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Research paper

Generation of recombinant guinea pig antibody fragments to the human $GABA_C$ receptor

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ABSTRACT

To generate monoclonal antibodies to the human ρ1 $GABA_C$ receptor, a ligand-gated chloride ion channel that is activated by the neurotransmitter γ-aminobutyric acid (GABA), we recovered the immunoglobulin variable heavy chain (V_H) and light chain (V_L) regions of a guinea pig immunized with a 14-mer peptide segment of the *N*-terminal extracellular domain of the ρ1 subunit. Oligonucleotide primers were designed and used to amplify the V_H and V_L regions of guinea pig RNA by the reverse transcriptase polymerase chain reaction. The amplified and cloned V_H and V_L regions were transferred together into a phagemid vector, yielding a library of 5×10^6 members, which displayed chimeric fragments of antigen binding (Fabs) with guinea pig variable and human constant regions fused to protein III of M13 bacteriophage. Through affinity selection of this phage-display library with the biotinylated 14-mer peptide segment of $GABA_C$, we isolated four different antibody fragments that bound specifically to the immunogenic peptide. Phage particles displaying two of these antibodies, but not negative controls, bound selectively to the surface of neuroblastoma cells expressing the ρ1 $GABA_C$ receptor. Such antibody fragments will be useful in future studies involving targeting of specific neural tissues that express the $GABA_C$ receptor.

1. Introduction

Phage libraries displaying various types of antibody fragments have been utilized extensively in research and for the development of therapeutics (Brissette and Goldstein, 2007; Siegel, 2008). Antibody fragments such as single-chain fragments of variable (V) regions (scFv), fragments of antigen binding (Fabs), or single-antigen binding variable domains of heavy-chain antibodies (i.e., V_{HH}), can be displayed on the surface of M13 bacteriophage particles, from which binders are isolated from libraries by affinity selection (Yamashita et al., 2007; Siegel, 2008; Bostrom and Fuh, 2009; Nieri et al., 2009; Pansri et al., 2009).

Phage-displayed libraries can be generated in several ways. First, it is possible to construct very large libraries (i.e., billions of clones) with variable domains captured from non-immune donors. Such libraries, considered naïve, are often good sources of binding clones with desired specificity and affinity to almost any protein target (de Haard et al., 1999; Schofield et al., 2007; Pansri et al., 2009; Hust et al., 2010). Similarly, naïve libraries have recently been constructed by randomizing the complementarity determining regions (CDRs) of an antibody scaffold, and have been a source of binders to a variety of targets (Barbas, 1995; Fellouse et al., 2005, 2007; Birtalan et al., 2008; Gao et al., 2009; Uysal et al., 2009). Alternatively, one can immunize animals and, after verifying an immunological response, recover the coding regions of immunoglobulins by reverse

Abbreviations: bp, base pairs; CDR, complementarity determining regions; cDNA, complementarity DNA; ELISA, enzyme linked immunosorbent assay; EDTA, Ethylenediaminetetraacetic acid; Fabs, Fragments of antigen binding; GABA, γ-aminobutyric acid; HRP, horseradish peroxidase; Ig, immunoglobulin; Kb, kilobase; PBS, phosphate buffered saline; PBST, PBS with 0.05% Tween 20; PEG, Poly(ethylene glycol); PCR, polymerase chain reaction; RT-PCR, reverse transcriptase-polymerase chain reaction; scFvs, single-chain Fragments of variable regions; V_H, variable domains of immunoglobulin heavy chain; V_L, variable domains of immunoglobulin light chain.

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transcribing the mRNA prepared from isolated spleens or circulating B cells, and then amplifying immunoglobulin variable heavy chain (V_H) and light chain (V_L) regions using the polymerase chain reaction (PCR) (Shen et al., 2007). While immunized libraries are generally limited in the spectrum of their diversity, they have the advantage of yielding affinity-matured antibodies with excellent affinity and specificity (Rader et al., 2000; Heitner et al., 2001).

The $GABA_C$ receptor, a subfamily of $GABA_A$ receptors, is a ligand-gated chloride ion channel that is activated *in vivo* by the inhibitory neurotransmitter γ-aminobutyric acid (GABA). $GABA_C$ receptors are expressed in the retina and in other tissues of the central nervous system (Enz et al., 1996; Euler and Wässle, 1998; Lukasiewicz and Shields, 1998; Shen and Slaughter, 2001; Gibbs and Johnston, 2005). Native $GABA_C$ receptors are pentamers that consist of a combination of ρ subunits, three types of which (ρ1, ρ2, and ρ3) have been cloned from mammalian retina (Zhang et al., 1995; Milligan et al., 2004; Pan and Qian, 2005). The ρ1 subunits are capable of forming fully functional homopentamers when expressed in model cell systems such as *Xenopus laevis* oocytes (Qian et al., 1998) or human embryonic kidney 293 cells (HEK-293) (Wotring et al., 1999). Given the biological importance of the $GABA_C$ receptor, antibodies to the receptor could be useful in modulating channel activity and in characterizing ion channel function (e.g., Tipps et al., 2010). In the present study, we report the recovery of antibody fragments from guinea pigs immunized with a peptide within the extracellular *N*-terminal region of the ρ1 subunit of the $GABA_C$ receptor (Gussin et al., 2008) and expression of chimeric (guinea pig variable and human constant regions) Fab antibodies against ρ1. Such anti-peptide antibodies, stabilized in the context of the Herceptin Fab antibody constant regions, bind to the ρ1 $GABA_C$ receptor expressed in a neuroblastoma cell line.

