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Production and Characterization of a Monoclonal Antibody against Human β_2-adrenergic receptor

Suk-jo KANG, Chan Young SHIN, Mi-ryoung SONG, Chung Jae LEE, Jae Hoon CHEONG[1], Sang Bong LEE[2] and Kwang Ho KO*

College of Pharmacy and Institute for Molecular Biology and Genetics, Seoul National University, Center for Biofunctional Molecules, POSTECH, Korea

[1]Department of Pharmacy, Samyook University, Seoul 139-742, Korea

[2]Cell Cycle and Signal Transduction Research Unit, Korea Research Institute of Bioscience and Biotechnology, Taejon 305-600, Korea

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Abstract – The purpose of the present study was to produce and characterize a monoclonal antibody against human β_2-adrenergic receptor. Male BALB/c mice were immunized with glutathione S-transferase (GST) fusion protein of the C-terminal portion of the human β_2-adrenergic receptor which was expressed in E.Coli. The immunized splenocytes were fused with myeloma SP2/0-Ag14 cells. The resulting hybridomas were screened for the production of a monoclonal antibody which can recognize human β_2-adrenergic receptor, and then subcloned by limiting dilution. The resulting monoclonal antibody was named as mAbβCO2. The monoclonal antibody βCO2 was determined as IgM subtype and then purified by anti-mouse IgM-agarose affinity chromatography. The results of ELISA, Western blot, and immunocytochemistry showed that mAbβCO2 recognized human β_2-adrenergic receptor in the β_2-adrenergic receptor-GST fusion protein and human epidermoid carcinoma cell line A431 with highly specific immunoreactivity. The monoclonal antibody βCO2 may provide useful tools for the study of the β-adrenergic receptor of human and other species including rats.

Keywords □ human β_2-adrenergic receptor, monoclonal antibody, fusion protein, cross-reactivity.

The analysis of membrane receptors for for hormones and neurotransmitters has progressed considerably through the use of specific antibodies (Bahouth *et al.*, 1991). In spite of advances in the purification and molecular characterization of receptors, the extremely low tissue concentration of physiologically relevant receptor molecules still make it laborious and tedious to elucidate the nature of receptors by pharmacological and biochemical methods. Thus, in order to further characterize receptors, it was of interest to raise anti-receptor antibody (Moxham *et al.*, 1988; Theveniau *et al.*, 1989; Wang *et al.*, 1989a, 1989b; Weiss *et al.*, 1987). For the production of anti-receptor antibody, partially purified receptors, synthetic peptide, or bacterially expressed receptor molecules could be used as immunogens (Moxham *et al.*, 1986; Strader *et al.*, 1987; Levey *et al.*, 1990). However, considering the low concentration of receptors in tissue or cells and the diversity and intensity of the antibody response, bacterially expressed receptor molecules seems to be the immunogens of choice. Among various bacterial expression system, glutathione-S-transferase (GST) fusion protein system is used extensively for high level expression and rapid purification of the fusion proteins by glutathione-agarose affinity chromatography under non-denaturing conditions (Smith and Johnson, 1988; Guan and Dixon, 1991, Frangioni and Neel, 1993). It has been reported that bacterial expression of human muscarinic and dopamine receptor using GST system and generation of anti-receptor antibody using the fusion protein were successful (Levey *et al.*, 1990; 1993).

Many physiological processes are regulated by catecholoamines via their interaction with β-adrenergic receptors (Stiles *et al.*, 1984). Like other G-protein coupled receptors, β-adrenergic receptors possess a common topological organization of seven transmembrane helices interspersed with hydrophilic extra- and intracellular loops, a glycosylated extracellular amino-terminal region, and a cytoplasmic carboxyl-terminal tail. In particular, the in-

*To whom correspondence should be addressed.

tracelluar cytosolic domain has been considered the binding site of G-protein and an important regulatory site. This hydrophilic domain is relatively heterogeneous in primary amino acid sequences among different receptors. Thus, it seems reasonable to choose this domain to produce monoclonal antibody against β-adrenergic receptor which is expected to be a good tool to determine β-adrenergic receptors.

