Primary Structure and Expression of the Human β-subunit and Related Proteins of the Rod Photoreceptor cGMP-gated Channel*

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The full-length cDNA for the β-subunit of the human rod photoreceptor cyclic nucleotide-gated channel has been shown to encode a 1251-amino acid (140 kDa) polypeptide which, like its bovine counterpart, has an unusual bipartite structure. The C-terminal part corresponds to the previously reported “subunit 2” of the human rod channel and contains the structural features of other cyclic nucleotide-gated channel subunits including six putative membrane spanning segments, a cyclic nucleotide binding domain, a voltage-sensor motif, and a pore region. The N-terminal part contains the human homolog of the bovine glutamic acid-rich protein called GARP. Western blots indicate that both the native and heterologously expressed human β-subunit migrate anomalously as a 220-kDa polypeptide by SDS-gel electrophoresis. Two other GARP variants, full-length GARP (f-GARP) and truncated GARP (t-GARP), are also present in human, bovine, and rat rod outer segments and migrate as 120–140- and 55–62-kDa polypeptides, respectively. The bovine f-GARP and t-GARP cDNAs code for proteins containing 590 amino acids and 299 amino acids, respectively. The first 571 amino acids of f-GARP and the first 291 amino acids of t-GARP are identical to the corresponding N-terminal amino acid sequence of the bovine β-subunit. The two GARP variants, themselves, are not tightly associated with the rod channel. These results indicate that mammalian rod outer segments contain three alternatively spliced variants of GARP, one of which constitutes the N-terminal part of the rod channel β-subunit.

Cyclic nucleotide-gated (CNG)1 channels comprise a family of nonselective cation channels found in a variety of tissues (1–4). In photoreceptor cells and olfactory neurons, these channels play a key role in signal transduction by controlling the flow of Na+ and Ca2+ ions into the cell in response to signal-induced changes in intracellular levels of cGMP or cAMP (5, 6). The rod photoreceptor CNG channel has been most extensively studied in both native rod outer segment (ROS) membranes and heterologous cell expression systems. It consists of two homologous subunits, generally referred to as the α- and β-subunits, which assemble into a hetero-oligomeric membrane protein complex. The cDNA for the bovine and human α-subunit encodes an 80-kDa polypeptide that forms a functional homooligomeric channel when expressed in Xenopus oocytes and HEK 293 cells (7–8). In ROS membrane preparations, the N-terminal 92 amino acids of the α-subunit are missing resulting in a truncated polypeptide that migrates as a 63-kDa polypeptide by SDS-gel electrophoresis (9, 10). More recently, the cDNA for the complete bovine β-subunit has been cloned and found to code for a 155-kDa polypeptide (11). Both the native and heterologously expressed bovine β-subunit migrate anomalously on SDS-polyacrylamide gels as a 240-kDa polypeptide. The β-subunit, itself, does not form a functional channel, but when coexpressed with the α-subunit, it assembles into a functional hetero-oligomeric channel having electrophysiological properties similar to those of the native rod CNG channel (11–13). The bovine β-subunit has an unusual bipartite structure (11). The N-terminal region is essentially identical to a previously cloned retinal glutamic acid-rich protein called GARP (14), whereas the C-terminal region contains structural features of other CNG channel subunits including six predicted transmembrane segments, a cyclic nucleotide binding domain, a voltage-sensor-like motif, and a pore region. The β-subunit also contains a binding site for calmodulin (13, 15).

The primary structure of the human rod β-subunit is less well resolved. Chen et al. (12) first isolated two cDNAs encoding 70-kDa and 102-kDa isoforms of the human β-subunit (or subunit 2) which differ only by an additional N-terminal segment in the latter. Polypeptides of this apparent Mr, however, are not present in CNG channel preparations immunoprecipitated from human ROS. Instead, a polypeptide of approximately 220-kDa is labeled by a β-subunit-specific antibody by Western blots (13). More recently, Ardell et al. (16) have cloned a cDNA from a human retinal library that encodes a truncated homolog of the bovine GARP protein. On the basis of its similarity to the GARP component of the bovine rod β-subunit, this protein has been suggested to be a third or γ-subunit of the human rod CNG channel. Since no evidence was presented to show that this polypeptide is associated with the human rod channel, questions remain whether this truncated GARP protein is, in fact, a true subunit of the human rod CNG channel.

