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Identification of Certain Sequences in the 3rd Cytoplasmic Loop of D_4 Dopamine Receptor that Suppress the Bacterial Expression

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To study the functional roles of dopamine receptors, we decided to raise antibodies against these proteins. To make antigen, we expressed the whole 3rd cytoplasmic loop of dopamine receptors in a fusion protein with glutathione-S-transferase (GST). For D_2 and D_3 receptors, it was successful to express and purify fusion proteins for the whole 3rd cytoplasmic loops. However, we could not express the fusion protein for the whole 3rd cytoplasmic loop of D_4 dopamine receptor in the bacteria. To study the causes that prevent the bacterial expression of the GST-fusion protein of the 3rd cytoplasmic loop of D_4 dopamine receptor, we conducted more detailed studies on D_4 dopamine receptor. To locate the region which prevents bacterial expression, we made sequential constructs in the 3rd cytoplasmic loop decreasing the size step by step, and confirmed their expressions in the SDS PAGE. It was found that certain regions of 3rd cytoplasmic loop of D_4 dopamine receptor, located in N-terminal side of the 3rd cytoplasmic loop of D_4 dopamine receptor suppress the bacterial expression of fusion protein.

Key words : D_4 dopamine receptor, GST fusion protein, Bacterial expression

INTRODUCTION

Brain dopaminergic nervous system has important roles both in physiological and pathological points of view. Disfunctioning of brain dopaminergic nervous system brings in motor disorders (Hornykiewicz, 1963) and mental disorders. Schizophrenia is believed to stem from abnormal increases in dopaminergic neurotransmission in limbic system (Defrance *et al.*, 1985; Matthysse, 1981; Stevens, 1979; Wise, 1987).

Numerous experimental data support the linkage between dopaminergic nervous system and mental disorders. For example, neuroleptics which block the dopamine receptors improve the patient status, but substances which increase the activity of dopamine nervous system, such as L-DOPA and amphetamine make it worse (Snyder, 1973).

Over past 20 years, dopamine was known to act through two dopamine receptors, D1 and D2 receptors (Brown and Markman, 1972; Kebabian and Calne, 1979). This, so called pharmacological classification of dopamine receptors, uses ligands which have high specificity to each dopamine receptor. However, introduction of molecular biological techniques in late 1980s revolutionized dopamine receptor works. D_2 and D_1 dopamine receptors were cloned in 1988 and 1990, respectively (Bunzow *et al.*, 1988; Dearry *et al.*, 1990; Sunahara *et al.*, 1990; Zhou *et al.*, 1990), followed by $\backsim_3$, D_4, D_5 (or D_{1B}) dopamine receptors (Sokoloff *et al.*, 1990; Sunahara *et al.*, 1991; Van Tol *et al.*, 1991).

Among these dopamine receptors, D_4 dopamine receptor is quite interesting in that its mRNA is mostly distributed in frontal cortex and limbic system (Van Tol *et al.*, 1991). These regions are believed to be important in the pathogenesis of schizophrenia. Also D_4 dopamine receptor has high affinity to clozapine which is efficient on treatment-resistant schizophrenics (Kane *et al.*, 1988; Durcan *et al.*, 1995).

We have been interested in brain dopamine system and we tried to raise antibodies against dopamine receptors. For this, we used the strategy to express the 3rd cytoplasmic loop of dopamine receptors in a fusion protein with glutathione-S-transferase (GST, Smith and Johnson, 1988). We could successfully express the entire 3rd cytoplasmic loops of D_2 and D_3 dopamine receptors, but not that of D_4 dopamine receptor. This, we thought, was interesting in that the expression level of D_4 dopamine receptor was far lower than those of D_2 or D_3 receptors even in mam-

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malian cell lines (Van Tol *et al.*, 1991; O'Mally *et al.*, 1992).

Thus, in this study, we assumed that certain sequences in the 3rd cytoplasmic loop of D_4 dopamine receptor prevent or lower the expression of glutathione-S-transferase/the 3rd cytoplasmic loop of D_4 dopamine receptor (GST-I_3D_4) in the bacteria, and tried to locate those particular sequences. For this, we made sequential GST-I_3D_4 constructs by PCR and tested their bacterial expression.

