, Burlingame, CA). Finally the specimens were examined under a confocal laser-scanning microscope (LSM410; Zeiss, Oberkochen, Germany) with appropriate excitation laser beams and emission filters (for Cy3, excitation at 543 nm, emission at ≫570 nm; for fluorescein, excitation at 488 nm, emission at 510–525 nm) [22].

3. Results

3.1. SPR is widely distributed in the human brain

The Western blot analysis of human and mouse liver lysates showed that the anti-hSPR antibody recognized a single major band of approximately 30 kDa and did not cross-react with other proteins (lanes 1 and 2 of Fig. 1). The hSPR migrated more slowly than the mouse one, probably due to differences in the amino acid composition of SPR proteins between the two species. We also elec-

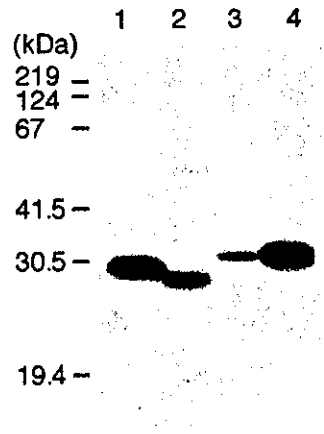

Fig. 1. Western blot analysis of human and mouse liver extract and of purified recombinant hSPR. Western blotting was performed with anti-hSPR antibody. One hundred micrograms of lysate protein of human (lane 1) and of mouse (lane 2) liver and 1 ng (lane 3) and 10 ng (lane 4) of His-hSPR were used.

trophoresed 1 and 10 ng of the purified His-hSPR and subjected these minute amounts to Western blot analysis. We could detect even 1 ng of the protein with the anti-hSPR antibody (lanes 3 and 4 of Fig. 1).

Next we examined the distribution of SPR in the human brain and compared it with that of TH. Human brain homogenates from the SN, CN, gray and white matters of the cerebral CTX, and dorsal and ventral parts of the MO were subjected to the Western blot analysis with anti-hSPR antibody (Fig. 2A) or with anti-TH antibody (Fig. 2B). Whereas TH protein showed a restricted localization mainly to the SN and CN, SPR protein was detected in all brain regions examined (Fig. 2). Further, we measured SPR activity in the brain homogenates (Table 1). The SPR activity was relatively high compared with the activity of GCH, the rate-limiting enzyme in the biosynthesis of BH4. The difference between the SPR and GCH activities in each brain region was less than 2-fold, while the relatively higher SPR activity was found in the SN and ventral part of the MO. The SPR activity was more widely distributed than the GCH activity (Table 1).

Table 1
Activities of SPR and GCH in the human brain regions

	Sepiapterin reductase activity (nmol/h per mg protein)	GTP cyclohydrolase I activity (pmol/h per mg protein)
Substantia nigra	44.5±2.8	3.82±0.25
Caudate nucleus	22.5±2.3	1.82±0.26
Cerebral cortex:		
Gray matter	23.7±0.2	N. D.
White matter	26.8±1.6	N. D.
Medulla oblongata:		
Dorsal part	26.5±0.6	4.15±0.64
Ventral part	42.0±1.1	2.54±0.28

Results represent the mean (S.D. from three independent experiments. N.D., not detectable (<0.1 pmol/h per mg protein).

3.2. Single-staining immunohistochemistry

3.2.1. Cerebral cortex

The pyramidal neurons in layers III and V of the cerebral CTX showed low to moderate SPR immunoreactivity (Fig. 3A,B), as did the granular cells.

3.2.2. Basal ganglia

The CN, putamen, and nucleus accumbens contained SPR-immunoreactive (-ir) cells and fibers (Fig. 3C). At high magnification, SPR-ir cell bodies and fibers were found around the fiber bundle of the internal capsule (ic) (Fig. 3D). In the globus pallidus, SPR-immunopositive neurons were sparse, with the staining intensity ranging from low to moderate. The neurons of the paraterminal gyrus and anterior olfactory nucleus also showed SPR immunoreactivity.

3.2.3. Hypothalamus

SPR-ir neurons were found in the dopaminergic fields of the posterior (A11) and caudal dorsal hypothalamic area (A13), as well as in the arcuate (A12) and periventricular (A14) zones [17,30] (not shown).

3.2.4. Midbrain

Moderately to intensely stained SPR-ir neuronal cell bodies were found in the retrorubral region, SN (Fig. 4A), and ventral tegmental area, corresponding to the A8–10 dopaminergic fields [49]. A number of positive neurons were also found in the dorsal raphe nucleus (DR: Fig. 3E,F), corresponding to the B7 serotonergic field [3].

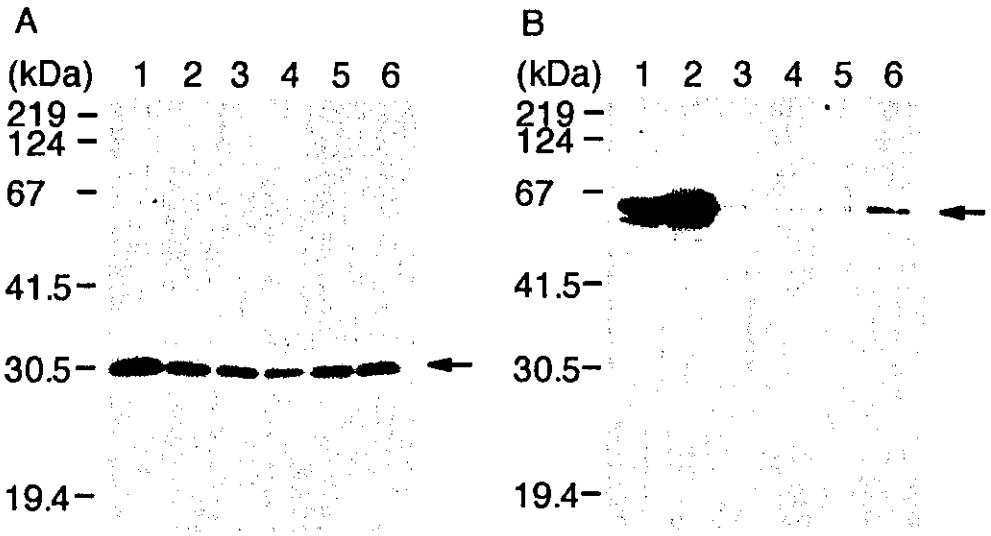

Fig. 2. Western blot analysis of human brain extract. Western blotting was performed by using anti-hSPR antibody (A) or anti-TH antibody (B). Twenty micrograms of lysate protein of the substantia nigra (SN) (lane 1), caudate nucleus (CN) (lane 2), cerebral gray matter (lane 3), cerebral white matter (lane 4), and dorsal (lane 5) and ventral (lane 6) parts of the medulla oblongata (MO) were used. The positions of SPR and TH are indicated by the arrows.

