A Novel Protein Containing cdc10/SWI6 Motifs Regulates Expression of mRNA Encoding Catecholamine Biosynthesizing Enzymes*

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Tohru Yamakuni‡, Toshifumi Yamamoto‡, Masato Hoshino, Si-Young Song†, Hideko Yamamoto**, Mayuko Kunikata-Sumitomo, Atsuko Minegishi, Misae Kubota, Mieko Ito, and Shiro Konishi‡

From the Department of Molecular Recognition, Graduate School of Integrated Science, Yokohama City University, 22-2 Seto, Kanazawa-ku, Yokohama 222-0022, Japan, the Department of Biochemical Cell Research, The Tokyo Metropolitan Institute of Medical Science, 18-22, Honkomagome 3-chome, Bunkyo-ku, Tokyo 113-0021, Japan, and the **Department of Psychopharmacology, Tokyo Institute of Psychiatry, 2-1-8 Kamikitazawa, Setagaya-ku, Tokyo 156-0057, Japan

Catecholaminergic (dopaminergic, noradrenergic, and adrenergic) transmitter phenotypes require the cooperative actions of four biosynthetic enzymes: tyrosine hydroxylase, aromatic L-amino acid decarboxylase, dopamine β-hydroxylase, and phenylethanolamine N-methyltransferase. Mechanisms that control expression of these enzymes in a transmitter phenotype-specific manner, however, are poorly understood. Here, we provide evidence that overexpression of a novel cdc10/SWI6 motif-containing protein, V-1, elicits the coordinate up-regulation of tyrosine hydroxylase, aromatic L-amino acid decarboxylase, and dopamine β-hydroxylase mRNAs in the neuronal cell line PIC12D, and as a result, catecholamine levels are increased. Furthermore, V-1 is strongly expressed in the cytoplasm of rat chromaffin cells of adrenal medulla. Thus, V-1 may act as a cytoplasmic protein/protein adapter and be involved in control of the catecholaminergic phenotype expression via an intracellular pathway signaling to the nucleus.

Abnormalities in catecholamine biosynthesis and neurotransmission have been implicated as the cause of neurological and psychiatric diseases (1). Signal transduction pathways involving protein kinase, including cAMP-dependent protein kinases, Ca2+/phospholipid-dependent protein kinase, and Ca2+/calmodulin-dependent protein kinase, that regulate the activity of TH and the transcription of genes encoding catecholamine biosynthesizing enzymes have been identified and extensively characterized (2–17). In addition, Mash1 and Phox2 transcription factors have been recently shown by gene targeting to be required for development of subpopulations of central nervous system and peripheral nervous system catecholaminergic neurons (18, 19). Little is known, however, about mechanisms that control expression of genes encoding the four enzymes that determine the catecholaminergic phenotype.

V-1 is a novel soluble protein consisting of 117 amino acids that contains 5 tandem repeats of the cdc10/SWI6 motif, also known as the ankyrin repeat (see Fig. 1A) (20, 21). This motif has been demonstrated to be crucial for protein-protein interactions (22, 23). Our recent studies have revealed a characteristic temporal profile for the expression of the V-1 gene in developing murine brain. During embryonic stages, expression of the V-1 gene is detectable but weak. After birth, expression of V-1 mRNA gradually increases to reach a maximal level during the first to second postnatal weeks, declining thereafter to adult levels by postnatal day 28.2 However, strong expression of the V-1 gene persists in regions of synaptic plasticity even after the second postnatal week (24). We have established stable transfectants that overexpress V-1 using the catecholamine-producing neuronal cell line PIC12D to examine functional roles of V-1 in neuronal cells and analyzed the neuronal phenotypes of these cells using techniques of molecular biology, neurochemistry, biochemistry, and electrophysiology. In this study, we also demonstrate that the V-1 protein is intensely co-expressed with TH protein in catecholamine-producing tissues in situ and provide evidence that V-1 functions in the control of the catecholaminergic transmitter phenotype.

EXPERIMENTAL PROCEDURES

Immunohistochemical Analyses—Immunohistochemistry was carried out as described previously (24–26).

