Differential expression of novel Gs α signal transduction protein cDNA species

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ABSTRACT

The Gs α guanine nucleotide-binding signal transduction protein is part of a heterotrimeric complex that is involved in the stimulation of adenylate cyclase upon activation of membrane receptors. We report the characterization of 16 Gs α cDNA clones isolated from the human adult retina and fetal eye libraries. Molecular heterogeneity in the 5'-region defines four novel Gs α cDNA species which are generated either by alternate splicing or by using alternative promoter. The novel exons upstream of exon 2 interrupt the highly conserved 'region A' in the Gs α polypeptide. Non-AUG codons in the novel 5'-exon can initiate translation of these Gs α species *in vitro*. Reverse transcription of total RNA coupled with polymerase chain reaction (RT-PCR) using specific primers and *in situ* hybridization to mRNA in baboon tissue sections with a specific oligonucleotide probe show a high level of expression of these species in retina and brain but not in liver. Differential expression of alternatively spliced Gs α species suggests novel signal transducing pathways.

INTRODUCTION

G-proteins consist of a highly conserved and widely distributed family of guanine nucleotide-binding proteins which mediate transmembrane signalling by coupling the extracellular receptors with intracellular effectors to modulate a number of biological events (1–4). Structural diversity of G protein subunits probably reflects the different intracellular signal transducing mechanisms. It is believed that the guanine nucleotide-binding α -subunit confers the functional specificity to a particular G protein. A number of distinct, yet highly homologous, cDNA clones representing various α -subunits have been obtained (4,5). The diversity has been further enhanced by the isolation of a novel olfactory neuron specific G_{olf} (6) and several new α -subunits using the polymerase chain reaction (7,8).

The adenylate cyclase pathway of signal transduction is activated by Gs protein which may contain four distinct α -subunit polypeptides encoded by alternatively spliced mRNA species (9–11). These Gs α mRNA (or cDNA) species differ from each other by the presence of an exon 3 and/or a CAG trinucleotide at the boundary of exons 3 and 4 (12). Each of these variant α -polypeptides are capable of stimulating both adenylate cyclase and calcium channels (13). Existence of a new Gs α mRNA species in canine ventricle has been described recently (14). Here we report the analysis and expression of four Gs α cDNA species identified from human adult retina and fetal eye libraries. The molecular heterogeneity in the 5' region of these mRNAs would generate novel, and as yet unidentified, Gs α polypeptides. The mRNA for these Gs α species is expressed at high levels in retina, brain and B-lymphocyte derived cell line, and at low levels in kidney but is undetectable in liver.

MATERIALS AND METHODS

Human Tissues and Cell Lines

The retina was dissected from the eyes of a 67 year old male donor. The eyes were obtained from the New Britain Eye Bank, New Britain, CT. Eyes from 4 different fetuses at 11 weeks of gestation were obtained at the University of Texas Health Science Center at San Antonio. The lymphoblastoid cell line JY was originally a gift from the laboratory of Dr. Jack Strominger of Harvard Medical School.

Preparation and Analysis of Nucleic Acids

The methods used in the study have been described earlier(15).

cDNA Libraries and Subtraction Cloning

The construction of directional cDNA libraries from the human adult retina, fetal eye and JY lymphoblastoid cells in Charon BS(–) phage vector (16) has been described elsewhere (17). MMLV–reverse transcriptase was used to synthesize the first

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strand of cDNA with poly A⁺ RNA as template and oligo (dT)₁₂₋₁₈ as primer. Double stranded (ds) cDNA was generated by cDNA synthesis system protocol from Bethesda Research Laboratories (Gaithersburg, MD). The ends of ds cDNA were polished by T4 DNA polymerase. Internal HindIII and EcoRI sites were protected by AluI and EcoRI methylases. Addition of a synthetic linker p(GCTTGAATTCAAGC) generated a HindIII site at 3'-ends of cDNAs. After digestion with HindIII and EcoRI and size fractionation by agarose gel electrophoresis, the cDNAs were ligated to Charon BS(-) arms generated by HindIII and EcoRI digestion. Subsequent packaging by Gigapack Gold (Stratagene Cloning System, LaJolla, CA) and amplification with K802 rec A⁻ *E. coli* cells as host generated the cDNA libraries. The retina cDNA library was enriched for specific clones by subtracting against the JY library, as described recently (18).