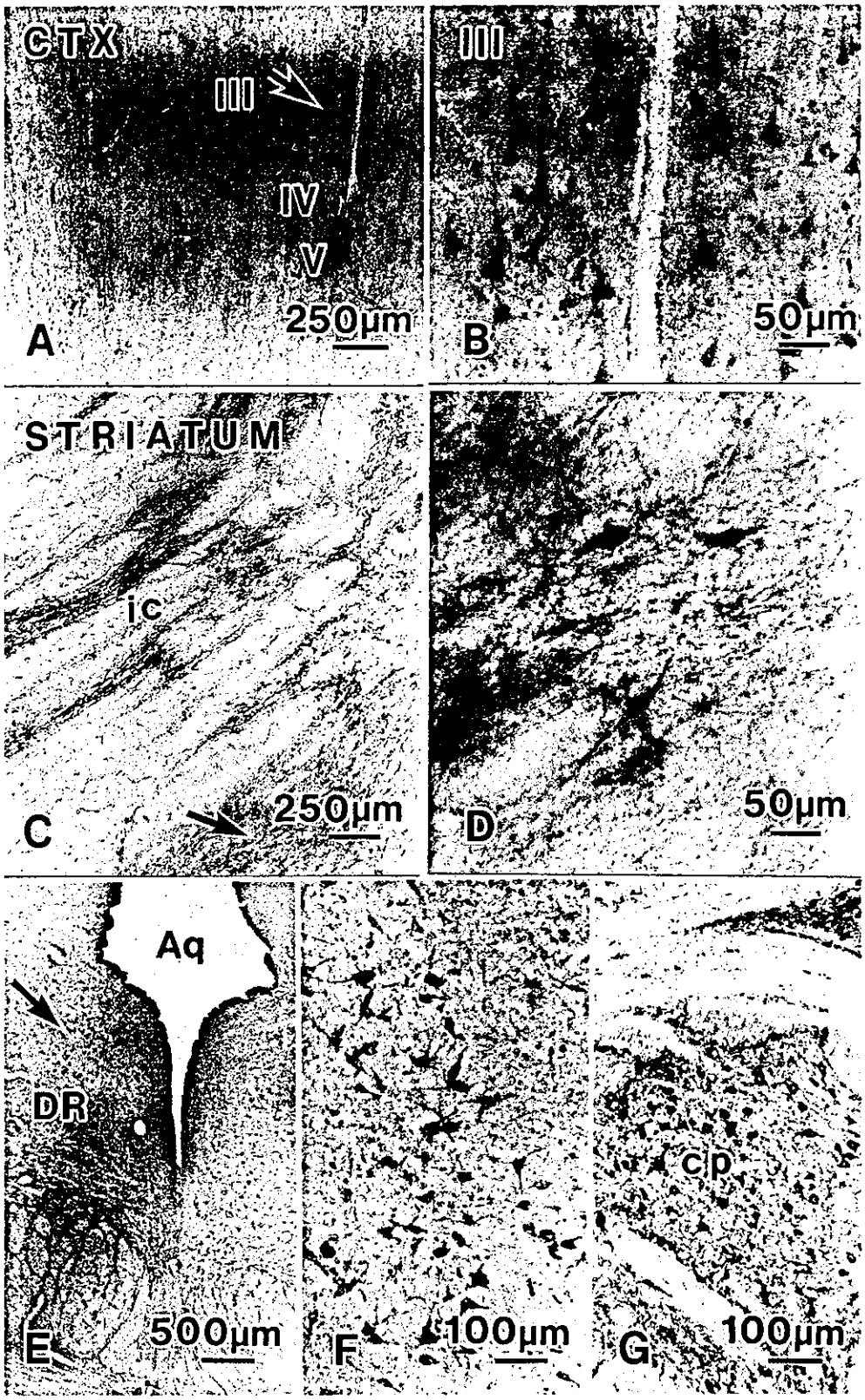

Fig. 3. Immunostained sections of SPR through the cerebral CTX (A,B), striatum (C,D), dorsal raphe nucleus (DR: E,F) and cerebral peduncle (cp: G). SPR-ir neurons indicated by an arrow in 'E' are magnified in 'F'. ic, internal capsule; Aq, cerebral aqueduct. Bar: 100 μm.

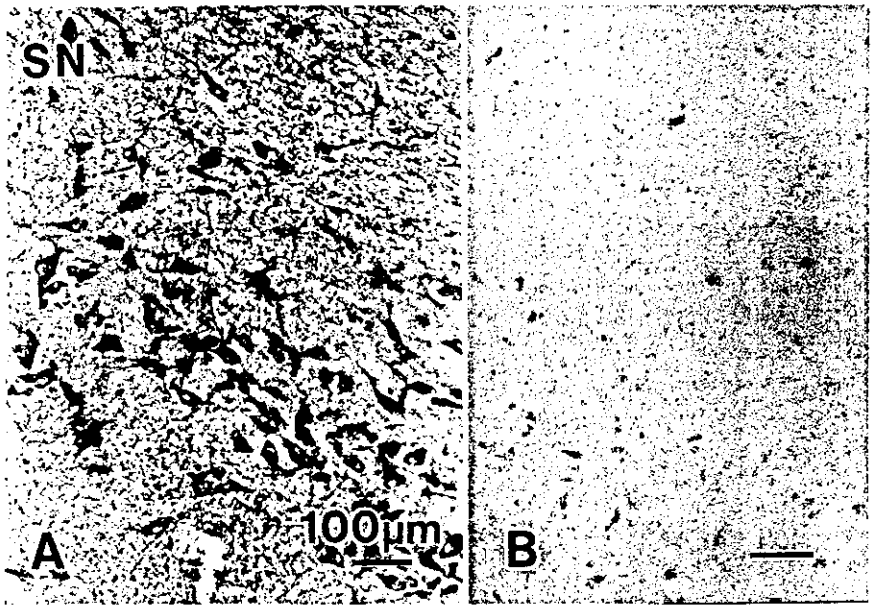

Fig. 4. (A) Immunostained sections of SPR through the SN. (B) Absorption test in the SN. No staining is shown in sections incubated with anti-human SPR serum preabsorbed with the human SPR antigen. Only neuromelanin pigments are seen. Bar: 100 μm.

