Journal of Immunological Methods 370 (2011) 14–23

Contents lists available at ScienceDirect

Journal of Immunological Methods

journal homepage: www.elsevier.com/locate/jim

Research paper

Novel antigen design for the generation of antibodies to G-protein-coupled receptors

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ARTICLE INFO

Article history:
Received 27 December 2010
Received in revised form 5 May 2011
Accepted 5 May 2011
Available online 12 May 2011

Keywords:
GPCR
Antibody
Antigen design

ABSTRACT

Antibodies are important tools for the study of G-protein-coupled receptors, key proteins in cellular signaling. Due to their large hydrophobic membrane spanning regions and often very short loops exposed on the surface of the cells, generation of antibodies able to recognize the receptors in the endogenous environment has been difficult. Here, we describe an antigen-design method where the extracellular loops and N-terminus are combined to a single antigen for generation of antibodies specific to three selected GPCRs: NPY5R, B2ARN and GLP1R. The design strategy enabled straightforward antigen production and antibody generation. Binding of the antibodies to intact receptors was analyzed using flow cytometry and immunofluorescence based confocal microscopy on A-431 cells overexpressing the respective GPCR. The antibody–antigen interactions were characterized using epitope mapping, and the antibodies were applied in immunohistochemical staining of human tissues. Most of the antibodies showed specific binding to their respective overexpressing cell line but not to the non-transfected cells, thus indicating binding to their respective target receptor. The epitope mapping showed that sub-populations within the purified antibody pool recognized different regions of the antigen. Hence, the genetic combination of several different epitopes enables efficient generation of specific antibodies with potential use in several applications for the study of endogenous receptors.

1. Introduction

G-protein-coupled receptors (GPCRs) belong to a protein family characterized by seven transmembrane helices connected by loops, an extracellular N-terminal and an intracellular C-terminal tail (Fig. 1A). This family of cellular receptor proteins is the largest protein family with more than 1000 members (Wess, 1997), regulating cellular function through interactions with a diverse selection of ligands, such as neurotransmitters, hormones, light, odors and taste (Lundström, 2005).

Due to the regulating activities, GPCRs are very interesting as drug targets and currently 60–70% of the drug development is focused on GPCRs (Lundström, 2005). However, because of difficulties in production and purification of these membrane proteins (Lundström, 2005; Sarkar et al., 2008), only a handful of solved structures exists in the Protein Data Bank, and thus information valuable for the drug development process is often lacking (Berman et al., 2000). In order to further understand and study GPCRs, genetic fusion to tags has commonly been used. To this end, small tags like His_6-, HA- or Flag have been expressed together with the target receptor (Jongsma et al., 2007). Hence, monoclonal antibodies recognizing the tag can

Abbreviations: GPCR, G-protein-coupled receptor; B2AR, beta 2-adrenergic receptor; GLP1R, glucagon-like peptide 1 receptor; NPY5R, neuropeptide Y receptor 5; ABP, albumin binding protein; HPA, Human Protein Atlas.

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doi:10.1016/j.jim.2011.05.001

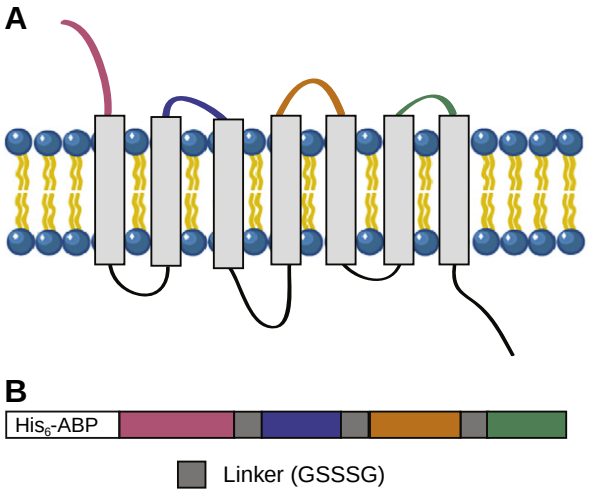

Fig. 1. Schematic representation of an intact G-protein-coupled receptor and outline of the antigen used for generation of GPCR-specific antibodies. (A) A seven transmembrane domain core is characteristic for all GPCRs. The N-terminus together with the extracellular loops is responsible for ligand binding and the intracellular loops and C-terminus for intracellular signaling. (B) The extracellular loops and N-terminus were assembled to a single antigen, separated by a polar linker (GSSSG) and N-terminally fused to a purification/immunization tag, His_6-ABP.

be utilized to elucidate localization and function of the receptors. Another approach is to express the receptor fused to a fluorescent group, such as green fluorescent protein (GFP) (Castro et al., 2005). In both these methods a genetic alteration is made by adding a protein tag, a procedure that may affect the behavior of the receptor regarding localization, quantity and activity. Therefore, specific antibodies recognizing the native forms of GPCRs are invaluable tools for the study of these cell surface receptors and their function (Michel et al., 2009).

