Expression and Localization of Recombinant Human B\textsubscript{2} Receptors in the Methylotrophic Yeast Pichia pastoris*

G. X. Yang, T. L. Liu, H. Zhang, C. Q. Wu, and D. L. Shen

State Key Laboratory of Genetic Engineering, Ministry of Education Key Laboratory for Biodiversity Science and Ecological Engineering, Institute of Biodiversity Science, School of Life Sciences, Fudan University, Shanghai 200433, China; fax: +86-21-65648376; e-mail: dlshen@fudan.edu.cn

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Abstract—The human bradykinin B\textsubscript{2} receptor (B\textsubscript{2}R) fused with green fluorescent protein (GFP) at the C-terminal has been expressed in the methylotrophic yeast Pichia pastoris. In the expression vector, B\textsubscript{2}R gene was driven under the highly inducible promoter of alcohol oxidase 1 gene of P. pastoris. By fluorescence activated cell sorting (FACS) analysis and Western blot analysis, it was proved that B\textsubscript{2}R recombinant receptor proteins were expressed at a high level in the yeast. Furthermore, the transformants of P. pastoris were monitored with confocal microscopy, a strong green fluorescence was checked out. The recombinant B\textsubscript{2}R receptor proteins were mainly located on the plasma membrane proved by immunofluorescence microscopy.

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INTRODUCTION

The human bradykinin receptor belongs to the superfamily of seven transmembrane segments G-protein coupled receptors (GPCRs). To date, two bradykinin receptors, human bradykinin B\textsubscript{1} receptor (B\textsubscript{1}R) and human bradykinin B\textsubscript{2} receptor (B\textsubscript{2}R), have been identified. These two receptors are believed to play a key role in a number of physiological and pathological processes, including vasodilatation, inflammatory pain, and algesia [1, 2]. Therefore, discovery of bradykinin agonists or antagonists might provide the basis for development of novel therapeutic agents.

Recently, methylotrophic yeast Pichia pastoris has been developed into a highly successful system for the large-scale production of a variety of heterologous proteins [9, 10]. Generally, the exogenous gene is tightly under the control of alcohol oxidase 1 (AOX1) promoter, which is highly inducible by methanol, which means that the synthesis of proteins is repressed if the cells are cultured on other carbon sources such as glucose or glycerol. Therefore, recombinant could grow to high densities providing with high concentrations of glycerol and the yield of exogenous protein is induced by switching carbon source to methanol. Several GPCRs have been expressed in P. pastoris, including 5HT\textsubscript{5A}-serotonin receptors [8], \mu- opioid receptors [11], and CB2 cannabinoid receptors [12].

Since the gfp cDNA from the jellyfish Aequorea Victoria was cloned in 1992 [13], the green fluorescence protein (GFP) has attracted considerable attention as a marker/reporter system [14]. The fluorescence of this protein is stable, species-independent, and can be monitored non-invasively in vivo. Fusing with GFP in their C/N-terminal part, several GPCRs have already been expressed in mammalian or yeast expression systems [11, 15, 16].

In order to develop a convenient and rapid assay for testing compounds which might be effective as B\textsubscript{2}R agonists or antagonists, we expressed the B\textsubscript{2}R in the yeast P. pastoris. In the present work, an enhanced green fluorescent protein was fused to B\textsubscript{2}R at the C-terminal to trace effect of B\textsubscript{2}R expression and the human bradykinin B\textsubscript{2} receptors were successfully expressed in P. pastoris.

MATERIALS AND METHODS

Materials. The yeast–E. coli shuttle vector pPIC9K and P. pastoris strain GS115 were purchased from Invitrogen (Carlsbad, United States). Plasmid pEGFP-N2 were purchased from Clontech (Palo Alto, United States). Escherichia coli strain DH5\textalpha, plasmid pGEM-T,
yeast nitrogen base, yeast extracts, peptone and tryptone were purchased from Promega (Madison, United States). Restriction enzymes and DNA ligase were obtained from New England Biolabs (Beverly, United States). Glass beads and all other chemicals including octyl-β-D-glucopyranoside (OG), bradykinin, benzamidine, pepstatin, leupeptin and phenylmethylsulfonyl fluoride (PMSF) were purchased from Sigma (St. Louis, United States). All antibodies used in this present experiments were purchased from Santa Cruz Biotechnology (Santa Cruz, United States) or from Clontech.