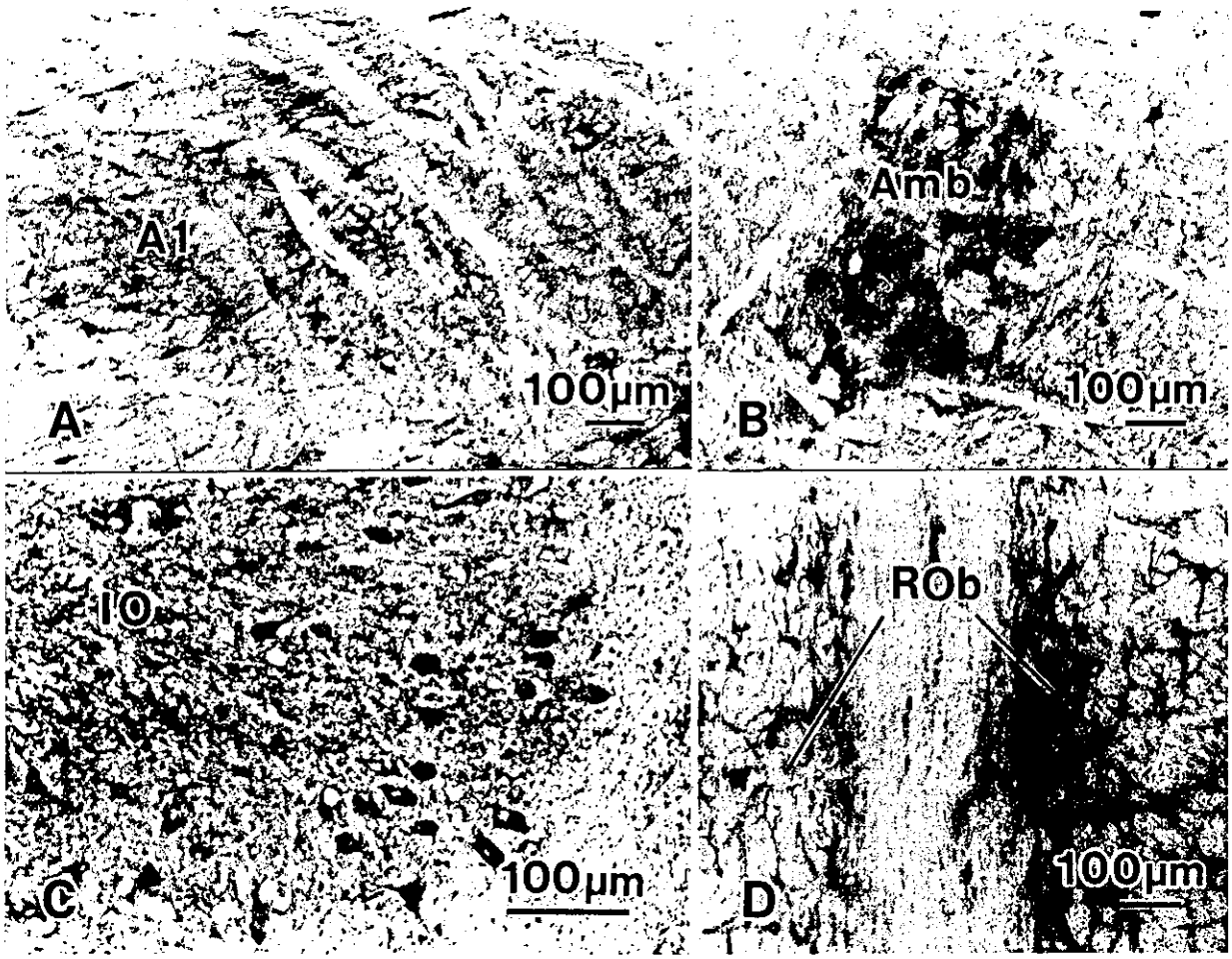

Fig. 5. SPR-immunostained sections through (A) the A1 of the ventrolateral medulla, (B) nucleus ambiguus (Amb), (C) olivary nucleus, and (D) raphe obscurus (ROb). IO, inferior olivary nucleus. Bar: 100 μm.