2. Materials and methods

2.1. Immunogen and guinea pig immunization

The immunogen was a 14-mer peptide (*N*-14), consisting of the amino acid sequence RQRREVHEDAHKQV, which is located within the *N*-terminal region of the human ρ1 subunit, outside the "core peptide" and within the potentially accessible "unstructured tail" of the protein (Gussin et al., 2008). Guinea pig was chosen as the species for antibody production, following earlier, unsuccessful attempts at obtaining specific anti-mammalian ρ1 $GABA_C$ antibodies in rabbit and chicken (H. A. Gussin and H. Qian, unpublished observations). Peptide synthesis (purity >85%), conjugation to keyhole limpet hemocyanin (KLH) immunogenic carrier protein, animal immunization and serum collection were contracted to an outside source (Covance, Inc., Denver, PA). For coating of enzyme linked immunosorbent assay (ELISA) plates, a form of the *N*-14 peptide biotinylated at the *N*-terminus was used (Research Resources Center, University of Illinois at Chicago, Chicago, IL).

2.2. Library construction

The strategy used for construction of the phage library is outlined in Fig. 1. At the moment, only a limited number of expressed V_H and V_L genes from guinea pig (*Cavia porcellus*) have been sequenced (Kabat et al., 1991), which prevented primer design for amplification of immunoglobulins (Ig) from guinea pig lymphoid tissue. Although whole genome shotgun sequences of guinea pig are available (National Center for Biotechnology Information projects 12582 and 12583; http://www.ncbi.nlm.nih.gov/sites/entrez?db=genomeprj&cmd=Retrieve&dopt=Overview&list_uids=12583), the sequences were not usable for primer design because annotation of leader peptide, V-D, and D-J junctions were lacking. In addition, it was unknown if the sequenced open reading frames (ORFs) in the genomic sequences were functional. Consequently, we decided to use degenerate primers, designed for amplification of murine V_L and V_H genes, as our starting point because guinea pig is closely related to mouse. However, these primers did not yield a PCR amplification product with guinea pig spleen cDNA. As an alternative approach, we designed degenerate primers based on human Ig sequences. Rabbit V_{H1} sequences (a1, a2, and a3) are slightly closer to human (nucleotide identity ~78%) than to mouse V_H gene (nucleotide identity ~73%) (Dr. K. L. Knight, personal communication), and we reasoned a similar situation might hold between guinea pig and human Ig sequences. The sequences of the degenerate primers (Fig. 2A and B) used to amplify and maximize the recovery of appropriate guinea pig V_H and V_L coding regions were designed using the database of human antibody sequences (http://vbase.mrc-cpe.cam.ac.uk/). Initially, several degenerate primers were used for PCR amplification of cDNA, prepared from RNA obtained from guinea pig B cells (described below), and DNA sequencing analysis of representative set clones showed that V_H forward and reverse primers were incorporated, whereas V_L clones contained forward primer sequences. We then modified our V_L primers based on the nucleotides sequences we had obtained, and amplified the V_L genes again. The resulting guinea pig V_H and V_L fragments were sequence-verified by comparison to mouse and human Ig (http://www.ncbi.nlm.nih.gov/igblast/), and to guinea pig genomic sequence (http://www.ncbi.nlm.nih.gov/nuccore/DS562855.1). Once we validated the output of our PCR reactions, they were scaled up for construction of the V_H and V_L libraries from the immunized guinea pig.

RNA was extracted from B cells of guinea pigs immunized with the *N*-14 peptide. Frozen B cells were provided by the vendor at a concentration 10^6 cells per mL, of which RNA was extracted from 100 μL, using the RNAaqueous®-4PCR kit (Ambion, Austin, TX), following the manufacturer's instructions. The quality of the RNA was assessed by agarose gel electrophoresis. First-strand cDNA was synthesized from 1 μg of total RNA with random decamers provided in the RNAqueous®-4PCR and RETROscript® kits (Ambion), and was directly used as a template for PCR. PCR conditions were as follows: 5 μL of 10× AccuPrime *Pfx* Reaction mix, containing either the V_H or V_L sets of primers at 0.3 μM, 5 μL of template cDNA (50–100 ng), 0.5 μL AccuPrime *Pfx* DNA polymerase, and water to 50 μL; amplification: 95 °C for 15 s, 58 °C for 30 s, and 68 °C for 1 min (35 cycles), final extension: 68 °C for 6 min. Fig. 2C shows the agarose gel electrophoresis of these PCR products (i.e., amplified guinea pig V_H and V_L coding regions). For cloning, 4 μL of each PCR reaction were incubated with the pCR® II-Blunt-TOPO® vector (Invitrogen, Carlsbad, CA), and after desalting, the DNA sample was transformed into the TG1 strain of