Antibodies are normally produced by immunization of an animal with the pure protein of interest. Due to the inherent problems in the production and purification of GPCRs, the use of the intact GPCR-proteins as antigen has been difficult. Hence, most antibodies recognizing GPCRs have so far been generated using synthetic peptide fragments of the receptor protein as antigens (Gupta and Devi, 2006; Mackrill, 2004). Zhang et al. (2004) further developed this strategy by synthetically producing cyclic peptides, thought to mimic the extracellular loops of the CCR5 receptor, for the selection of single-chain Fv (scFv) fragments. These approaches for production of antigens have given rise to functional antibodies and scFv-fragments.

To facilitate the production of antigens and allow the exposure of optional loop regions, we present an alternative method for antigen design of GPCRs. This antigen design strategy has the potential to be used as a general method for the generation of GPCR specific antibodies. As a proof of concept, three different receptors were chosen, and the N-terminal tail together with the three extracellular loops were genetically linked together to form a continuous antigen, where the different segments were separated by polar and flexible glycine/serine (GSSSG) containing linkers. The acquired antibodies were purified using the antigen as affinity ligand and successfully validated for target specificity in a number of different applications such as flow cytometry, immunofluorescence-based confocal microscopy and immunohistochemistry using human cell lines and tissues.

2. Materials and methods

2.1. Materials

The oligonucleotides for assembly of the GPCR constructs were synthesized by Thermo Electron Corporation and Operon. All primers were purchased from Thermo Fisher Scientific. Full-length GPCR cDNA clones were acquired from the Mammalian Gene Collection via geneservice (glucagon peptide 1 receptor, GLP1R, AccNo: BC113493 and beta2-adrenogenic receptor, B2AR, AccNo: BC063486) and imaGenes (neuropeptide Y receptor, NPY5R, AccNo: BC042416). Commercial antibodies used were: rabbit anti-B2AR (Abcam, ab13300); mouse anti-GLP1R (R&D systems, MAB28141); rabbit anti-GLP1R (Strategic Diagnostics, GA1940) and rabbit anti-GLP1R (Chemicon, AB9433). For NPY5R, two additional rabbit antibodies produced by the Human Protein Atlas (HPA) project were used: HPA013790 and HPA014056.

2.2. Plasmid construction

The extracellular parts of the GPCRs were cloned in series, separated by a polar five amino acid linker—GSSSG. The GPCR constructs were generated using solid-phase gene assembly (Nguyen et al., 1994; Ståhl et al., 1993), where a longer DNA sequence was built on a solid support from shorter oligonucleotides by parallel ligations. The oligonucleotides (Thermo Electron Corporation and Operon) were 50 bases long with 10 bases overlap. In brief, 300 μg of streptavidin-coated DynaBeads (DynaBeads M-280 streptavidin (10 mg/ml), Dynal Biotech) was used to couple 80 pmol of the first biotinylated segment. After incubation for 1 h at RT and subsequent washing, 40 pmol each of the remaining segments was mixed for 30 min at 65 °C. T4 DNA ligase (Invitrogen) was then added, and the ligation reaction proceeded for 4 h at RT. The assembled constructs were amplified using nested PCR with AmpliTaq Gold DNA polymerase (Applied Biosystems). Due to the long N-terminal region of GLP1R, it was PCR amplified separately and ligated to the rest of the construct using a naturally occurring *Pst*I site. The assembled GPCR constructs were *Not*I/*Asc*I digested and ligated into the expression vector pAff8c (Larsson et al., 2000) in frame with a hexahistidine (His_6) purification tag, as well as an Albumin binding protein (ABP) derived from streptococcal protein G used for immunization purposes.

2.3. Protein production and immunization

BL21(DE3) cells, containing the expression vector encoding the antigen, were grown, harvested and lysated essentially as formerly described (Agaton et al., 2003), and the lysed cell cultures were IMAC-purified under denaturing conditions using an automated set-up for affinity chromatography (Steen et al., 2006). Moreover, the fusion proteins were analyzed with SDS-PAGE (BioRad), and the