DNA Sequencing

The clones in Bluescript plasmid (Stratagene Cloning System, LaJolla, CA) were directly sequenced using the Sequenase kit from U.S. Biochemicals, Cleveland, OH (15).

In situ hybridization to RNA in baboon tissue sections

Two ³⁵S-labelled synthetic oligonucleotides (1988 and 2103) were used as probes for *in situ* hybridization to mRNA in baboon retina sections. The oligonucleotide 1988 extends from bp 30 to 49 (20mer) in sense orientation, whereas oligonucleotide 2103 represents the antisense strand of this sequence (bp 49-30, 20 mer). These oligonucleotides are specific for the novel exon A of the Gsα cDNA clones reported here. The oligonucleotides were labelled with ³⁵S-dATP using terminal transferase (19) to a specific activity of 1×10⁹ cpm/μg. The method for the preparation of tissue and *in situ* hybridization is essentially as described earlier (17), with certain modifications: there was no RNase treatment; hybridization was done at 55°C; and the slides were washed five times with 2×SSC containing 50% formamide at 55°C for 45 min each.

RESULTS

Isolation and analysis of Gsα cDNA Clones

Forty random cDNA clones from two subtracted retina libraries were initially isolated, and the sequence of the 3'- and 5'- ends from these clones was obtained with T3 and T7 primers respectively (18). A search of the GenBank database revealed that an approximately 250 bp long 3'-end sequence of one of the cDNA clones, AS175, matched completely to that of human Gsα cDNAs (9,10). However, the homology in the 5'-end sequence began only from exon 2 of Gsα (12) and there was a novel upstream sequence. To investigate this further, the cDNA libraries from human adult retina and fetal eye were screened with the full length AS175 cDNA and a 207 bp 5'-fragment of this cDNA. Fifteen cDNA clones (9 from the retina library and 6 from the fetal eye library) were plaque purified and converted to plasmid clones as described (16). The number of cDNA clones that hybridized to the 5'- fragment was less than 5% of those detected by the full length AS175 cDNA. Restriction analysis of the fifteen cDNA clones revealed a similarity to either the AS175 cDNA or the known Gsα cDNAs (9), except in the case of 1752FE. The clones were analysed further by DNA sequencing.

The composite nucleotide sequence for the AS175 and a longer 1758FE cDNA has been deposited to the EMBL database (accession number X56009). Almost complete identity between the AS175 and known Gsα cDNAs is observed after bp 216 which is the beginning of exon 2. There is only one difference in this region: AS175 has an A at position 1469 (in the 3'-untranslated region) while the reported Gsα genomic sequence (12) and all other cDNA clones reported here have a G. The sequence from bp 1-215 has an open reading frame (ORF) that is continuous with the reported Gsα coding region in later exons (i.e. exon 2 onwards). Confirmation of the existence of such Gsα species is provided by the sequence analysis of 15 additional independent cDNA clones from retina and fetal eye libraries. The results of the analysis are summarized in Table 1. A simple explanation of 5'-heterogeneity is that the sequence from bp

Table 1. Summary of the sequence analysis of Gsα cDNA clones isolated from human adult retina and fetal eye libraries