The purpose of the present study was to produce a monoclonal antibody directed against human β_2-adrenergic receptor carboxylic domain by immunizing BALB/c mice with β_2-adrenergic receptor-GST fusion protein and to characterize its immunological properties.

MATERIALS AND METHODS

Materials

Sepharose CL-4B were obtained from Pharmacia (Uppsala, Sweden). Electrophoresis reagents were obtained from Gibco BRL (Gaithersburg, MD). Restriction enzymes and other enzymes used in molecular cloning were purchased from Promega (Madison, WI). Isopropyl-β-D-thiogalactopyranoside (IPTG) was purchased from Boehringer Mannheim (Mannheim, Germany). Freund's Adjuvants and other immunochemical reagents including horseradish peroxidase-conjugated goat anti-mouse IgG or IgM were purchased from Pierce (Rockford, IL). Reagents for the hybridoma cell culture and myeloma-spleen cell fusion including polyethyleneglycol, HT, HAT media supplement was obtained from Sigma (St. Louis, MO). Sigma ImmunoType™ was purchased from Sigma (St. Louis, MO). Prestained protein molecular weight marker (myosin, phosphorylase B, bovine serum albumin, ovalbumin, carbonic anhydrase, β-lactoglobulin, lysozyme) and iron supplemented calf serum were obtained from Gibco (Gaithersburg, MD). All other reagents were obtained from Sigma Chemicals (St. Louis, MO) and were of the highest grade commercially available.

Expression and purification of the human β_2-adrenergic receptor-GST fusion protein

A 3'-590 bp DNA fragment coding third intracellular loop and cytoplasmic tail region of human β_2-adrenergic receptor was removed with *Bgl*II and *Acc*I from pBSβAd that contains entire cDNA of human β_2-adrenergic receptor. The DNA fragment was ligated with bacteriophage T4 ligase into pGEXAcc that was double digested with *Bam*HI and *Acc*I, which resulted the expression plasmid, pGEXβC. Transformants with pGEXβC were identified by restriction analysis. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot was carried out to confirm the expression of β_2-adrenergic receptor-GST fusion protein. For the protein induction, IPTG was added at a final concentration of 0.5 mM into the overnight cultures of *E. coli* DH5α that was transformed with pGEXAcc or pGEXβC. After a further 4 h of incubation, cells were pelleted and resuspended in 10 ml of ice-cold STE buffer (150 mM NaCl, 10 mM Tris HCl, 1 mM EDTA, pH 8.0) containing 5 mM dithiothreitol (DTT) and 1 mM PMSF. N-lauroylsarcosine was added to a final concentration of 1.5% from 10% stock solution in STE buffer. Cells were sonicated on ice and centrifuged at 4℃ for 20 min at 40,000 × g to remove the cell debris. The supernatant containing solubilized fusion proteins was adjusted to 2% Triton X-100 and incubated with glutathione-Sepharose CL-4B beads for 30 min. The beads were collected by brief centrifugation at 500 × g and washed with phosphate buffered saline (PBS) containing 0.1% Triton X-100 by repeated centrifugation. The fusion proteins were eluted with STE buffer, pH 8.0 containing 10 mM reduced glutathione, and the eluents were analyzed by SDS-PAGE.

Immunization and production of monoclonal antibody

The purified fusion proteins were used as immunogens for BALB/c mice. Male BALB/c mice (8 weeks of age) were immunized by intraperitoneal injections of 0.4 ml of the purified fusion proteins (30 μg/animal) emulsified in an equal volume of Complete Freund's Adjuvant (CFA). Booster immunizations were administered at 3-week interval with Incomplete Freund's Adjuvant (IFA). Three days prior to the spleen and myeloma cell fusion, the animal was injected with 0.4 ml of the purified fusion proteins alone. The monoclonal antibody was produced by the procedure of Köhler and Milstein (1975). Positive hybridomas were screened by ELISA and were cloned by two consecutive rounds of limiting dilution. Among the three clones finally obtained, mAbβCO2 showed strongest immunological signal and was further characterized.