MATERIALS AND METHODS

Bacterial expression of 3rd cytoplasmic loop of D_2 and D_3 dopamine receptors

The whole 3rd cytoplasmic loops of D_2 and D_3 dopamine receptors were PCR amplified using primers shown in Table I from cDNAs of D_2 and D_3 dopamine receptors. PCR products were digested with *Bam*HI/*Eco*RI, and subcloned into pGEX-2T (Pharmacia). Saturated bacterial culture was diluted 100 times and grown for two hours. Protein expression was induced by adding IPTG (final concentration, 0.5 mM), and was confirmed on SDS-PAGE. Couple of expressing clones were selected and were sequenced.

Bacterial expression of 3rd cytoplasmic loop of D_4 dopamine receptor

The 3rd cytoplasmic loop of D_4 dopamine receptor was bacterially expressed in a fusion protein with glutathione-S-transferase (Smith and Johnson, 1988). Since the D_4 dopamine receptor is highly expressed in the heart tissue (Van Tol *et al.*, 1991; O'Malley *et al.*, 1992), mRNA was prepared from whole rat heart tissue using RNA preparation kit (Invitrogen). First strand cDNA was synthesized using a primer specific for D_4 receptor (TGCACAGGCTTGG) with cDNA cycle kit (Invitrogen).

PCR primers were chosen for the whole 3rd cytoplasmic loop of D_4 dopamine receptor (Primer a vs. f, Table I and Fig. 2). For bacterial expression of fusion proteins, exactly the same procedure was followed as in those of D_2 and D_3 dopamine receptors.

Table I. Primers used for PCR amplification of the 3rd cytoplasmic loops of dopamine receptors

1. D_2 receptor
TTTTGGATCCAGCAGTCGAGCTTTCAGAGCCAA
AAAAGAATTCTCACTTCTGCTGGGAGAGCTTTCTGCG

2. D_3 receptor
TTTTGGATCCCAGAACAGCCAGTGCATCAGT
TTTTGAATTCTCAAACCTCTAAGCTGAGCTTGGG

3. D_4 receptor
a. TTTTGGATCCGCCGCTTTCCGTGGCTTGCGGCG
b. TTAAGGATCCTGGGAGGCAGCCCGGCACACCAAG
c. ATATGGATCCCCGCGCCGACCCAGCGGCCC
d. TTAAGGATCCGTGTCGGACCCTACTCAGGGTCCC
e. AAAAGAATTCTCATGCATCAGGGAGCAGACACCCGGG
f. AAAAGAATTCTCATCCAGTGATCTTGGCGCCTCTCTT

*underlineds represent restriction sites.

RESULTS AND DISCUSSION

Bacterial expression of the whole 3rd cytoplasmic loops of D_2, D_3, and D_4 receptors

Dopamine receptors share high homology with other G protein-coupled receptors in transmembrane regions. Meanwhile, the 3rd cytoplasmic loops and carboxy terminals are relatively long and have distinct sequences among G protein coupled receptors. D_2, D_3, D_4 receptors which belong to D2 receptor family have

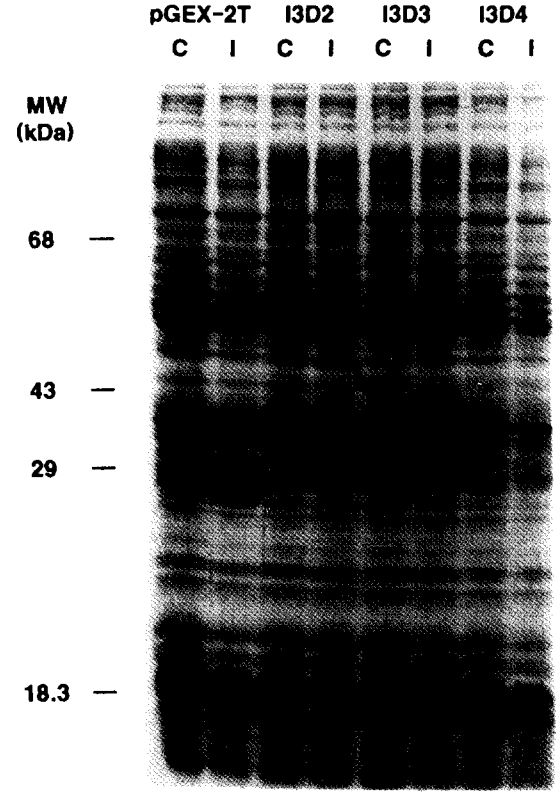

Fig. 1. Bacterial expression of GST fusion proteins of 3rd cytoplasmic loops of D_2, D_3, and D_4 receptors. Clones which have correct sequences were cultured overnight. Next morning, cultures were 100 times diluted and cultured for 2 hours. Proteins were induced by adding IPTG at 0.5 mM final concentration for 2 hours. Cells were harvested by centrifugation and were resuspended in SDS sample buffer. After boiling for 5 min, cell extracts were loaded onto 10% SDS PAGE. 'C' represents 'control' and 'I' represents 'induced'. Arrows represent proteins induced by IPTG.

