C-terminal Truncation Mutant of the Human β_2-adrenergic Receptor Expressed in *E. coli* as a Fusion Protein Retains Ligand Binding Affinity

Jin Chul Shin[1], Sang Derk Lee[2], Chan Young Shin[2], Sang Bong Lee[3] and Kwang Ho Ko[2]*

[1]*Research Institute of Dong Shin Pharmaceutics, Seoul 135-280, Korea*

[2]*College of Pharmacy and Institute for Molecular Biology and Genetics, Seoul National University, Seoul 151-742, Korea*

[3]*Lab. of Cell Signaling, National Institute of Health, Bethesda, MD 20982, U.S.A.*

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Abstract—To investigate whether human β_2-adrenergic receptor devoid of the C-terminal two transmembrane helices retain its ligand binding activity and specificity, 5' 780-bp DNA fragment of the receptor gene which encodes amino acid 1-260 of human β_2-adrenergic receptor was subcloned into the bacterial fusion protein expression vector and expressed as a form of glutathione-S-transferase (GST) fusion protein in *E. coli* DH5α. The receptor fusion protein was expressed as a membrane bound form which was verified by SDS-PAGE and Western blot. The fusion protein expressed in this study specifically bound β-adrenergic receptor ligand [^{3}H] Dihydroalprenolol. In saturation ligand binding assay, the K_d value was 7.6 nM which was similar to that of intact β_2-adrenergic receptor in normal animal tissue (K_d=1~2 nM) and the B_{max} value was 266 fmol/mg membrane protein. In competition binding assay, the order of binding affinity of various adrenergic receptor agonists to the fusion protein was isoproterenol≫epinephrine≧norepinephrine, which was similar to that of intact receptor in normal animal tissue. These results suggest that N-terminal five transmembrane helices of the β_2-adrenergic receptor be sufficient to determine the ligand binding activity and specificity, irrespective of the presence or absence of the C-terminal two transmembrane helices.

Keywords□ [^{3}H]DHA, *E. coli*, β_2-adrenergic receptor, fusion protein, C-tetminal truncation mutant, binding activity

For the study of many receptor-mediated transmembrane signal transduction, the receptors for the catecholamines especially, β-adrenergic receptor was regarded as an excellent model system. The cloning and sequence determination of the β-adrenergic receptor protein shows that this receptor has seven hydrophobic stretches of 20-25 amino acids, which might form seven transmembrane helices connected by alternating extracellular and intracellular loops (Mashida *et al.*, 1990).

A lot of biochemical evidences suggest that no single transmembrane domain is the dominant contact site for the ligand binding and that determinants of the subtype specific ligand binding are found on several membrane spanning domains (Dohlman *et al.*, 1991; Lefkowitz *et al.*, 1988).

The ligand-binding site for the β-adrenergic receptor is suggested to be buried deep beneath the surface of the receptor (Tota *et al.*, 1990). The amino-terminus and carboxyl-terminus, the hydrophilic loop regions of β-adrenergic receptor could be truncated or removed without affecting the ability to bind either the agonist isoproterenol or the antagonist iodocyanopindolol (ICYP), therefore these regions within the hydrophilic loops of the a-drenergic receptor seem not to be the essential regions either for protein folding, membrane insertion or ligand binding (Parker *et al.*, 1991). It was also shown that the extensive trypsinization of the purified adrenergic receptor which also has the seven transmembrane helices, yields a hydrophobic core which has not the hydrophilic loop regions. This hydrophobic core has the intact normal ligand-binding activity (Wilson *et al.*, 1990). Among the entire seven transmembrane helices, the first five helices are suggested to be important in ligand binding in several recent experiments. Single amino acid substitution of the conserved residues in the transmembrane domain of the β-adrenergic receptor with other amino acid residues have suggested that Ser 204, Ser 207 are important in ligand binding and the side chain of the Asp 113 in the third transmembrane helix is the counterion for the amine

* To whom correspondence should be addressed.

groups of β-adrenergic agonists and antagonists (Strader *et al.*, 1989a, b; Dixon *et al.*, 1988).