Enzyme-linked Immunosorbent Assay (ELISA)

The purified fusion proteins diluted in PBS were added to wells of microtiter assay plates (Falcon) and allowed to adsorb for 1 h at room temperature. After blocking with 5% nonfat dried milk in PBS plus 0.2% Tween 20 (Blocking solution), the wells were then incubated for

1 h at 32℃ with 50 μl of the supernatant from hybridoma culture which was serially diluted in blocking solution. The wells were then probed with 100 μl of horseradish peroxidase-conjugated goat anti-mouse IgM diluted 1:3,000 in blocking solution for 1 h at 32℃. Each well was developed with 50 μl of soluble substrate solution (0.1 mg/ml 3,3'5,5'-tetramethylbenzidine in 0.1 M sodium acetate, pH 6.0, 0.01% H_2O_2) and the absorbance was read at 450 nm. In some cases, human epidermoid carcinoma A431 cells were cultured on 96-well culture plates and used as antigens. A431 ATCC (human epidermoid carcinoma cell line) cells were cultured in Dulbecco's modified Eagle's meduim (DMEM) supplemented with 10% fetal bovine serum at 37℃ in a 5% CO_2/water-saturated atmosphere. A 431 cells were fixed with 1:1 mixture of acetone and methanol. After extensive washing with PBS, the wells were subjected to ELISA as above.

Immunoblot Analysis

Membrane preparations of A431 cells which naturally express human β_2-adrenergic receptors were subjected to gel electrophoresis and then electrophoretically transferred onto nitrocellulose membrane in methanol/glycine/Tris buffer as described (Towbin *et al.*, 1979). After blocking, the nitrocellulose strips were incubated for 2 h at 32℃ with hybridoma culture supernatants diluted 1:10 in blocking solution. After washing, they were incubated with horseradish peroxidase-conjugated goat anti-mouse IgM diluted 1:3,000 in blocking solution for 1 h at 32℃. Blots were washed and the immunoreactivity was visualized with 3,3'-diaminobenzidine substrate solution (9 mg in 10 ml of 50 mM Tris-HCl, pH 7.5, 0.01% H_2O_2).

RESULTS

Expression and purification of the human β_2-adrenergic receptor-GST fusion protein

The fusion proteins incorporating the C-terminal region of the human β_2-adrenergic receptor were obtained in high yield (more than 10 mg/250 ml) from cultures of *E. coli* DH5α transformed with the pGEXβC as shown in Fig. 1. The fusion protein migrated at apparent molecular weight of 45 kDa, which was predicted from primary sequence. Expression of the fusion protein was further confirmed by Western blot analysis using monoclonal anti-GST antibody (Fig. 1).

The β_2-adrenergic receptor-GST fusion protein was expressed as the form of inclusion body and was not solubilized with Triton X-100. The detergent N-lauroylsarcosine effectively solubilized the fusion proteins and that treatment of Triton X-100 before applying to glutathione agarose affinity matrix resulted in increased and saturable binding to affinity matrix. The purified fusion protein was used as immunogens without further modification.

Preparation and Characterization of Monoclonal Antibody

The monoclonal antibody directed against the human β_2-aderenergic receptor was produced by immunization of BALB/c mice with the purified human β_2-adrenergic receptor-GST fusion protein. The monoclonal antibody produced in this study was named as mAbβCO2. The immunoglobulin subclass of mAbβCO2 was determined as IgM using Sigma Isotying kit (St. Louis, MO). For the purification of mAbβCO2, anti-mouse IgM-agarose affinity chromatography was used. The purified mAbβCO2 consists of 78 and 25 kDa protein bands which were identical with the molecular weight of heavy chain and light chain, respectively (Fig. 2). Western blot made it clear that the 78 kDa protein was a heavy chain, which could be probed with goat anti-mouse IgM (Fig. 2).