Stable Cell Transfections—The PC12D subclone of rat pheochromocytoma cells (PC12) was cultured as described previously (27). For stable transfection experiments, 3 × 105 cells/100-mm dish were incubated overnight in 10 ml of the culture medium. The following day, cells were transfected with 10–12 μg/dish pEFSAneo (28, 29), a modified version of the pcDNA3 vector (Invitrogen). Eighty G418-resistant clones transfected with the DNA construct directing V-1 overproduction were screened by Western blot analysis with anti-V-1 antibody as described below, and several V-1-overexpressing clones were thereby isolated. For the subsequent experiments, two clones highly expressing V-1, V1–46, and V1–69 were used. Similarly, two stable transfectants with the vector alone (C-7 and C-9) were isolated and used as vector control clones.

Western Blot Analyses—For Western blot analysis, 1 × 106 cells were plated on 60-mm tissue culture dishes and cultured for 48 h. Cells were lysed in SDS-PAGE sample buffer (2% SDS, 10% glycerol, 62.5 mM Tris-HCl (pH 6.8)) including protease inhibitors (30). The lysates were analyzed by immunoblotting with anti-V-1 antibody (24, 25), a monoclonal antibody to TH (26), anti-14–3–3 β protein antibody (sc-628, Santa Cruz Biotechnology), and anti-erb 2 monoclonal antibody (Upstate Biotechnology, Inc.), and signals were visualized with enhanced

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‡ To whom correspondence should be addressed. Tel.: 81-427-24-6262; Fax: 81-427-24-6316; E-mail: yamakuni@libra.la.m-kagaku.co.jp.
Our extensive immunohistochemical analyses indicated that the V-1 protein is expressed in a wide variety of different central neurons (24) as well as in rat ovary (25) and other rat tissues including thymus, spleen, and kidney. Interestingly, strong expression of the V-1 protein coincided with TH expression in the cytoplasm of catecholaminergic cells, including adrenergic chromaffin cells of adrenal gland (Fig. 1B) and noradrenergic neurons of the sympathetic ganglia and locus coeruleus. To examine the potential role of the V-1 protein in the regulation of catecholaminergic phenotype, we generated stable transfectants that overexpress the V-1 protein using PC12D cells. Among stable transfectants harboring V-1 expression constructs, two clones V1–46 and V1–69 were chosen for phenotype analyses. V-1 protein levels of the second enzyme AADC could be detected (Fig. 3, B and C). Three mRNA species of the third enzyme DBH were detected in all transfectants. The expression of two DBH mRNA species of higher molecular weight was differentially up-regulated 6.2–19.3-fold in the V-1-overexpressing clones compared with those in the control clones (Fig. 3, A and B). We initially examined the functional role of V-1 in the regulation of expression of genes encoding TH (36), AADC (37), DBH (38), and PNMT (39, 40), which catalyze the four synthetic successive steps from tyrosine to dopa, dopamine, norepinephrine, and epinephrine. As shown in Fig. 3, A and B), mRNA expression of the first enzyme, TH, was increased in both V-1-overexpressing clones from 2.4- to 8.1-fold compared with those in the control clones. Two sizes of mRNA species of the second enzyme AADC could be detected (Fig. 3, A and B). Levels of the larger AADC mRNA species were increased 20.2–68.2-fold in V-1-overexpressing clones compared with those of the control clones, where AADC mRNA expression appeared to be down-regulated in comparison with that of parent cell (Fig. 3, A and B). 

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detectable in the parent cell by Northern analysis, consistent with previous data showing that there is no detectable level of PNMT enzymatic activity in PC12 cells (41). PNMT mRNA was also not detected in the V-1-overexpressing clones. These results suggest that the V-1 protein positively controls the coordinate expression of TH, AADC, and DBH mRNA in PC12D cells.