In a recent day, the human β_2-adrenergic receptor gene was expressed as a fusion protein form in *E. coli* with intracellular-galactosidase (Marullo *et al.*, 1988), lam B gene of *E. coli* K12 which encodes an outer membrane protein (Chapot *et al.*, 1990). All of the expressed fusion protein of receptor retained their ligand binding activities and pharmacological specificities. These results suggest that the membrane environment required by human β_2-adrenergic receptor for ligand binding activity is conserved in bacteria and that bacterial and eukaryotic membrane-embedded receptors might share a common transmembrane organization (Chapot *et al.*, 1990; Marullo *et al.*, 1988). These results suggest that for the molecular biological study of receptor ligand interaction, *E. coli* system can be used instead of eukaryotic expression system. *E. coli* cells do not express β-adrenergic receptors and G proteins, so regarding background noise, *E. coli* system seems to be superior to eukaryotic expression system.

Although relative importance of transmembrane helical regions of β_2-adrenergic receptor was suggested in many reports, there is no clear demonstration whether N-terminal five transmembrane helices of β_2-adrenergic receptor out of entire seven transmembrane helices is sufficient for the receptor ligand interaction. In the present study, C-terminal two transmembrane helices truncated form of human β_2-adrenergic receptor which contained only N-terminal five transmembrane helice were expressed in *E. coli* DH5α as a glutathione-S-transferase (GST) fusion protein (Hakes *et al.*, 1992; Guan *et al.*, 1991). The effects of this truncation mutation on receptor ligand binding specificities and selectivities were investigated.

Materials and Methods

Materials

Restriction enzymes and other enzymes used in molecular cloning were purchased from Promega (Madison, WI, U.S.A.). Isopropyl-β-D-thio-galactopyranoside (IPTG) was purchased from Boehringer Mannheim (Mannheim, Germany). Prestained electrophoresis molecular weight standards (myosin, phosphorylase B, bovine serum albumin, ovalbumin, carbonic anhydrase, β-galacto globulin, lysozyme) and electrophoresis reagents were obtained from GIBCO-BRL (Gaithersberg, MD, U.S.A.) or Sigma chemicals (St. Louis, MO, U.S.A.). Horse radish peroxidase labelled goat anti-mouse IgG antibody was obtained from Pierce (Rockford, IL, U.S.A.). Sepharose CL-4B was obtained from Pharmacia (Uppsala, Swedden) or from Sigma chemicals (St. Louis, MO, USA). All other chemicals were obtained from Sigma Chemicals and Pharmacia (Uppsala, Swedden) and were highest commertially available grade.

Construction of expression vector pGEXβN

Human β_2-adrenergic receptor gene was amplified by PCR and subcloned into pBS-KS resulting in a cloning vector pBS Ad. This plasmid was digested with BamHI and BglII restriction enzymes. The DNA fragment encodes amino acids 1-260 of the human β_2-adrenergic receptor devoid of the last C-terminal two transmembrane helices. Expression vector pGEX-CS was digested with BamHI and 5'-terminal phosphate residues were removed by digestion with alkaline phosphatase. The receptor gene fragment was ligated with the dephosphorylated pGEX-CS with bacteriophage T4 ligase. The resulting β_2-adrenergic receptor expression vector was named as pGEXβN.

Transformation and expression of fusion protein

Competent *E. coli* DH5α was transformed with Heat Shock Method (Maniatis *et al.*, 1989). A single colony was grown overnight in 4 m*l* LB media containing 50 μg/m*l* ampicillin, 1% glucose. Overnight cultures of transformed cells were diluted 1:100 in 400 m*l* of fresh medium containing 50 μg/m*l* ampicillin, 0.2% glucose and were grown for additional 4 hours until O.D. 250 value of 0.5 was reached. After addition of 0.3 mM IPTG, the culture was incubated further for 4 hours for the maximum induction of the fusion protein.

Identification of the fusion protein expression

Expression vector pGEXβN was identified by the restriction analysis. After the culture was harvested, plasmid DNA was prepared and digested with BamHI and EcoRI simultaneously and resulting fragment profile was analyzed by 1% agarose gel electrophoresis. For the identification of the expression of fusion protein, equivalents to 100 μ*l* culture of *E. coli* cell membrane fractions transformed with pGEXβN were electrophoresed, followed by staining with Coomassie brilliant blue. For Western blot analysis, the protein bands were electrically transferred to nitrocellulose membrane and treated with monoclonal anti-GST antibody and probed with HRP-labelled goat anti-mouse IgG antibody.