For the characterization of mAbβCO2, ELISA and Western blot were performed. In ELISA, mAbβCO2 showed strong and specific immunoreactivity against the β_2-adr-

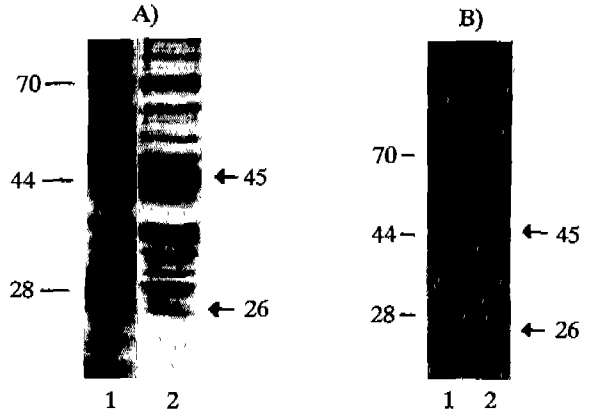

Fig. 1. Identification of the β_2-adrenergic receptor-GST fusion protein expression by A) SDS-PAGE: *E. coli* DH5α transformed with pGEXAcc or pGEXβC were grown in LB broth until OD_{600} of 1 was reached. The cultures were induced with 0.5 mM IPTG for 4 h at 37℃. An aliquot of the culture (100 μl) was electrophoresed on 10% polyacrylamide gel and the protein bands were visualized by staining with Coomassie brilliant blue. B) Western blot: After SDS-PAGE, the protein bands were electrically transferred on nitrocellulose membrane. The strips were incubated with monoclonal anti-GST antibody for 2 h at room temperature and probed with HRP-conjugated goat anti-mouse IgG (1:3,000 dilution). Bands were visualized with diaminobenzidine. lane 1: pGEXAcc, lane 2: pGEXβC.

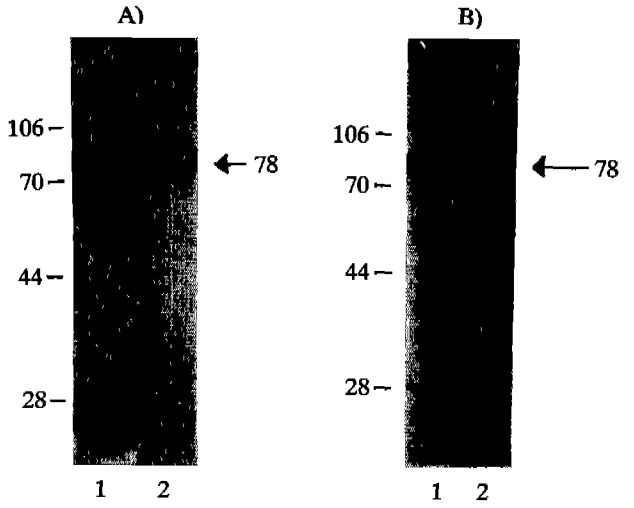

Fig. 2. Purification of mAbβCO2 by immunoaffinity chromatography. Monoclonal antibody producing culture supernatants were precipitated with ammonium sulfate and the precipitates were dialyzed against PBS and were loaded onto anti-mouse IgM-agarose affinity column. After extensive washing with high salt buffer (0.5 M NaCl in PBS), the bound immuno- globulin was eluted with 0.1 M glycin pH 2.5. The eluates were analyzed by A) 10% SDS-PAGE and B) Western blot with HRP-conjugated anti-mouse IgM. A 78 kDa of μ-heavy chain was indicated by an arrow. lane 1: ammonium sulfate precipitates lane 2: purified mAbβC02.

energic receptor-GST fusion protein in a concentration-dependent manner as shown in Fig. 3. It was calculated that mAbβCO2 has an apparent titer of the range of 1: 100 dilution for hybridoma culture supernatant at the antigen concentration of 1 μg/ml. Furthermore, it could recognize the β_2-adrenergic receptor on human epidermoid carcinoma A431 cell membrane as shown in Fig. 3. The specificity of mAbβCO2 for the β_2-adrenergic receptor was further demonstrated by Western blot. As shown in Fig. 4, mAbβCO2 didn't react with the GST which was used as negative control but recognized specifically the human β_2-adrenergic receptor C-terminal fusion protein at 45 kDa. In addition, when A431 cells were subjected to Western blot, a 65 kDa band of human β_2-adrenergic receptor was observed (Fig. 4).