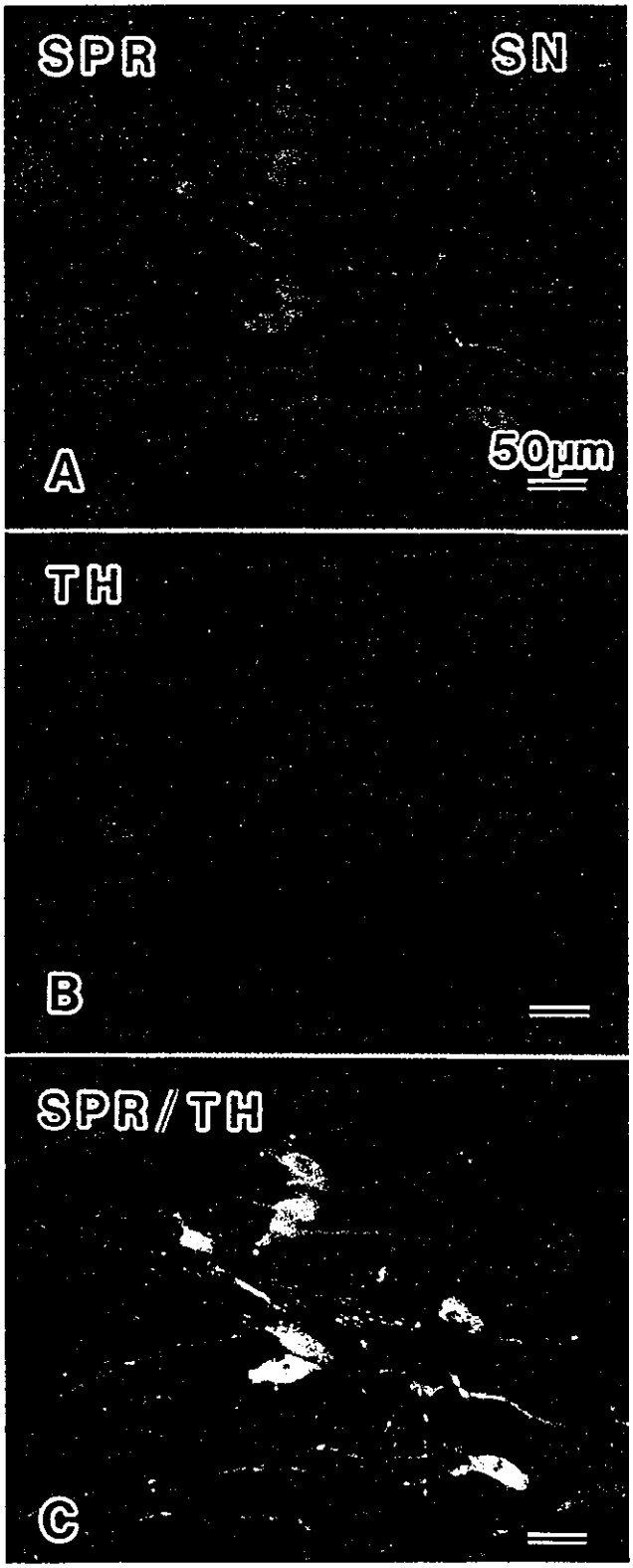

Fig. 6. Double-immunostaining: (A,B) SPR (red)- and TH (green) immunoreactivities in the SN. (C) SPR (+)/TH (+) neurons are shown in yellow. Bar: 25 μm.

There were many SPR-ir neurons packed within the fiber bundle of the cerebral peduncle (cp: Fig. 3G). No staining could be obtained in sections incubated in anti-hSPR antiserum preabsorbed with the protein antigen (Fig. 4B).

3.2.5. Pons

In the locus coeruleus (LC) neurons, corresponding to the A6 noradrenergic field [49], intense SPR immunoreactivity was found (not shown).

3.2.6. Medulla oblongata (MO)

SPR-ir neurons were seen in the A1 or C1 of the ventrolateral medulla (Fig. 5A), and in the A2 or C2 of the solitary tract nucleus [49]. The neuronal cell bodies in the nucleus ambiguus (Amb: Fig. 5B), raphe obscurus (ROb: Fig. 5D), and the olivary nucleus (Fig. 5C) showed intense SPR immunoreactivity.

3.3. Double immunostaining

Double staining for TH and SPR in the neurons of the SN showed many neurons doubly labeled by anti-TH and anti-SPR (Fig. 6A–C).

4. Discussion

The present study showed that SPR protein and SPR activity were widely distributed in the human brain. As expected from its role as the enzyme in the final step of BH4 synthesis. SPR could be detected in the neuronal fields where pteridine-requiring enzymes such as TPH [2], TH [49,57] and NOS [10,12,13,43,44,61] are known to be located. The present study also showed that the distribution of GCH activity was rather restricted mainly to the monoaminergic neurons. This finding coincides with our previous observations using human materials [42].

4.1. Localization of SPR in relation to monoamine neurons

The present study demonstrated SPR protein and SPR activity by biochemical methods in the human brain areas where monoaminergic neurons are distributed. The SPR immunohistochemical results confirmed SPR immunoreactivity in the hypothalamic and midbrain dopaminergic fields, locus coeruleus noradrenergic region, and medullary C1–2 adrenergic and A1–2 noradrenergic regions [17,30,49]. In these areas of the human brain, immunoreactivity of GCH, the first-step and rate-limiting BH4-synthesizing enzyme was earlier described [42]. Some of the serotonin neurons in the dorsal raphe nucleus were previously shown to contain NOS [25,32,60]. In our observation, the number of SPR-ir neurons was greater than that of GCH-ir neurons [42], suggesting the existence of SPR-positive, GCH-negative neurons.

4.2. Relationship between the distribution of NOS and SPR

Previous studies on human brain specimens reported NOS-ir non-pyramidal neurons in layers IV–VI of the cerebral CTX, as well as in the striatum, nucleus ambiguus, nucleus tractus solitarii, raphe nucleus, and inferior olivary nucleus [10,12,13,43,44,61]. The present study showed SPR-ir neurons in these areas, though the previous studies did not describe GCH immunoreactivity or GCH mRNA expression in them [33,40,41]. This indicates that there might be an unknown biosynthetic pathway of BH4 via SPR where GCH is not involved, or that NOS requires only a very small amount of BH4 that is synthesized by an undetectable level of GCH. It is unclear whether PTPS is involved in this possible biosynthetic pathway. In some neurological and psychiatric diseases, NOS is supposed to be involved in their etiology [4,5,9,18,44]. It remains unclear whether SPR is also involved in the pathogenesis of these diseases. Recently, patients with SPR deficiency were reported [6]: and so the pathognomonic mechanism of SPR deficiency should be elucidated.

4.3. Some other unknown functions of SPR?

The present study showed SPR immunoreactivity in the pyramidal neurons of the cerebral CTX in which immunoreactivity of dopamine, noradrenaline, serotonin or NOS has not been described. Although TH has been reported in the pyramidal-like neurons in the human cerebral CTX, the number is small [14,23] and such cells are not catecholaminergic neurons [23]. It is unclear whether SPR in the human cerebral CTX synthesizes BH4, a cofactor of TH, TPH, PAH, and NOS. Thus, it is possible that SPR might have some yet unidentified function(s) that remains to be elucidated.

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Enhanced expression of GTP cyclohydrolase I in V-1-overexpressing PC12D cells☆

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Abstract

Three of the catecholamine-synthesizing enzymes, i.e., tyrosine hydroxylase (TH), aromatic L-amino acid decarboxylase, and dopamine β-hydroxylase, were earlier shown to be up-regulated in cloned PC12D cells overexpressing V-1, a cdc10/SWI6 motif-containing protein. GTP cyclohydrolase I (GCH) is the rate-limiting enzyme for the biosynthesis of tetrahydrobiopterin (BH_4), known as an essential cofactor for TH; and here we found the increased expression of GCH in V-1-overexpressing clones. Both GCH activity and total biopterin content were highly increased in the V-1 clones; whereas the activity of sepiapterin reductase, enzyme in the final step of the BH_4 biosynthesis, was not altered. Biochemical analyses revealed increased levels of GCH protein, mRNA, and transcription in the V-1 clones. Promoter analysis showed increased reporter activity in the construct with 150 bp of the promoter region of the human GCH gene, suggesting the involvement of cAMP-responsive element-mediated transcriptional regulation.