Purification of the fusion proteins

IPTG induced culture of *E. coli* was harvested and

resuspended in 10 m*l* of STE buffer (150 mM NaCl, Tris·HCl, 1 mM EDTA, pH 8.0) containing 5 mM dithiothreitol(DTT), 10 μg/m*l* trasylol, 10 μg/m*l* leupeptin, 1 mM phenylmethylsulphonylfluoride (PMSF). Anionic detergent N-lauroylsarcosine was added to the final concentration of 1.5%. After sonication, the solution was incubated for 10 minutes on ice and was centrifuged for 10 minutes at 40,000×g. The supernatants containing solubilized fusion proteins were adjusted to 2% Triton X-100, vortexed and incubated with glutathione-Sepharose CL-4B. After washing with phosphate buffered saline (PBS) containing 0.1% Triton X-100, the fusion proteins were eluted with STE buffer containing 10 mM reduced glutathione and the fractions were analyzed by SDS-PAGE.

Preparation of *E. coli* crude membranes for ligand binding study

Transformed *E. coli* cells were harvested and resuspended in 20 m*l* buffer (20 mM Tris, 5 mM EDTA, pH 7.4). Lysozyme and DNase I were added to a final concentration of 0.5 mg/m*l* and 30 μg/m*l* respectively and incubated for 30 minutes on ice. After sonication, unbroken cells were removed by centrifugation of the suspension at 1000×g for 10 minutes at 4°C. The supernatants were centrifuged at 40000×g for 30 minutes and the resulting pellet was resuspended in buffer (50 mM Tris, 5 mM EDTA, pH 7.4, 10 μg/m*l* trasylol, 10 μg/m*l* leupeptin) and again centrifuged. The final pellets were resuspended in the same volume of buffer and were used for the receptor ligand binding studies.

Saturation ligand binding assay

Saturation ligand binding assay was performed in triplicate, in a volume of 0.5 m*l* containing 100 μg of the crude membrane fraction in 50 mM Tris, 5 mM EDTA pH 7.4. The concentration of radioligand [^{3}H]Dihydroalprenolol was varied from 0.2 nM to 40 nM. After incubation for 30 minutes at 23°C with vigorous shaking, receptor-ligand complex was separated from free ligands through 0.3% polyethyleneimine pretreated Whatman GF/B filters by the vacuum filtration procedure. The filters containing the receptor-ligand complex were dried, suspended in 10 m*l* of toluene based scintillation fluid cocktail and counted by liquid scintillation counter. Non-specific binding was defined as the counting value not inhibited by 10 μM *dl*-propranolol. The saturation binding assay data were analyzed by iterative curve fitting computer program 'LIGAND'(Munson *et al.*, 1980).

Competitive ligand binding assay

Competitive binding assay was performed in a volume of 0.5 ml containing 200 μg membrane protein fraction, 1 mM ascorbate, 10 nM [^{3}H]Dihydroalprenolol, various competing drug 1 μM, 10 μM and 100 μM respectively in 50 mM Tris, 5 mM EDTA, pH 7.4. The competing drugs used were l-isoproterenol, epinephrine and norepinephrine as agonists and alprenolol and *dl*-propranolol as antagonists. The competitive ligand binding assay data were compared with each drug by the inhibition % of the [^{3}H]Dihydroalprenolol binding to the receptor by competing drugs. In comparing the results the inhibition % by 100 μM alprenolol was regarded as 100% inhibition.

Determination of protein

Content of protein was estimated by the procedure of Bradford (1976), using Bovine Serum Albumin as the standard.

Results

The fusion protein including N-terminal five transmembrane helices of the human β_2-adrenergic receptor was obtained from the culture of *E. coli* DH5α transformed with the expression vector pGEXβN. The molecular weight of the fusion protein was calculated to be 57 kDa from primary sequence. In SDS-PAGE, the fusion protein band was detected at 57 kDa (Fig. 1). Also, in Western blot, 57