long 3rd cytoplasmic loops but short carboxy terminals (Bunzow *et al.*, 1988; Sokoloff *et al.*, 1990; Van Tol *et al.*, 1991). With these reasons, the 3rd cytoplasmic loop is the good target to raise specific antibodies for each subtype of dopamine receptors.

We tried to express the whole 3rd cytoplasmic loops of D_2, D_3, D_4 receptors in the bacteria in the form of fusion proteins with glutathione-S-transferase (Smith and Jhonson, 1988; Kim *et al.*, 1995). Fig. 1 shows the bacterially expressed proteins on the SDS-PAGE. The vector itself, pGEX-2T (Pharmcia), which contains sequences for GST and linker portion, produces about 27.5 kDa protein when bacteria was induced by IPTG. The fusion proteins between GST and 3rd cytoplasmic loops of D_2, D_3 receptors produced proteins with expected molecular weight of 45 and 40 kDa, respectively.

Because of the difficulties in cloning cDNA of D_4 receptor (Van Tol *et al.*, 1991; O'Mally *et al.*, 1992), we decided to amplify the 3rd cytoplasmic loop of D_4 receptor directly from tissue. Since rat D_4 receptor is known to be highly expressed in heart (O'Mally *et al.*, 1992), we prepared mRNA from rat heart tissue. After synthesizing single-stranded cDNA from mRNA prepared, 3rd cytoplasmic loop was PCR amplified using primer a and f shown in Table I (DMSO was added up to 10% of PCR reaction to compensate high G-C contents). After transformation, 5 clones were selected and exactly the same procedures as in D_2 and D_3 receptors, were followed to pick out clones which express fusion proteins. However, unlike D_2 and D_3 receptors, none of these clones expressed fusion protein of GST/3rd cytoplasmic loop of D_4 receptors (construct A in Fig. 2 & 3, expected molecular weight, 38 kDa). This was not due to PCR mistakes because the sequence was identical with previously reported one. Because D2 like receptors other than D_4 receptor were successfully expressed (Fig. 1), it was very likely that certain parts of D_4 receptor prevent bacterial expression of fusion protein. Therefore, we decided to locate regions in the 3rd cytoplasmic loop of D_4 receptor which suppress the bacterial expression of this construct.

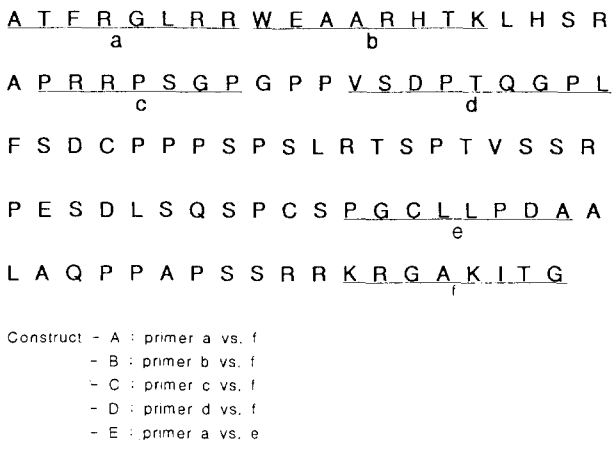

Fig. 2. The amino acid sequences of 3rd cytoplasmic loop of D_4 dopamine receptor. Underlineds represent the regions where primers were made. a~f are names of primers used for PCR amplification. The first amino acid (alanine) is the 208th amino acid from the amino terminal.