In order to resolve the uncertainties surrounding the subunit composition of the human rod CNG channel and, in particular the primary structure of the β-subunit, we have cloned and characterized the full-length cDNA for the human β-subunit. In this paper we show that the human rod CNG β-subunit, like the bovine subunit, has a bipartite structure with a GARP component comprising the N-terminal part of this subunit. Two other GARP variants, which are not subunits of the channel,
have also been cloned and found to be expressed specifically in outer segments of rod photoreceptors.

**EXPERIMENTAL PROCEDURES**

**cDNA Libraries and cDNA Clones for the β-Subunit and GARP Proteins—**A human retinal cDNA Agt-10 library and a bovine retinal cDNA ZAP library were generously provided by Drs. Jeremy Nathans and Wolfgang Baehr, respectively. The cDNAs for the bovine β-subunit (phRCNCCβb) and bovine full-length GARP (pSP14GARP) have been previously described (11). The cDNA for the human truncated GARP protein was cloned from the human Agt-10 library and found to be identical in sequence to the clone reported by Ardell et al. (16). The hRCNCC2β cDNA for the human rod subunit 2β (12) was a generous gift of Dr. K.-W. Yau.

**Library Screening—**The bovine retinal cDNA A ZAP library was screened with two radiolabeled, PCR-amplified fragments (BG1 and BG2) of bovine GARP (11, 14). The primers used to generate the fragments were located at positions 1–21/827–807 (BG1) and at 1039–1062/124–1410 (BG2) of the bovine sequence. Screening of approximately 250,000 plaque-forming units of the bovine library with BG1 yielded 150 positive clones, 10 of which were further characterized by re-screening with the BG2 probe. Six clones were labeled with BG1, but not BG2; two clones were sequenced in both directions.

**Poly(A) mRNA Isolation, cDNA Synthesis, and PCR—**Poly(A) mRNA was isolated from human retinas by a fast detergent-lysis oligo(dT) adsorption method (Fast Track, Invitrogen). For first strand cDNA synthesis, 5 μg of total human RNA was incubated with 0.025 A260 units of random hexamers, and 40 units of avian myeloblastosis virus reverse transcriptase (Boehringer Mannheim) according to the manufacturer’s protocol. PCR reactions, unless stated otherwise, were performed in 50-μl volumes containing 1–5 μl of diluted template, 20 pmol of each primer, 120 μM of each dNTP and 0.5 mM MgCl2, routinely 30 cycles of 94°C (15 sec), 55°C (30 sec), and 72°C (1–2 min) were carried out, followed by a 10-min final extension.

**5'-RACE—**A 5′-end of the cDNA encoding the human β-subunit was amplified using a 5′-RACE kit (Life Technologies, Inc.). First strand cDNA synthesis was performed on human mRNA (1 μg) using a primer corresponding to nucleotides 500–474 (amino acid residues 124–132) of the hrNCNCC2β cDNA (12). Subsequent PCR was performed using primers corresponding to nucleotides 1–21 of the “truncated” human GARP sequence (16) and nucleotides 131–108 of hRCNCC2b, 35 cycles of amplification were carried out using an annealing temperature of 55°C. All PCR products were subcloned into pBluescript (Stratagene) for sequencing. At least three different clones were sequenced in both directions.

**Generation of an Anti-GARP Polyclonal Antibody—**An oligonucleotide encoding the 16 N-terminal amino acids of the truncated human GARP sequence was annealed to a complementary oligonucleotide and ligated into pGEX2 (Pharmacia Biotech Inc.). Glutathione S-transferase fusion protein purified by standard methods (17) was injected into New Zealand White rabbits for the production of the PPc 6N antibody. The JH 486 anti-human β-subunit antibody (12) was a generous gift of Dr. K.-W. Yau.

**Heterologous Expression of cDNAs in COS-1 Cells—**The cDNAs were subcloned into pcDNAI (Invitrogen) and transiently expressed in COS-1 cells (18). Cells were dissolved in phosphate-buffered saline containing 1% CHAPS and 0.1 mg/ml phenylmethylsulfonyl fluoride, and the cell extracts were analyzed by SDS-gel electrophoresis (19) and Western blotting as described previously (11).

**Immunofluorescence Microscopy of COS-1 Cells—**COS-1 cells transfected with the various PCR primers used for amplification are shown in Fig. 2. The constructs were transiently transfected into COS-1 cells using Lipofectin (Life Technologies, Inc.). The cells were stained by immunofluorescence microscopy as described previously (10).