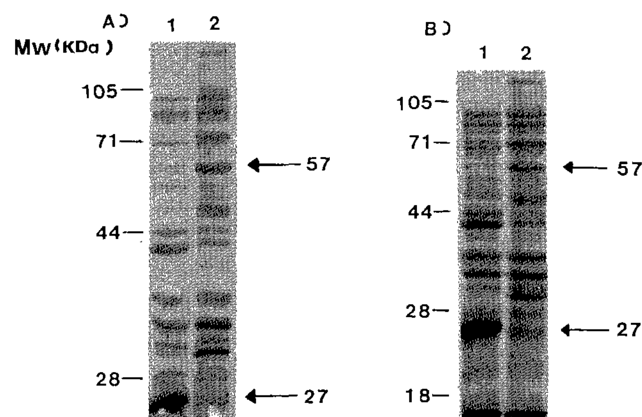

Fig. 1. Identification of β_2-adrenergic receptor-GST fusion protein expression.
by SDS-PAGE ; *E. coli* DH5 transformed with pGEX-CS or pGEXβN were grown in LB broth containing 0.2% glucose until O.D. 250 of 0.5 was reached. The culture was induced with 0.3 mM IPTG for 4 hours at 37°C. 100 μl equivalents of induced cells were treated with 2 X SDS sample treatment buffer and electrophoresed. (a) 10% gel (b) 12 % gel. lane 1; pGEX-CS, lane 2; pGEXβN. A 57 kDa of β-adrenergic receptor-GST fusion protein band was indicated by an arrow.

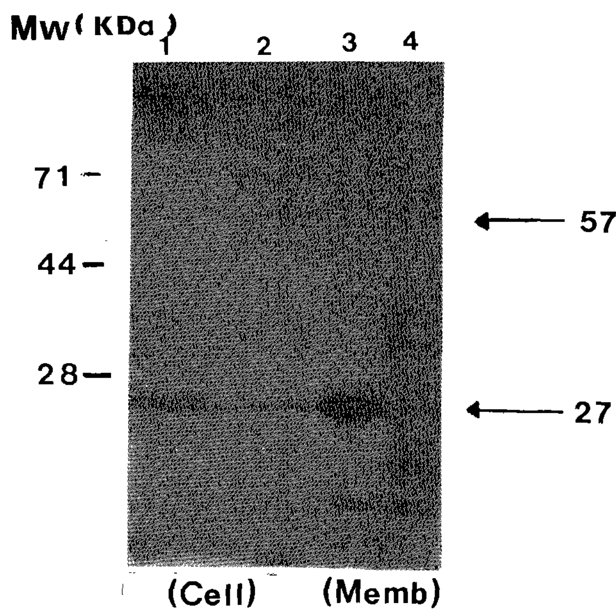

Fig. 2. Identification of β_2-adrenergic receptor-GST fusion protein expression. by Western blot; *E. coli* DH5 transformed with pGEX-CS or pGEXβN were grown in LB broth containing 0.2% glucose until O.D. 250 of 0.5 was reached. The culture was induced with 0.3 mM IPTG for 4 hours at 37°C. The whole cell (lane 1, 2) and crude membrane preparation (lane 3, 4) of control and fusion protein expressing cell were electrophoresed and the protein bands were electrically transferred to nitrocellulose membrane. The strips were incubated with monoclonal anti-GST antibody for 1 hours at room temperature and probed with HRP-labelled goat anti-mouse IgG. lane 1, 3; pGEX-CS, lane 2, 4; pGEXβN. A 57 kDa of β-adrenergic receptor-GST fusion protein band was indicated by an arrow.

kDa band was identified (Fig. 2). There also exist bands of lower molecular weight supposed to be the degradation fragments of the receptor-GST fusion protein.

The standard protocol for isolation of GST fusion protein relies on the lysis of bacteria with a nonionic detergent such as Triton X-100. This procedure works quite well for soluble GST fusion proteins. But in this study receptor-GST fusion protein was not solubilized with Triton X-100 alone. Of the many detergents we tried, only the anionic detergent N-lauroylsarcosine successfully solubilized the fusion protein. The purified fusion protein fraction was loaded on 14% polyacrylamide gel and was identified as a band of molecular weight of 57 kDa (Fig. 3).