The result of the immunocytochemical localization of the human β_2-adrenergic receptor was shown in Fig. 5. The monoclonal antibody βC02 recognized the human β_2-adrenergic receptors on fixed A431 cells.

DISCUSSION

In the present study, a monoclonal antibody, mAbβCo$_2$,

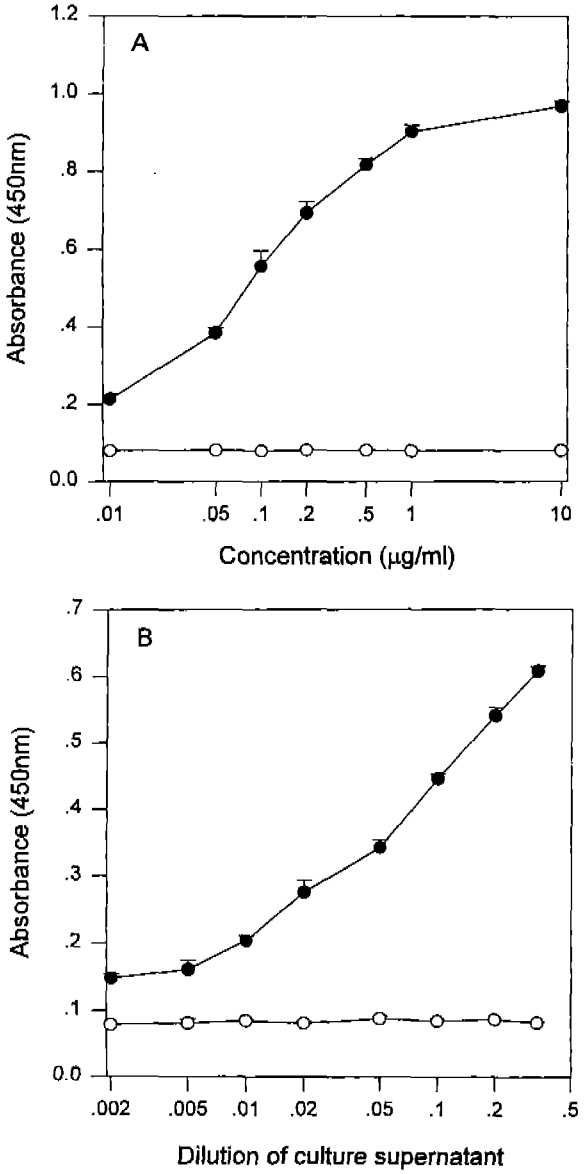

Fig. 3. Enzyme linked immunosorbent assay of the purified mAbβCO2 with the β_2-adrenergic receptor-GST fusion protein. A) Various concentrations of the purified β_2-adrenergic receptor-GST fusion protein or GST were prepared in PBS and 100 μl aliquots of each sample were adsorbed onto 96-well microtiter plates. The plates were incubated with culture supernatants of mAbβCO2 and then probed with HRP-conjugated goat anti-mouse IgM. 3,3',5,5'-tetramethylbenzidine was used for soluble substrate and the absorbance was read at 450 nm. The data shown are the means±S.D. from triplicate determinations. ●: β_2-adrenergic receptor-GST fusion protein, ○: GST B) A431 cells were cultured in 96-well culture plates (4×10^4 cells/well) and fixed with 1:1 mixture of acetone and methanol. The plates were incubated with culture supernatants of mAbβCO2 and the immunoreactivity was determined as above. For negative control, myeloma culture supernatants were used as primary antibodies. The data shown are the means±S.D. from triplicate determinations. ●: mAbβCO2 ○: control.