**Immunoblotting of COS-1 Cells—**COS-1 cells were transfected with the various PCR primers used for amplification are shown in Fig. 2. The constructs were transiently transfected into COS-1 cells using Lipofectin (Life Technologies, Inc.). The cells were stained by immunofluorescence microscopy as described previously (10).

**RESULTS**

**Cloning and Sequence Analysis of the Full-length Human β-Subunit—**Previous studies have shown that a glutamic acid-rich protein (GARP) exists as both a free protein (14) and the N-terminal 571 amino acids of the β-subunit of the bovine rod CNG channel (11). A truncated human homolog of GARP has recently been cloned from a human retinal cDNA library (16), but its relationship to the β-subunit of the human CNG channel has not been established. In order to determine if a GARP-like component is part of the human β-subunit, PCR was used to amplify regions of human cDNA that contain sequences specific for both the human truncated GARP protein and human rod subunit 2b (12). After having obtained such a “junction sequence,” 5′-RACE was carried out on human mRNA to amplify the 5′ end of the cDNA. A schematic representation of the relationship between the 5′-RACE product, the “junction sequence,” and the hRCNCC2b clone and the relative positions of the various PCR primers used for amplification are shown in Fig. 1.

**Fig. 1. Construction of human β-subunit cDNA. PCR primers corresponding to nucleotides 857–876 (amino acid residues 286–292) of the truncated human GARP (t-GARP) species (primer A) and nucleotides 500–473 (amino acid residues 124–132) of the human rod subunit 2 clone hRCNCC2b (primer B) and a random-primer human cDNA template were used to amplify the “junction sequence.” This 566-base pair fragment encoded a stretch of 57 amino acids showing some alignment with sequence downstream of the primer A-binding site in the bovine GARP sequence, followed by the first 132 amino acid residues of hRCNCC2b (12). Primer B was subsequently used for 5′-RACE cDNA synthesis, and primers encoding nucleotides 131–108 of hRCNCC2b (primer C) and nucleotides 1–21 of the human truncated GARP sequence (primer D) were used to amplify the 5′ end of the cDNA clone. The 5′-RACE product, the junction sequence, and hRCNCC2b were then used to construct the full-length cDNA for the human β-subunit CNG. Numbers correspond to nucleotide positions as they occur in the full-length sequence, with the A of the ATG start codon being designated as nucleotide 1.

**These DNA fragments were used to construct the full-length cDNA (phRCNCGCβ) for the human β-subunit. The deduced amino acid sequence shown in Fig. 2 is aligned with the bovine β-subunit sequence (11). The human cDNA encodes a protein consisting of 1251 amino acids (calculated Mr 140,000), 143 residues shorter than its bovine counterpart (calculated Mr 155,000). Like the bovine protein, the human β-subunit can be viewed as having a bipartite structure with a C-terminal or β-part containing the membrane spanning segments and structural features of other CNG channel subunits and the N-terminal part containing a GARP component. The N-terminal 291 amino acids are in fact identical to a truncated form of human GARP cloned independently by Ardell et al. (16) and this laboratory. While the human and bovine β-subunits exhibit a high degree of identity in the C-terminal “β-part” (86%), there is only a 55% overall identity in the GARP region. However, smaller segments of much higher identity are apparent within the human and bovine GARP sequences; the most obvi
ous is a stretch of 25 amino acids at the N terminus which is 92% identical. The most striking difference between the human and bovine β-subunit sequences is the absence of the glutamic acid-rich repeat region in the human homolog. This is the main reason for the different lengths of the human and bovine subunits. The GARP portion of the human β-subunit, however, is...
Expression of the \( \beta \)-Subunit in Rod Outer Segments and COS-1 Cells—In order to determine if the human \( \beta \)-subunit cDNA (phRCNGC\( \beta \)) codes for the full-length \( \beta \)-subunit found in native rod photoreceptors, Western blots of human ROS, immunoaffinity purified channel, and membrane extracts of transfected COS-1 cells were probed with two \( \beta \)-subunit antibodies. As shown in Fig. 3A, the JH 486 antibody specific for the C terminus of the human \( \beta \)-subunit (12) labeled a 220-kDa polypeptide in both human ROS and membrane extracts of COS-1 cells transfected with the \( \beta \)-subunit cDNA. This antibody has previously been shown to label a similar size polypeptide in immunoprecipitated human rod CNG channel preparations (13). The PPC6N antibody directed against a conserved epitope within the N-terminal 16 amino acid segment of human GARP. The channel fraction from ROS was immunoprecipitated with the \( \alpha \)-subunit-specific antibody PMc 1D1 bound to Sepharose.