Clone #	5'-End (bp)	5'-End (fig 2, ref. 12)	Polyadenylation Site (bp)	EXONS							Subtype (see table 2)
				A	B	1	2	3	CAG	4	
AS 175	52		1562	+	+	-	+	+	-	+	VI
1751 AR	52		1462	+	+	-	+	-	-	+	VIII
1752 AR	80		1586	+	-	-	+	+	-	+	X
1753 AR		-314	ND	-	-	+	+	-	-	+	IV
1754 AR		-288	1504	-	-	+	+	+	-	+	II
1755 AR		-318	ND	-	-	+	+	+	-	+	II
1756 AR		-281	1586	-	-	+	+	+	+	+	I
1757 AR		-115	1601	-	-	+	+	+	-	+	II
1758 AR		-319	1504	-	-	+	+	+	-	+	II
1759 AR		-314	ND	-	-	+	+	+	-	+	II
1751 FE	51		1601	+	+	-	+	+	-	+	VI
1752 FE	52		*	+	+	-	+	-	+	+	*
1754 FE	55		1618	+	+	-	+	+	-	+	VI
1756 FE	83		1444	+	-	-	+	-	-	+	XII
1757 FE	28		1546	+	+	-	+	+	-	+	VI
1758 FE	1		1618	+	+	-	+	-	-	+	VIII

*1752 FE cDNA has a different 3'-sequence that is not homologous to any known sequence in the Genbank database. Therefore, it does not belong to any subtype listed in Table 2. ND=not done.

Sequence has been submitted to the EMBL data base (accession number X56009).

1–215 results from at least 2 exons, as evident by the absence of a block of 21 nucleotides (represented as exon B) in cDNA clones 1752 AR and 1756FE. These clones contain nucleotides 1–194 (shown as exon A), joined to exon 2. None of the cDNA clones with exon A and/or exon B contains exon 1, which is present in all previously reported Gs α cDNA species. Conversely, the cDNA clones containing exon 1 did not possess either exon A or B sequences. Further diversity in the novel Gs α cDNA clones is generated by the presence or absence of exon 3. Table 2 lists the possible Gs α subtypes that may be generated by alternative splicing of the 5'-exons: A, B, 1, 3 and CAG trinucleotide. Of the 12 subtypes, seven are represented in the total 16 cDNA clones analyzed in this study. Interestingly exon 2, that encodes part of the conserved region of the phosphate binding loop (20), is present in all the cDNA species. Partial DNA sequencing and restriction analysis show that exon 4 and the downstream exons are present in all Gs α cDNAs except 1752 FE.

The extended ORF sequence in novel Gs α species does not contain an AUG codon, suggesting that the translation of these mRNAs is initiated either from a further upstream and as yet unidentified AUG codon, a non-AUG codon or from an in-frame exon 2 AUG. To clarify the nature of the putative protein product of the novel Gs α species, *in vitro* translation experiments were performed (Fig 1). 1757 AR and 1753 AR cDNAs are for previously reported species, whereas 1757 FE and 1758 FE encode novel species (see Table 1). 1753 AR and 1758 FE lack exon 3 and therefore encode a 15 amino acid shorter polypeptide. 1757 AR and 1753 AR lanes show 43 kD and 41.5 kD products respectively, as predicted by the cDNA sequence (394 and 379 amino acids). Smaller products of 38.5 and 37 kD are obtained with these RNAs because of the initiation of translation from an

Fig. 1. *In vitro* translation of human Gs α species: The Gs α cDNA clones in KSM13(–) Bluescript plasmid were linearized with HindIII and used to synthesize sense strand RNA with T7 RNA polymerase. Equal amount of RNA for each Gs α clone was translated *in vitro* using rabbit reticulocyte lysate (Promega Biotec, Madison, WI). The ³⁵S-labelled products were analysed by SDS-polyacrylamide gel electrophoresis, followed by fluorography. Lanes: 1, no RNA; 2, 1757 AR; 3, 1757 FE; 4, 1758 FE; and 5, 1753 AR (see Table 1). Molecular weight standards are shown on the left.

in-frame AUG in second exon (335 and 320 amino acids respectively). These results are similar to those reported previously (14,21). The novel Gs α species 1757 FE and 1758 FE also show 38.5 and 37 kD products apparently because of the initiation of translation from the exon 2 AUG. However, two additional higher molecular weight products (of 45 and 42 kD in 1757 FE; and of 43 and 41 kD in 1758 FE) are observed in these species. This suggests the use of non-AUG codons in the novel 5' exon A during the translation *in vitro*. It should be noted that human fibroblast growth factor gene and murine pim-1 oncogene encode alternative polypeptide products that are initiated from CUG codon (22,23). It may be possible that the in-frame CUGs in the novel exon A are initiating the translation of the higher molecular weight products at least *in vitro*.