Keywords: Catecholamine; cAMP-responsive element; GTP cyclohydrolase I; Gene expression; PC12 cells; Tetrahydrobiopterin; Transcription; V-1

Catecholamines (dopamine, norepinephrine, and epinephrine) are neurotransmitters and they play crucial roles in a variety of physiological functions in the brain. Catecholamines are also known to be involved in many neurological and neuropsychiatric diseases such as Parkinson's disease, manic-depressive illness, and schizophrenia. Catecholamines are synthesized from L-tyrosine by the sequential action of four enzymes: tyrosine is converted to DOPA by tyrosine hydroxylase (TH), DOPA to dopamine by aromatic L-amino acid decarboxylase (AADC), dopamine to norepinephrine by dopamine β-hydroxylase (DBH), and norepinephrine to epinephrine by phenylethanolamine *N*-methyltransferase. Regulation of the gene expression of catecholamine-synthesizing enzymes is important in brain function under physiological and pathological conditions as well as in the determination of the expression of neurotransmitters during brain development; however the molecular mechanisms of this regulation are poorly understood.

V-1 is a novel protein that may regulate the gene expression of catecholamine-synthesizing enzymes. It was originally identified in the rat cerebellum as one of the proteins the expression of which was transiently increased during the initial stage of postnatal development [1,2]. A high level of the V-1 expression was found to persist in the regions of synaptic plasticity even after the postnatal stage [3]. It is also endogenously expressed in chromaffin cells and overexpression of V-1 in PC12D cells elicited the coordinate up-regulation of the expression 3 of the catecholamine-synthesizing enzymes, i.e., TH, AADC, and DBH, resulting in remarkable

☆ *Abbreviations:* AADC, aromatic L-amino acid decarboxylase; BH_4, tetrahydrobiopterin; CRE, cAMP-responsive element; DBH, dopamine β-hydroxylase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GCH, GTP cyclohydrolase I; PTPS, 6-pyruvoyl tetrahydropterin synthase; SPR, sepiapterin reductase; TH, tyrosine hydroxylase.

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increases in dopamine and norepinephrine contents [4]. The V-1 protein consists of 117 amino acids, ~ 73% of which represents 2.5 tandem repeats of the cdc10/SWI6 motif, also known as the ankyrin repeat [1]. While a role for the ankyrin repeat in protein–protein interaction has been proposed (reviewed in [5]), no protein has been identified yet as one interacting with the V-1 protein.

Tetrahydrobiopterin (BH_4) synthesis is essential for catecholamine biosynthesis, because BH_4 is a cofactor for TH. BH_4 is synthesized from GTP in mammals via three enzymatic reactions: GTP cyclohydrolase I (GCH) catalyzes the formation of D-*erythro*-7,8-dihydroneopterin triphosphate from GTP; and this intermediate is further metabolized by 6-pyruvoyltetrahydropterin synthase (PTPS) and then finally converted to BH_4 by sepiapterin reductase (SPR). GCH is the rate-limiting enzyme for the de novo synthesis of BH_4. Recently, we found that alteration of the BH_4 content regulates catecholamine and serotonin biosyntheses differently [6].

The GCH enzymatic activity and gene expression are increased in response to various stimuli, whereas those of PTPS and SPR are relatively unaffected (reviewed in [7]). It has been suggested that stimuli that increase expression of the TH gene can often enhance the GCH expression [8–10]. However, the molecular mechanism for co-induction of TH and GCH is unclear.

In this study, we examined BH_4 synthesis in the V-1-overexpressing PC12D cell clones to know whether the biosynthesis of BH_4, like that of the catecholamine-synthesizing enzymes, was up-regulated in the V-1 clonal cells. We found the enhanced expression of GCH and the elevated level of biopterin content in the V-1 clones. In these cells, GCH enzymatic activity, protein level, and mRNA level were all elevated. We further demonstrated that GCH promoter activity was enhanced, indicating GCH gene transcription.

Materials and methods

Cell culture. Two PC12D clones stably and highly expressing V-1, named V1-46 and V1-69, and two vector control clones, termed C-7 and C-9, were established, as described previously [4]. Parental PC12D cells were cultured in DMEM containing 10% horse serum and 5% fetal bovine serum. The stable clones were cultured in medium containing 280 μg/ml G418 (GIBCO).

Preparation of cell lysates. Cells were washed three times and suspended in ice-cold phosphate-buffered saline and then pelleted in a microcentrifuge at 300*g* for 3 min. For the preparation of cell lysates for the measurement of enzymatic activities and immunoblotting of GCH and SPR proteins, the cell pellet was suspended in 20 mM Tris–HCl buffer (pH 7.5) containing 100 mM KCl, 1 mM EDTA, 1 mM DTT, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 1 mM PMSF. The cell suspension was sonicated and the sonicate was centrifuged at 1500*g* for 10 min; and then the supernatant was immediately collected as the cell lysate. Protein concentration was determined by the Bradford [11] method, with bovine γ-globulin used as the standard. The cell lysate was stored at −80 °C in small aliquots until assayed.

Measurement of GCH activity. GCH activity was assayed as described previously with a slight modification [12]. The incubation mixture (total volume of 100 μl) contained 100 mM Tris–HCl (pH 8.0), 300 mM KCl, 2.5 mM EDTA, 10% glycerol, and 1 mM GTP as substrate. The reaction was carried out at 37 °C for 1 h. The product, D-*erythro*-7,8-dihydroneopterin triphosphate, was oxidized by iodine solution and dephosphorylated with alkaline phosphatase to neopterin. Neopterin was separated by HPLC on a reverse-phase HPLC column and detected fluorometrically.

Measurement of SPR activity. SPR activity was assayed as described previously with a slight modification [13]. The incubation mixture (total volume of 50 μl) contained 100 mM potassium phosphate buffer (pH 6.4), 0.1 mM NADPH, and 0.15 mM sepiapterin as substrate. The reaction was carried out at 37 °C for 1 h. Then the reaction was terminated by the addition of iodine solution and kept at room temperature in the dark to oxidize the product, 7,8-dihydrobiopterin, to biopterin. Biopterin was separated and detected by the same method described above for neopterin.

Measurement of intracellular BH_4 content. The amount of BH_4 in the cell lysate was measured as biopterin by HPLC with fluorescence detection after iodine oxidation as described above.

Measurement of TH activity. TH activity was determined based on the measurement of L-DOPA formed from L-tyrosine by HPLC with electrochemical detection as described previously with a slight modification [14]. The incubation mixture (200 μl) consisted of 200 mM sodium acetate (pH 6.0), 1 mM BH_4, 0.1 M 2-mercaptoethanol, and 0.2 mg/ml catalase. The reaction was carried out at 37 °C for 10 min in air.