To obtain additional evidence at the enzymatic and functional levels, we assayed protein levels and enzymatic activities of TH and the catecholamine contents of cells. Western blot analysis using an antibody specific for TH showed that the levels of TH protein were 1.8–5.3 times higher in the V-1-overexpressing clones compared with control clones (Fig. 3, C and D). TH enzymatic activities in homogenates prepared from the V-1-overexpressing clones were also 1.8–3.8-fold higher than those prepared from control clones (Fig. 4A), indicating that increases in TH enzymatic activities parallel the changes in the protein and mRNA levels of TH. Dopamine levels in V-1-overexpressing clones were 6–36-fold higher, and norepinephrine levels were 10–100-fold higher compared with those in the control cells (Fig. 4B). The results of our biochemical and neurochemical experiments suggest that up-regulation of the expression of TH, AADC and DBH mRNAs observed in the V-1-overexpressing clones is indeed responsible for the increases in dopamine and norepinephrine production.

In addition, among the V-1-overexpressing clones and the control clones, there were no discernible changes in the magnitudes of Na\(^+\) and Ca\(^{2+}\) channel currents, as determined by the whole cell voltage-clamp recording method 7 days after treatment with nerve growth factor. We therefore conclude that the remarkable increases in catecholamine production observed in both V1–46 and V1–69 clones are the result of a change in phenotype induced by V-1 overproduction.

To our knowledge, the present study is the first to indicate

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**FIG. 3.** Up-regulation of TH, AADC, and DBH mRNAs (A and B), and TH protein levels (C and D) in the V-1-overexpressing clones. Each photograph is representative of three and five independent Northern and Western analyses, respectively. The histograms (B and D) indicate results compiled from these experiments. Data represent the means ± S.E. A, two autoradiographic images for DBH mRNA were obtained at different exposure times. Total cellular RNAs (10 μg) of transfectedants were subjected to Northern blotting. Staining gels with ethidium bromide (bottom panel) and probing blots with mouse Phox2 cDNA were performed to confirm equivalent loading. B, differences in TH, AADC, and DBH mRNA levels among the V-1-overexpressing clones and control clones are significant at p < 0.05. C, cell lysates (1.2 μg of protein) of these clones were subjected to 10% SDS-PAGE, blotted onto polyvinylidine difluoride membrane, and probed with anti-TH antibody and successively with anti-erk 2 (as an internal control) antibody. Differences in TH protein levels among the V-1-overexpressing clones and control clones are significant at p < 0.05 (paired t test).

**FIG. 4.** Effects of V-1 overexpression on TH enzymatic activity and catecholamine biosynthesis. A, TH enzymatic activities in homogenates. Values denote the means ± S.E. (n = 6). Significant differences from two vector control clones are indicated, at p < 0.05 (*) and p < 0.01 (**), respectively (Student’s t test). B, cellular contents of dopamine and norepinephrine. Values indicate the means ± S.E. (n = 5). Asterisks denote significant differences (***, p < 0.005; ****, p < 0.001) from two control clones (Student’s t test).

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\(^4\) S. Konishi, unpublished observations.
the involvement of V-1 in the control of a neuronal function, i.e. regulation of catecholamine biosynthesis. The fact that the V-1 protein is expressed in various rat tissues as described above raises the possibility that V-1 may have additional functions.

There are neither putative DNA binding domains nor other functional domains that seem to be involved in gene expression regulation within the V-1 protein (20, 21). The V-1 protein also could not be detected in nuclear extracts prepared from the V-1-overexpressing clones, as assayed by Western blot analysis, which suggests that the V-1 protein may function in the cytoplasm. Nevertheless, overexpression of V-1 resulted in an increase in mRNA expression for three enzymes that determine cytoplasm. Nevertheless, overexpression of V-1 resulted in an increase in mRNA expression for three enzymes that determine catecholaminergic phenotype. An unusual characteristic of the V-1 protein is that about 73% of the entire molecule is composed of 2.5 contiguous repeats of the cdc10/SWI6 motif. Therefore, we cannot exclude the possibility that V-1 could not be detected in nuclear extracts prepared from the V-1-overexpressing clones, as assayed by Western blot analysis, which suggests that the V-1 protein may function in the cytoplasm. Nevertheless, overexpression of V-1 resulted in an increase in mRNA expression for three enzymes that determine catecholaminergic phenotype. An unusual characteristic of the V-1 protein is that about 73% of the entire molecule is composed of 2.5 contiguous repeats of the cdc10/SWI6 motif.

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