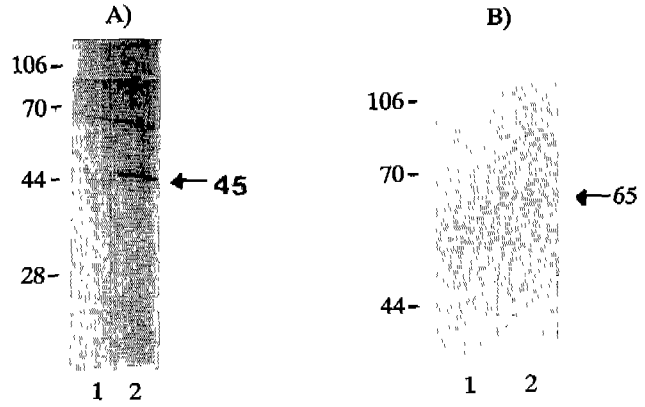

Fig. 4. Immunoblot analysis of the β_2-adrenergic receptor-GST fusion protein and A431 cell membrane with mAbβCO2. A) An aliquot of the purified GST (lane 1) or β-adrenergic receptor-GST fusion protein (lane 2) was electrophoresed and the protein bands were electrically transferred onto nitrocellulose membrane. The strips were incubated with $mAb\beta CO_2$ for 1 h at 32℃ and probed with HRP-conjugated goat anti-mouse IgM. The bands were visualized with diaminobenzidine. The β_2-adrenergic receptor-GST fusion protein (Mw 45 kDa) was indicated by an arrow. The monoclonal antibody βCO_2 did not show any immunoreactivity against GST-carrier moiety (lane 1). B) A431 cell membrane preparation (50 μg protein) was electrophoresed and the protein band was electrically transferred onto NC paper. After blocking the strip was incubated with $mAb\beta CO_2$ (lane 2) or myeloma culture supernatant (negative control, lane 1) for 2 h at 32℃. The strip was probed with HRP-conjugated goat anti-mouse IgM and was visualized with diaminobenzidine. The human β_2-adrenergic receptor (Mw 65 kDa) was indicated by an arrow.

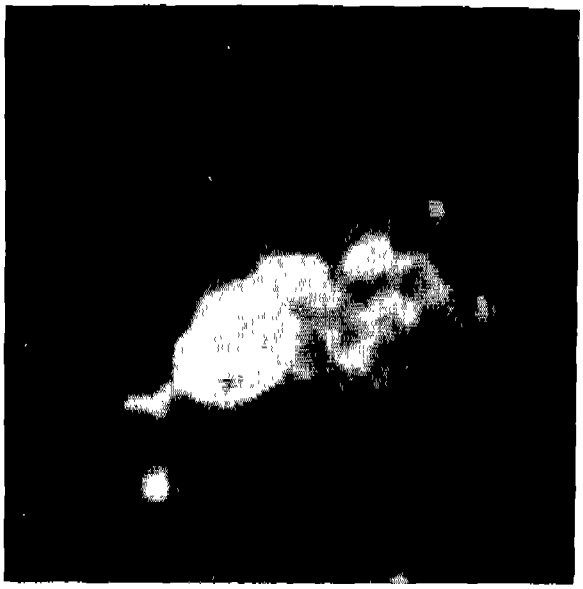

Fig. 5. Immunocytochemical localization of the human β_2-adrenergic receptor on A431 cells with mAbβCO2. Rapidly growing culture of human epidermoid carcinoma A431 cell line was fixed with 4% paraformaldehyde containing 0.2% Triton X-100 and then probed with mAbβCO2 for 2 h at 32℃. For negative control, myeloma culture supernatants were used as primary antibodies. The cell was then probed with FITC-conjugated anti-mouse Immunoglobulin for 30 min at 32℃. The immunoreactivity was observed under a confocal microscope and photographed.

was raised against human β_2-adrenergic receptor by immunizing BALB/c mice with human β_2-adrenergic receptor-GST fusion protein. The monoclonal antibody was characterized by ELISA, Western blot, and immunohistochemistry.