Production of anti-SPR antibody. A recombinant human SPR was expressed in *Escherichia coli* and purified as previously described [15] and a rabbit polyclonal antibody was then raised against the purified human SPR. For immunization, a solution containing the purified recombinant protein (1 mg/ml) was emulsified with Freund's complete adjuvant. A dose of 0.5 mg of the protein in Freund's incomplete adjuvant was then given subcutaneously as a booster injection at 2-week intervals until a suitable titer had been achieved.

Immunoblot analysis. A polyclonal antibody against GCH was raised, as described previously [12]. In the experiments on GCH and SPR, the cell lysate was separated by SDS–PAGE (12% polyacrylamide gel) and transferred to a PVDF membrane (Bio-Rad). Proteins were visualized with ECL plus (Amersham Biosciences).

Quantitative real-time PCR analysis. Total RNA was isolated from each clone by using the TRIZOL reagent (GIBCO). The total RNA was subjected to reverse transcription by using Superscript II (GIBCO). Analysis of GCH transcripts by quantitative real-time PCR was performed on a LightCycler using a LightCycler-FastStart DNA Master SYBR Green I Kit (Roche). $MgCl_2$ was added to a final concentration of 4 mM, and two oligonucleotide primers, AGCATCACCTGGTCCCATTTG (forward) and TTCCACAATCCTG GCAAGTTTG (reverse), were added to a final concentration of 500 nM each. In parallel, we analyzed the 18S rRNA as an internal control for normalization. Real-time PCR of 18S rRNA was performed on an ABI PRISM 7700 using TaqMan Universal PCR Master Mix (Applied Biosystems).

Isolation of mRNA and Northern blot analysis. Cells were harvested and mRNA was partially purified by oligo(dT)-cellulose affinity chromatography using a Quick-prep mRNA Purification Kit (Amersham Biosciences) following manufacturer's protocol. About 30–50% of the purified preparation was expected to be mRNA. The preparations of stable PC12D clones (10 μg of RNA) were electrophoresed in a 1% agarose gel, transferred to Hybond N^+ membrane (Amersham Biosciences) by capillary diffusion, and then hybridized with ^{32}P-labeled mouse GCH cDNA [16] or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA probes. Radioactivity was visualized by using a BAS-1000 Bio-imaging analyzer (Fuji Photo Film, Tokyo).

Reporter plasmids. We previously isolated the human GCH gene [17] and cloned a fragment containing 8.1 kb of its 5′-flanking

promoter region into pBluescript plasmid (hGCHpro8.1-pBS). To generate hGCHpro5.2-Luc containing the region −5236 bp from the transcriptional start site [18] to the translational start site upstream of the firefly luciferase cDNA, we double digested hGCHpro8.1-pBS with *Eco*RV and *Nco*I and then inserted the fragment between *Sma*I and *Nco*I sites of PGV-B2. To generate hGCHpro0.45-Luc containing −452 bp of the GCH 5′-flanking region, we double digested hGCHpro8.1-pBS with *Hin*dIII and *Nco*I and inserted the fragment between the same sites of PGV-B2. hGCHpro0.15-Luc containing −149 bp of the GCH 5′-flanking region was selected from deletion constructs of hGCHpro0.45-Luc, which were generated by digesting with *Hin*dIII and *Kpn*I and deleting with exonuclease III and mung bean nuclease.

DNA transfection and luciferase assay. Seapansy luciferase vector, pRL-CMV (Toyoink, Tokyo), was used as an internal control to normalize for variations in transfection efficiency. Cells were transfected by lipofection using LipofectAMINE 2000 (GIBCO) according to manufacturer's instructions of the manufacturer. One day prior to transfection, the cells were plated in 24-well plates and transfected at ~80% confluence with 0.75 μg firefly reporter plasmids and 0.05 μg pRL-CMV per well. At 48 h after transfection, the cells were harvested and assayed for firefly and seapansy luciferase activities by using a PicaGene Dual Luciferase Assay Kit (Toyoink, Tokyo).

Statistics. Student's *t* test was used for statistical evaluations. A level of $P < 0.05$ was accepted as statistically significant.

Results

Increased levels of GCH activity and BH_4 content in V-1-overexpressing PC12D cells

We first measured the TH activity (Fig. 1A) and confirmed that TH activity in V-1-overexpressing clones (V1-46 and V1-69) was significantly higher than that in control clones (C-7 and C-9), as described previously [4]. Because BH_4 is an important regulator of TH activity and GCH is the enzyme in the first and rate-limiting step for BH_4 biosynthesis, we assayed the GCH activity and BH_4 content in the V-1 clones. The GCH activity in the V-1-overexpressing clones was increased 8- to 9-fold compared with that in the control clones (Fig. 1B). Reflecting the elevated GCH activity, the BH_4 content in the V-1 clones was 5- to 6-fold higher than that in the control clones (Fig. 1C). In contrast, the activity of SPR, the enzyme in the final step of the BH_4 biosynthesis, remained unchanged (Fig. 1D).