Construction of expression plasmids. The cDNA for the B2R was isolated by reverse transcription and PCR-amplification from human umbilical vein endothelial cell (HUVEC) RNA using oligonucleotides based on the clone described previously [6]. Sense oligonucleotide primer 5′-AAGTGAATTCGACCACTGCT-CAATGTCAACCTTGCAAGCCACTCTTA AC-3′ and antisense oligonucleotide primer 5′-GTGGATCCGACCCGGTGCAGTCTGCTTCC-3′, which introduce EcoRI and BamHI restriction sites at the 5′ end and the 3′ end (underlined), and replace the stop codon (TGA) with TGT. The product was cloned into the pGEM-T vector and sequenced. The sequence of B2R cDNA was blasted and identified as in [5].

To construct the GFP expression vector pPIC9K-B2R-GFP, in which the GFP fragment was fused to the C end of the B2R gene, first the plasmid pGEM-T-B2R was digested with EcoRI and BamHI and then B2R cDNA was inserted to the equivalent sites of plasmid pEGFP-N2, so that vector pEGFP-B2R was constructed. Secondly, a fragment of B2R-GFP fused gene from vector pEGFP-B2R was cut out with EcoRI and NotI, and then inserted into the same restriction enzyme sites of plasmid pPIC9K to yield pPIC9K-B2R-GFP. This cloning step leads to an in-frame fusion of the GFP tagged B2R gene with no intervening in-frame stop codons to the α-mating factor signal sequence of Saccharomyces cerevisiae, which is encoded by the pPIC9K vector.

Yeast transformation and culture. The plasmid pPIC9K-B2R-GFP was linearized with SalI and transformed into P. pastoris strain GS115 by electroporation using the Gene Pulser apparatus (BioRad, Hercules, United States). Transformants were selected on MD plates, then cultured in buffered glycerol-complex (BMGY) medium and expression of recombinant proteins was induced in buffered minimal methanol-complex medium (BMMY) as described in the Pastoris Expression Kit (Version F) (Invitrogen, United States).

Fluorescence microscopy experiments. Transformed yeast cells expressing the B2R-GFP protein were washed twice with PBS and fixed in 3.7% paraformaldehyde for 30 min at room temperature. Cells were then washed three times in PBS and GFP fluorescence was visualized using a standard microscope (Leica DMRB).

SDS–PAGE and Western blot analysis. Yeast cell suspension expressing the fusion receptors in breaking buffer (50 mM sodium phosphate, pH 7.4, 1 mM EDTA, 5% glycerol, and freshly added protease inhibitors including 1 mM PMSF, 10 mM benzamidine, 20 µg/ml aprotinin, 20 µg/ml leupeptin, and 10 µg/ml pepstatin) was mixed with an equal volume of ice-cold and acid-washed glass beads (425–600 µm). The mixture was vigorously vortexed eight times for 30 s each time with an interval of 30 s on ice and centrifuged at 1500 g for 5 min, then the supernatant was collected and centrifuged at 100 000 g for 1 h. The crude membrane pellet was resuspended in solubilization buffer (50 mM Tris-HCl, pH 7.4, 500 mM NaCl, 1.0% OG, and freshly added protease inhibitors as in breaking buffer) and incubated at 4°C for 2 h under stirring, then centrifuged at 100 000 g for 1 h. Solubilized fractions were treated with SDS-loading buffer and proteins were electrophoresed on a 10% SDS-polyacrylamide gel electrophoresis. After electrophoresis, the gel was blotted onto a Nylon membrane (Amersham Pharmacia Biotech, United Kingdom) using semi-dry electrophoresis transfer (BioRad, United States). The Nylon membranes were blocked with 5% non-fat dried milk in TBS-T for 1 h and then incubated overnight at 4°C with primary antibodies. Subsequently, the membranes were washed twice for 10 min each time with TBS-T buffer and incubated with AP-conjugated secondary antibodies for 1 h at room temperature. The membranes were then washed three times with TBS-T buffer for 10 min each time and the antibody-recognized protein band was then detected by 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium (Roche, United States).

Immunofluorescence microscopy. Fixation, permeabilization and immunofluorescence labeling were performed as described previously [17, 18]. For localization of the recombinant receptor proteins, the permeabilized cells were incubated with the primary anti-B2R goat polyclonal antibody (1 h, diluted 1 : 250) and the secondary rhodamin-conjugated rabbit antigen antibody. A confocal laser scanning microscope was used to view and analyze the stained cells.

RESULTS

Expression of B2 Receptors in P. pastoris as Membrane Proteins

Plasmids for the heterologous expression of B2 receptors in P. pastoris were constructed. The plasmid pPIC9K-B2R-GFP encodes a fused protein of B2R with GFP at C-terminal. The N-terminal of B2R was fused to S. cerevisiae α-factor signal sequence that existed in the parent expression vector and was used to enhance the membrane protein expression.

The expression of B2 receptors in P. pastoris GS115 cells was analyzed by testing GFP fluorescence. Strong green fluorescence could be detected under fluorescence microscopy with an excitation wavelength of