It has been reported that a variety of eucaryotic polypeptides can be expressed in *E. coli* as GST fusion proteins and that these fusion proteins can be readily purified using glutathione-agarose affinity chromatography under non-denaturing condition (Smith and Johnson, 1988; Guan and Dixon, 1991; Hakes and Dixon, 1992). The plasmid pGEXAcc directs the synthesis of glutathione-S-transferase (GST) in *E. coli* under the control of the IPTG-inducible *tac* promoter. Through a series of manipulations, pGEXAcc was modified so that C-terminal intracellular domain of human β_2-adernergic receptor could be expressed as fusion protein with GST (Fig. 1). Since this protein was not expressed prior to induction with IPTG, this protein was evidently encoded by the pGEXβC.

In practice, a major limitation of this system is the fact that many GST fusion proteins, even relatively short ones (40~50 kDa), are partially or completely insoluble after lysis in nonionic detergents. The standard protocol for isolation of GST fusion protein relies on lysis of bacteria with a nonionic detergent such as Triton X-100. This procedure works quite well for freely soluble GST fusion proteins. The relatively hydrophobic nature of β-adrenergic receptor made one to predict that the GST fusion protein containing β-adrenergic receptor should be highly insoluble and aggregate as inclusion body. In this study, only relatively hydrophilic C-terminal portion of β_2-adrenergic receptor was coupled to the end of GST and it was expected that it should be soluble. However, far from our expectation, the fusion protein was observed as inclusion body in *E. coli*. In order to circumvent this problem, we took advantage of the ability of sarcosyl to inhibit coaggregation of proteins (Frankel *et al.*, 1991; Frangioni *et al.*, 1993) and successfully solubilized and purified the fusion protein.

In ELISA and Western blot, mAbβCO2 showed specific and quantitative binding to the β_2-adrenergic recep-

tor portion of GST fusion protein and human β_2-adrenergic receptor on A431 cell. A431 cells have been reported to have large numbers of functionally active β_2-adrenergic receptors and display β_2-adrenergic agonist stimulation of adenylate cyclase and agonist-promoted desensitization of transmembrane signaling (Delavier-Klutchko *et al.*, 1984; Guillet *et al.*, 1985). In Western blot, mAbβCO2 recognize only the β_2-adrenergic receptor portion of the fusion protein and the calculated molecular weight of C-terminal portion was close to the one estimated from the nucleotide length. Furthermore, Western blot analysis revealed predominant immunoreactive staining of β_2-adrenergic receptor with molecular weight of 65,000 in blots of membrane preparation of A 431 cell line. The molecular weight of the polypeptide probed with mAbβCO2 was identical to the molecular weight of 65,000 of β_2-receptor on A431 cell membrane reported by Kaveri *et al.*, (1987) and Wang *et al.*, (1989b). This molecular weight is also in close agreement with those of mammalian β-adrenergic receptors from other sources: S49 mouse lymphoma cell (Weiss *et al.*, 1987), rat fat cell (Cubero and Malbon, 1984), rat and frog erythrocytes (Rashidbaigi and Ruoho, 1982), hamster lung (Benovic *et al.*, 1984), and rat hepatic cell (Graziano *et al.*, 1985). As evidenced from all above results, mAbβCO2 showed a strong and specific immunoreactivity against human β_2-adrenergic receptor.

Fig. 4 showed the immunocytochemical localization of β_2-adrenergic receptor on A431 cell using mAbβCO2. While no significant signal was obtained with nonfixed cells (data not shown), specific immunostaining of detergent-permeabilized fixed cells was observed. These results reflect the staining of a cytoplasmic domain of β_2-adrenergic receptor that was not accessible to the antibody in intact cells.

In conclusion, mAbβCO2 was characterized by ELISA, Western blot, and immunocytochemistry. It is highly specific for human β_2-adrenergic receptor. Therefore, mAbβ CO2 will be useful for future studies of human β_2-adrenergic receptor.

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