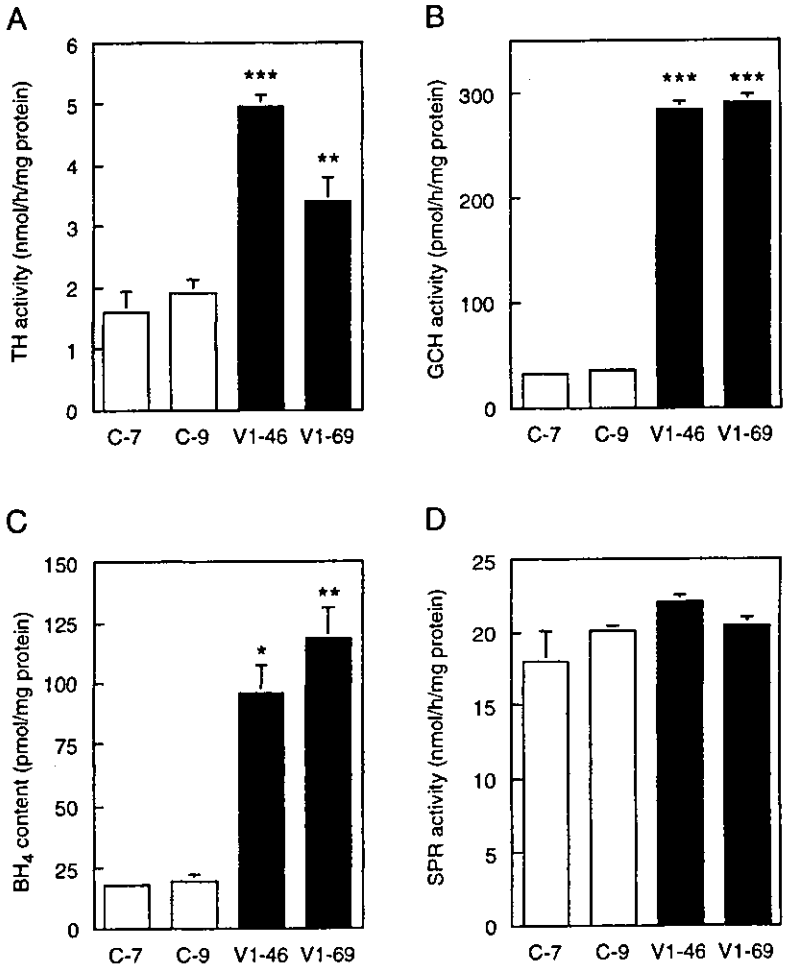

Fig. 1. Increased levels of GCH activity and BH_4 content in V-1-overexpressing PC12D cell clones. Enzymatic activities of TH (A), GCH (B) and SPR (D), and cellular contents of BH_4 (C) in cell lysates prepared from V-1-overexpressing clones (V1-46 and V1-69; closed bars) and control clones (C-7 and C-9; open bars) were measured as described in "Materials and methods". Data are the means ± SD values from three independent experiments. Values of *p* were calculated based on either value of the control clones: $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

Up-regulation of GCH protein and mRNA in the V-1 clones

As shown in Fig. 2A, Western blot analysis revealed that the amount of GCH protein was much increased in the V-1 clones, paralleling the increase in the enzymatic activity. The level of SPR protein was unaffected (Fig. 2A).

Next, we determined the GCH mRNA level by the quantitative real-time PCR method. We also quantified 18S rRNA as an internal control for normalization. The amount of GCH mRNA relative to that of 18S rRNA in the V-1 clones was increased 3- to 5-fold compared with that for the parental PC12D cells, whereas the vector control clones and parental cells were at the same level (Fig. 2B).

Because two transcripts for GCH (about 1.2 and 3.0 kb) were reported to exist in the rat adrenal medulla and PC12 cells [19], we performed a Northern blot analysis. It turned out that both sizes of GCH mRNAs

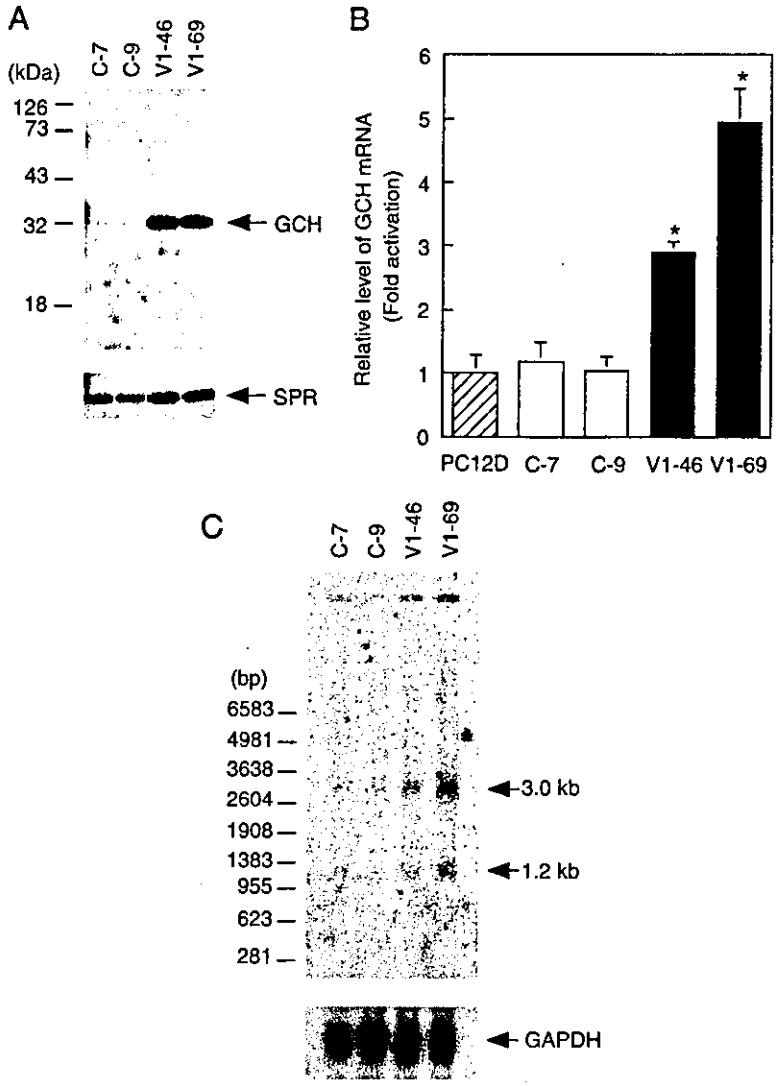

Fig. 2. Up-regulation of GCH protein and mRNA levels in the V-1 overexpressing clones. Analyses by Western blotting (A), real-time quantitative PCR (B), and Northern blotting (C) were performed as described in "Materials and methods". A, Western blotting was performed by using anti-GCH (upper panel) and anti-SPR (lower panel) antibodies as described in "Materials and methods". Fifteen microgram amounts of cell lysate protein of the V-1-overexpressing clones and control clones were used. The position of GCH is indicated by the arrow. B, levels of GCH mRNA in the V-1-overexpressing clones (closed bars) and those in the control clones (open bars) are expressed as fold activation relative to those in the parental PC12D cells (hatched bar). 18S rRNA was used for the internal control of normalization. Data are the means ± SD values from three or four independent experiments. Values of p were calculated compared with the values of the parental PC12D cells: $^{*}p < 0.01$. C, Partially purified polyA^{+} RNA preparation of stable clones (5 μg) was used for Northern blotting. An analysis using the mouse GCH cDNA probe is shown in the upper panel and a subsequent reprobing of the blot with the mouse GAPDH cDNA probe is shown in the bottom panel.