When the ligand binding assay was performed with the whole cell, the presence of ligand binding site was identified. When the ligand binding assay was performed with crude membrane fraction, cytosol and culture supernatant fraction respectively, the receptor ligand bind-

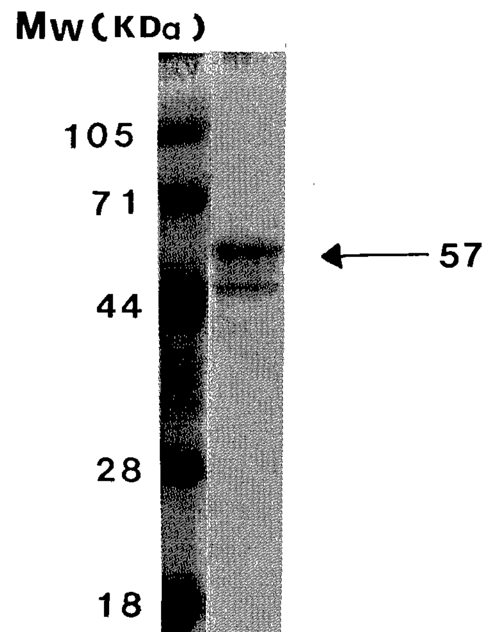

Fig. 3. Purification of human β_2-adrenergic receptor-GST fusion protein. Crude membrane fraction of *E. coli* culture transformed by pGEXβN was solubilized with 1.5% N-lauroylsarcosine. The soluble fraction was adjusted with Triton X-100 to the final concentration of 2% in phosphate buffered saline (PBS) and incubated with glutathione-agarose affinity gel for 30 minutes. The mixture was washed three times with 0.1% Triton X-100 in PBS. 10 μl portion of affinity matrix was treated with 2 X SDS sample buffer and electrophoresed. A 57 kDa of β-adrenergic receptor-GST fusion protein band was indicated by an arrow.

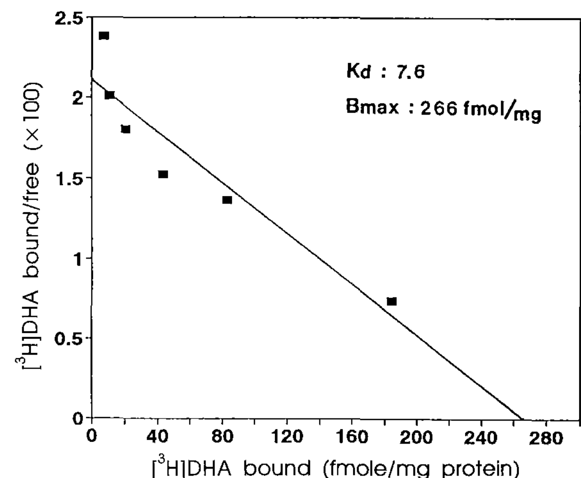

Fig. 4. Scatchard Analysis of [^{3}H]DHA binding to the *E. coli* crude membrane preparation transformed with pGEXβN. 100 μg crude membrane preparation was incubated with [^{3}H]DHA for 30 minutes at 23°C. [^{3}H]DHA concentration was varied up to 40 nM. After incubation, the bound and the free form of the radioligand were separated by rapid vacuum filtration. The data were analyzed by iterative curve fitting computer program 'LIGAND'. The experiments were repeated three times and other experiments gave similar results.

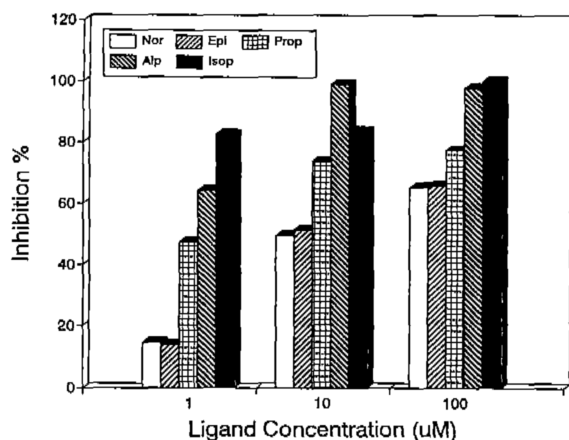

Fig. 5. Inhibition of the [^{3}H]DHA binding to the *E. coli* crude membrane preparation which was transformed with pGEXβN by various β-adrenergic agonists and antagonists. 200 μg crude membrane preparation of *E. coli* was incubated with 10 nM [^{3}H]DHA, competing drug 1 μM, 10 μM and 100 μM respectively for 30 minutes at 23°C. The competing drugs used were isoproterenol, epinephrine and norepinephrine as agonists and alprenolol and propranolol as antagnists. After incubation, the bound and the free form of the radioligand were separated by rapid vacuum filtration. The experiments were done in triplicate and each bar represents the mean±S. D. (n=3).

ing activity was localized only at the crude membrane fraction. In Scatchard analysis of saturation binding assay, the Kd value was 7.6 nM and the Bmax value was 266 fmole/mg membrane protein (Fig. 4).