In addition to labeling the \( \beta \)-subunit, the GARP-specific PPC 6N antibody also labeled several polypeptides with lower apparent molecular weights that migrate with an apparent molecular mass of 120–140- and 55–62-kDa in human and bovine ROS (Fig. 3A and B). A significant amount of these polypeptides partitioned with the supernatant fraction of hypotonically lysed ROS indicating that they are soluble or peripheral membrane proteins. Although these polypeptides may represent proteolytic fragments of the \( \beta \)-subunits, more likely they correspond to products of differentially spliced bovine and human GARP transcripts (see below). Interestingly, these polypeptides are not present in purified channel preparations indicating that they are not tightly associated with the rod CNG channel.

Cloning of a Truncated GARP Protein from Bovine Retina—The finding that the GARP-specific antibody PPC 6N labels a 62-kDa polypeptide on Western blots of bovine ROS suggested the possibility that there exists a bovine truncated form of GARP analogous to human truncated GARP (t-GARP). This was examined by screening a bovine retinal cDNA library with two probes to full-length bovine GARP protein (f-GARP), one corresponding to the N-terminal sequence and another to the glutamic acid-rich repeat region near the C terminus; the idea is that t-GARP clones should hybridize with the N-terminal encoded probe but not the glutamic acid-rich repeat clone. Using this approach a bovine t-GARP cDNA clone was indeed isolated and sequenced. It codes for a protein of 299 amino acids (calculated \( M_r \sim 32,000 \) which has the same length and is 60% identical in sequence to the human t-GARP homolog. The first 291 residues of the bovine and human t-GARP are identical to the N-terminal 291 amino acids of the corresponding \( \beta \)-subunits of the channel (see Fig. 1). The remaining C-terminal 8 amino acids, however, are unique (Fig. 4). Although the bovine and human t-GARP proteins do not contain the extended stretches of glutamic acid residues found in the GARP component of the rod CNG \( \beta \)-subunit and f-GARP, nonetheless they contain a relatively high glutamic acid content (\( \sim 13\% \)). The relationship between the primary structures of the three cloned bovine GARP-containing proteins and two cloned human GARP-containing proteins is shown in Fig. 4.

Expression of GARP Variants in ROS and Transfected COS-1 Cells—The human and bovine t-GARP proteins and the full-length bovine f-GARP protein were expressed in COS-1 cells in order to compare their electrophoretic mobility (apparent \( M_r \)) with GARP proteins in ROS. As shown in Fig. 5A, the heterologously expressed bovine CNG \( \beta \)-subunit, t-GARP, and t-GARP migrate as polypeptides having apparent molecular
masses of 240, 140, and 62-kDa, respectively, the same apparent molecular masses observed for the three major GARP-containing polypeptides in bovine ROS. The PMs 4B2 monoclonal antibody that recognizes an epitope within the glutamic acid-rich repeat region of bovine f-GARP also labeled the expressed bovine rod β-subunit and f-GARP proteins in both native ROS membranes and heterologously expressed systems but did not label t-GARP which lacks this epitope (data not shown). Similarly, Western blots of human ROS and COS-1 cell extracts labeled with the Ppc 6N antibody (Fig. 5B) indicate that the expressed human t-GARP protein exhibits the same relative mobility \((M_r \sim 55,000)\) as a GARP-containing protein in human ROS. An additional less intensely labeled 120-kDa polypeptide in human ROS most likely corresponds to a full-length version of human GARP which has yet to be cloned.

Together these results indicate that bovine and human ROS contain three major GARP-containing proteins, β-subunit of the rod CNG channel, f-GARP, and t-GARP. These three GARP variants are also present in rat ROS (data not shown), indicating that these proteins are likely found in most, if not all, mammalian rod photoreceptor cells.

**Distribution of GARP Proteins in Retina**—Cryosections of human retina were labeled with the Ppc 6N antibody to study the expression and distribution of GARP proteins by immunofluorescence microscopy. As shown in Fig. 6A, staining was only observed in the photoreceptor outer segment layer of the retina tissue. Cone photoreceptors were not labeled with this antibody. Labeling was specific since no staining was observed when the antibody was preincubated with a glutathione S-transferase fusion protein containing the Ppc 6N epitope (data not shown). Similar results were obtained for bovine and rat retina tissue. These studies indicate that the f-GARP and t-GARP proteins along with the β-subunit of the rod CNG channel are predominantly, if not exclusively, localized to the outer segment of mammalian rod photoreceptors.