Localization of certain sequences in the 3rd cytoplasmic loop of D_4 receptors which inhibit bacterial expression

Several shortened constructs were made from construct A in order to locate sequences which inhibit the bacterial expression of the GST-3rd cytoplasmic loop of D_4 receptors (Fig. 2). To roughly determine which part (5' or 3') of the 3rd cytoplasmic loop inhibits bacterial expression, two constructs in which either 5' or 3' part was shortened were made (constructs D and E, respectively). As shown in Fig. 3, construct E in which 20 amino acids on carboxy terminal side of the 3rd cytoplasmic loop were deleted from construct A (Fig. 2, calculated MW, 35.9 kDa) did not express fusion protein. On the other hand, construct D in which 31 amino acids in amino terminal side were deleted from construct A (calculated MW, 34.45 kDa) was well expressed. These results suggest that it is the certain sequence in amino terminal side of the 3rd cytoplasmic loop that inhibits protein expression.

Based on these results, two more constructs were

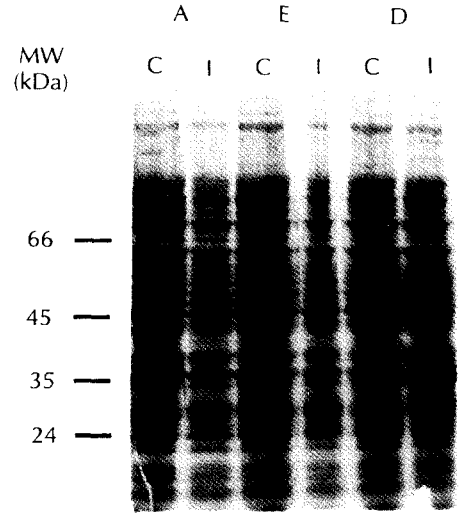

Fig. 3. Bacterial expression of GST fusion proteins of 3rd cytoplasmic loops of rat D_4 dopamine receptor. A, E, and D represent constructs made from the 3rd cytoplasmic loop of rat D_4 receptor as defined in Fig. 2. For the preparation of samples and running gel, exactly the same procedures were followed as in Fig. 1. Arrow represents fusion protein induced by IPTG.

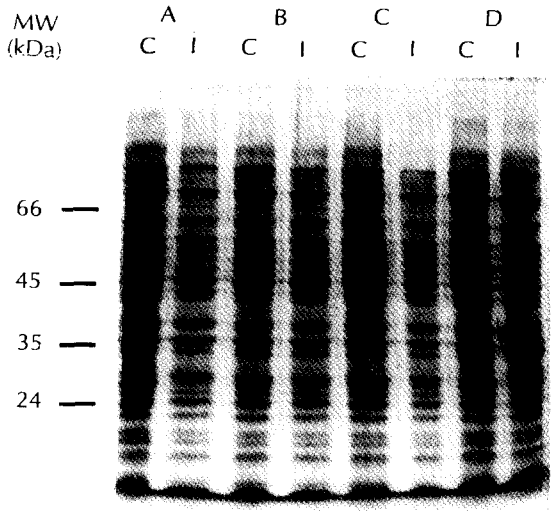

Fig. 4. Bacterial expression of GST fusion proteins of 3rd cytoplasmic loops of rat D_4 receptor. A, B, C, D represent constructs defined in Fig. 2. For the preparation of samples and running gel, exactly the same procedures were followed as in Fig. 1. Arrow represents fusion protein induced by IPTG.

made to locate the particular sequences more precisely by giving two more primers between primer a and d. Construct B in which 8 amino acids in amino terminal side were deleted from construct A (calculated MW 37 kDa) did not express proteins. Construct C in which 13 amino acids in amino terminal side were deleted from the construct B still did not express proteins. These results suggest that certain sequences within primer C or between primer C and D inhibit bacterial expression of fusion protein. Furthermore, this region seems to be toxic to the cells in that whole protein content was reduced when cells were treated with IPTG (Fig. 4).

Using 12 amino acids sequences as a probe, HTKLHSRAPRRP, the SwissProtein bank was searched. Seveal sequences showed some homology with the given 12 amino-acid sequence. Among these, some proteins from virus such as human immunodeiciency virus type 2 GAG Polyprotein (Core proteins p16 and p26, homology in RAPRR) and vaccinia virus minor core protein (protein V, homology in APRR) showed homologies in particular regions. Also yeast polymixin B resistance protein showed a high homology to these regions (homology in RAPRR).

Further systemic studies are needed to locate the exact sequence which inhibits bacterial expression and to test whether this has some thing to do with low level of expression in mammalian cell lines. But still it could be a good idea to avoid these sequences when we design constructs for bacterial expression of proteins.

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