The presence of ligand binding specificity of the receptor fusion protein was identified by competitive ligand binding assay. The order of affinity of various adrenergic agonists to the receptor-GST fusion proteins was almost similar to that of the intact β_2-adrenergic receptor in normal animal tissue. In case of agonists, the affinity order was isoproterenol≫epinephrine≧norepinephrine and in case of antagonists, the order was alprenonlol≧propranolol (Fig. 5).

Discussion

In the present study, human β_2-adrenergic receptor devoid of the C-terminal two transmembrane helices was expressed as a fusion protein form with GST in *E. coli.* The expression of the receptor-GST fusion protein as a membrane protein was identified by SDS-PAGE, Western blot and purification by glutathione affinity chromatography. The ligand binding activity of the fusion protein was identified by ligand binding assay using [^{3}H] DHA as a radioligand. The localization of the active β_2-adrenergic receptor-GST fusion protein was identified in the crude membrane fraction. The inner membrane probably seems to be the target site of the receptor-GST fusion protein. Contrast to the outer membrane, the bacterial inner membrane is phospholipid bilayer like the eukaryotic plasma membrane (Marullo *et al.*, 1988). Therefore, most parts of the β_2-adrenergic receptor-GST fusion protein seem to be folded correctly in the membrane and this bacterial membrane-embedded receptor protein might share a common transmembrane organization with that of eukaryotic cell (Chapot *et al.*, 1990; Marullo *et al.*, 1988). The ligand binding affinity of the receptor-GST fusion protein (K_d=7.6 nM) was similar to that of the intact receptor in animal tissue (K_d=1~2 nM). Bmax value (266 fmole/mg protein) seems to be short to represent the total number of the receptor-GST fusion proteins because there may be a partial protein degradation by the *E. coli* proteases, conformational distortion or disability of insertion into the membrane by the effect of GST portion of the fusion protein (Pacaud *et al.*, 1981; Sreedhara *et al.*, 1981). The receptor protein in animal tissue is normally located at the membrane but its fusion with the soluble cytoplasmic GST protein could somewhat inhibit the correct insertion into the bacterial membrane (Marullo *et al.*, 1988).

Western blot revealed that the receptor-GST fusion protein might be degraded into several fragments by some proteases in the bacterial cell. Since several of these enzymes have been found in the cytoplasm and the inner membrane, such proteolytic activity is presumably responsible for the complex degradation pattern observed in Western blot with monoclonal anti-GST antibody (Pacaud *et al.*, 1981, Hsien-Yu *et al.*, 1991).

In bacterial cell, the procedure of the co-, post-translational processing of protein does not exist, but the lack of glycosylation and palmitoylation does not seem to have dramatic effects on the ligand binding or functional activity of the β-adrenergic receptor expressed in the form of fusion protein. In case of the eukaryotic cell, blocking of receptor glycosylation, either by inhibition of glycosylating enzymes in the cell or by site-directed mutagenesis of the possible glycosylation or palmitoylation site in the sequence of β_2-adrenergic receptor (Rands *et al.*, 1990) seems to have almost no effects on the ligand binding activity when expressed in the plasma membrane (Rands *et al.*, 1990; Marullo *et al.*, 1988).

The ligand binding specificity of the fusion protein

was investigated by the competitive ligand binding assay. In this study, the sixth and seventh membrane region of the β_2-adrenergic receptor was truncated, but the order of the affinity of the agonists to the β_2-adrenergic receptor-GST fusion protein was isoproterenol$\gg$epinephrine$\geqq$ norepinephrine, which was similar to that of the intact β_2-adrenergic receptor in tissue (U'Prichard *et al.*, 1978). These results suggest that N-terminal five transmembrane region of the β_2-adrenergic receptor is largely involved in the determination of the ligand binding specificity.

In summary, the present study shows that the C-terminal two transmembrane helices truncated form of human β_2-adrenergic receptor which was expressed as GST fusion protein in *E. coli* retain its ligand binding activity and specificity. The region of the N-terminal five transmembrane helices of human β_2-adrenergic receptor is supposed to be sufficient determinant of the receptor ligand binding activity and specificity, irrespective of the absence of the C-terminal two transmembrane helices.

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