The distribution of GARP-containing proteins in ROS membranes was also visualized using pre-embedding immunogold labeling for electron microscopy. As shown in Fig. 6B, the Ppc 6N antibody preferentially labeled the cytoplasmic side of the ROS plasma membrane in the region where the discs come in close contact to the plasma membrane. A similar pattern of labeling has been previously observed for rod CNG-specific antibodies that recognize epitopes exposed on the cytoplasmic surface of the ROS plasma membrane (10, 21). In addition, Ppc 6N labeling was occasionally observed on discs; this labeling may represent the f-GARP or t-GARP variants that have not been released from the membranes during the lysis of the ROS.

**DISCUSSION**

In this study we have cloned and expressed the full-length β-subunit of the human rod CNG channel. Evidence for this is based on the following observations: 1) this polypeptide when expressed in COS-1 cells co-migrates with the 220-kDa polypeptide (β-subunit) found in immunoaffinity purified channel preparations from human ROS; 2) it binds both N-terminal and C-terminal anti-β-subunit-specific antibodies; and 3) the full-length human β-subunit has structural features found for the bovine rod β-subunit. These results clarify several unresolved issues surrounding the β-subunit of the human rod CNG channel. In particular the hRCNC2a and hRCNC2b clones, which were first reported by Chen et al. (12) to code for the second subunit of the rod CNG channel, are most likely either incomplete clones or alternatively spliced forms of the β-subunit that are expressed in low abundance in the retina. This is based on the finding that although the polypeptides encoded by these subunit 2 clones assemble into functional "native-like" channels when coexpressed with the human rod α-subunit in HEK 293 cells, they have significantly lower apparent \(M_r\) than the human β-subunit cloned in this study and found in ROS (13). More recently, Biel et al. (22) have cloned several CNG β-subunit variants from bovine testes. These variants are identical in sequence to the β'-part of the bovine rod channel, but have N-terminal extensions of −105 amino acids corresponding to the C-terminal region of GARP. It is possible, therefore, that the human subunit 2b variant is an analogous spliced variant of the human β-subunit, perhaps expressed in another retinal cell. The report of Ardell et al. (16) indicating that a human
glutamic acid-rich protein is a third or γ-subunit of the human rod CNG channel is somewhat misleading. Although this GARP component is translated as the N-terminal 291 amino acids of the full-length β-subunit, in its free form, it is not associated with the purified rod CNG channel complex. Accordingly, it cannot be defined as a third subunit of the channel.

The full-length β-subunit of the human rod CNG channel, like its bovine counterpart, has a bipartite structure. The C-terminal part contains the previously cloned human rod CNG subunit 2 and exhibits the structural features of other CNG channel subunits including the six predicted transmembrane segments, a voltage-sensor motif, and a pore region. This part of the β-subunit also contains the calmodulin binding site that modulates the apparent affinity of the channel for cGMP. Most, if not all, of the information required for channel activity is contained within this part of the subunit, since the functional properties of the native rod CNG channel are reproduced when this part of the β-subunit is co-expressed with the α-subunit (11–13). The N-terminal 457 amino acids of the human β-subunit appear to be the human homolog of the bovine f-GARP protein. Sequence comparisons indicate that the GARP part of the β-subunit can be further subdivided into two regions. The N-terminal 291 amino acids of the human and bovine homologs are most similar, being ~60% identical in sequence and corresponding to the truncated GARP forms expressed as individual polypeptides in ROS. This region has a relatively high glutamic acid content of ~13%, but interestingly, it has an even higher content of proline residues of ~15–17%. To date related proteins have not been found in the Protein Data Bases. The C-terminal regions of the human and bovine f-GARP that contain a stretch of glutamic acid residues is less highly conserved between human and bovine proteins. In fact two repetitive regions found in the bovine sequence are missing, whereas the inner segment (is) and nuclear layers (n) are not.

In summary the studies reported here indicate that mammalian rod photoreceptors contain three GARP variants, a GARP component that constitutes the N-terminal part of the rod CNG β-subunit, f-GARP, and t-GARP. Structural features of these GARP components appear to contribute to their anomalous migration on SDS gels. Studies are now being carried out to determine the role of these GARP components in the structure and function of rod photoreceptor cells and their possible role in retinal degenerative diseases.

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