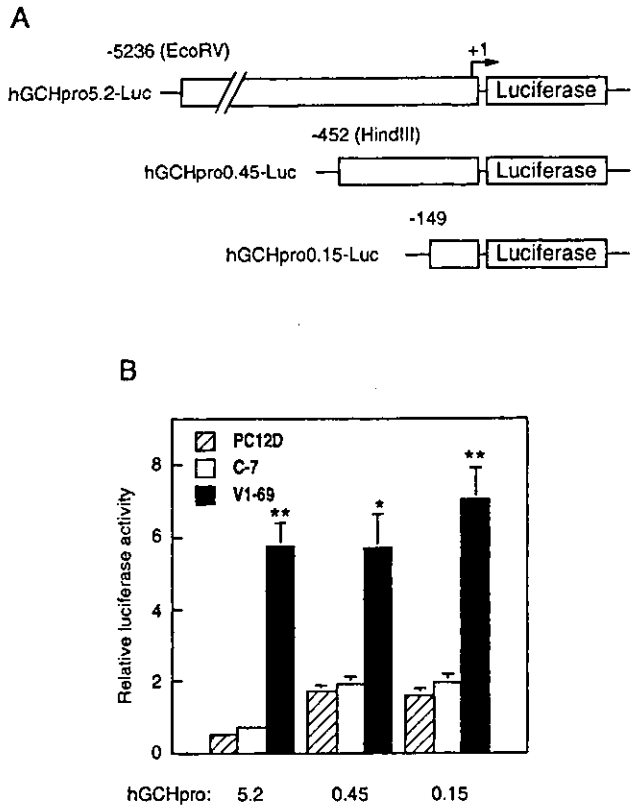

Fig. 3. Enhancement of GCH promoter activity in the V-1 clones. Reporter activities of GCH promoter plasmids (hGCHpro5.2-Luc, hGCHpro0.45-Luc, and hGCHpro0.15-Luc) in the V-1-overexpressing clone (V1-69, closed bars), the control clone (C-7, open bars), and the parental cells (PC12D, hatched bar) were measured as described in "Materials and methods." A seapansy luciferase vector, pRL-CMV, was used as an internal control to normalize for variations in transfection efficiency. Data are the means ± SD of triplicate cultures and are representative of those of two other independent experiments. Values of p were calculated based on the value of either the parental cells or the control clone: $^{*}p < 0.05$, $^{**}p < 0.01$.

in the V-1 clones were increased compared with their levels in the control clones (Fig. 2C).

Increased promoter activity of the GCH gene in the V-1-overexpressing clones

To examine the transcription of the GCH gene, we transfected the parental and cloned PC12 cells with plasmid constructs containing 5.2, 0.45, and 0.15 kb of the human GCH 5′-flanking region fused to a luciferase reporter gene. Reporter activities of all constructs relative to the activity of pRL-CMV were significantly increased in the V-1 clone (V1-69) compared with those in the control clone (C-7) and the parental cells (Fig. 3). Similar activation was observed using the other V-1 clone (V1-46, data not shown). These data suggest that the increased level of GCH mRNA in the V-1 clones was mainly due to an increased transcriptional rate.

Discussion

In the present study, we showed an increased BH_4 content and enhanced expression of GCH in the V-1-overexpressing clones of the PC12D cell line. The data indicate that GCH is up-regulated along with three of the catecholamine-synthesizing enzymes, i.e., TH, AADC, and DBH, in the V-1 clones to elevate the BH_4 level.

To characterize the transcriptional machinery acting for the expression of these genes including GCH in the V-1 clones, we performed transient transfection assays with various lengths of the 5′-upstream region of the GCH gene. The results of the promoter analysis showed that 0.15 kb of the GCH promoter region was sufficient for the increased reporter activity both in the V1-69 clone (Fig. 3) and in the V1-46 clone (data not shown). This construct is nearly of the same size as the minimum promoter, which was recently identified as the region contributing to basal and cyclic AMP-induced transcriptional activities and containing a noncanonical CRE [10,20]. Recently, using a reporter construct having the canonical CRE, we demonstrated that the CRE-mediated transcriptional activity was enhanced in the V-1 clones (Suzuki et al., manuscript in preparation). These data suggest that the enhanced expression of GCH may be due to the elevation of the CRE-mediated transcription in the V-1 clones.

Whereas GCH activity and BH_4 content were increased in the V-1 clones, SPR activity and the protein level were little affected (Figs. 1 and 2). Previous reports described the induction of GCH activity by various hormones or cytokines with the constitutive expression of SPR in various cell types [21,22]. Our data confirm that GCH is more highly regulated than SPR and suggest that the regulation of GCH gene expression is closely related to the modulation of the BH_4 content.

Our finding of the increased biopterin content in the V-1 clones suggests that the up-regulation of the BH_4 cofactor contributes to the increased levels of dopamine and norepinephrine [4]. BH_4 is not only essential for TH enzymatic activity but also for the stability of the TH protein [6]. In addition, it was reported that the gene expression of GCH was induced along with that of TH by various stimuli that also prompted elevation of the BH_4 level [8–10]. Therefore, our data also suggest that elevation of the BH_4 level by GCH induction is essential when TH gene expression is induced to increase catecholamine levels. This suggestion is supported by the findings of a previous study in gene therapy showing that triple transduction with TH, AADC, and GCH genes in adeno-associated virus vectors improved the rotational behavior of parkinsonian rats more effectively and resulted in a greater dopamine production in 293 cells than did double transduction with TH and AADC genes [23].

In the V-1-overexpresing cells, the mechanism(s) elevating the TH, AADC, and DBH mRNA levels is still unknown. However, our data showing the enhanced transcription of the GCH gene suggest that any transcriptional machinery could be expected to play key roles in the concordant expression of these enzymes, even though the expression of each enzyme is governed by different mechanisms. In particular, the concordant gene expression of all catecholamine-synthesizing enzymes should be required at the developmental stage at which catecholaminergic neurons arise. Since the expression pattern of V-1 in the brain seems to be closely related to the brain development and neural plasticity [1–3], the present data imply the existence of transcriptional machinery for the concordant gene expression of the catecholamine-synthesizing enzymes including GCH in developing catecholaminergic neurons.

Although V-1 contains 2.5 tandem repeats of the ankyrin repeat, which may serve as an interface for protein–protein interaction [5], no V-1-associating protein has been identified yet. V-1 has no putative DNA binding domain and it likely acts as an adapter protein in the cytosol [4]. To clarify the entire cascade of the V-1 overexpression for the up-regulation of the catecholamine synthesis, we are now investigating proteins interacting with V-1 as well as regulatory proteins that enhance the expression of the catecholamine-synthesizing enzyme genes including GCH.

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