Table 1 also shows the polyadenylation sites in various Gs α cDNA species. Multiple polyadenylation sites generate further complexity. There appears to be no correlation between the presence of a particular 5'-exon and use of a specific polyadenylation signal.

Genomic Southern Analysis

The Southern blot analysis with the 5'-probes from AS175 and 1758 AR detects common fragments on digestion of human genomic DNA with several enzymes, indicating that the various

Fig. 2. RTPCR analysis: Total RNA (8–12 μ g) was used to synthesize first strand of cDNA with oligo dT primer (cDNA cycle kit, Invitrogen, San Diego, CA). An aliquot of the first strand cDNA was used for polymerase chain reaction (94°C-1 min, 55°C-1 min, 72°C-2 min; 40 cycles) with oligonucleotide primers 1988 (bp 30–49, 20mer) and 1919 (bp 525–505, 21 mer). The products were analysed by electrophoresis on 1.5% agarose gel and transferred to Duralon membrane (Stratagene cloning system, LaJolla, CA). The blot was probed with an internal oligonucleotide 1443 (bp 251–271, 21 mer), labelled with ³²P using T4 polynucleotide kinase. (1) human brain (75 years); (2) human kidney (2 months); (3) human liver (2 days); (4) human retina (61 years); (5) human B-lymphocyte derived cell line, JY. A strong signal at around 0.45 kb is observed in lanes with brain, retina and JY RNA. Kidney RNA shows a weak hybridization. JY contains an additional smaller hybridizing species.

Table 2. Possible subtypes of Gs α cDNAs generated by alternative splicing of 5'-exons (this report and ref. 12)

Subtype/Exon	A	B	1	2	3	CAG	4→13
I	–	–	+	+	+	+	+
II	–	–	+	+	+	–	+
III	–	–	+	+	–	+	+
IV	–	–	+	+	–	–	+
V	+	+	–	+	+	+	+
VI	+	+	–	+	+	–	+
VII	+	+	–	+	–	+	+
VIII	+	+	–	+	–	–	+
IX	+	–	–	+	+	+	+
X	+	–	–	+	+	–	+
XI	+	–	–	+	–	+	+
XII	+	–	–	+	–	–	+

Fig. 3. In situ hybridization to RNA in baboon tissue sections: (A, C and E) sense oligonucleotide 1988 (bp 30–49, 20 mer) as probe; (B, D and F) antisense oligonucleotide 2103 (bp 49–30, 20 mer) as probe. (A, B) Retina; (C, D) Brain; (E, F) Liver. (P) Photoreceptors; (O) Outer nuclear layer; (I) Inner nuclear layer; and (G) Ganglion cell layer.

5'-exons are part of the same gene (or gene family) (data not shown).

Expression and localization of novel Gs α mRNAs

Because of the high GC content, the expression studies using total human RNA for northern analysis with complete exon A/B probe were not definitive. To determine the expression of these novel Gs α species, we performed RTPCR with total RNA from human tissues/cell lines and *in situ* hybridizations to RNA in baboon tissue sections using exon-specific oligonucleotides. As shown in fig. 2, the mRNA is present in high amounts in retina, brain and a B-cell derived lymphoblastoid cell line. A lesser expression is obtained in kidney but no signal is detectable in liver. The RTPCR results are substantiated by the *in situ* hybridization study (Fig. 3). Strong signal is detected with exon A-specific antisense oligonucleotide in retina and brain. The mRNA is present in all neural retinal cells (outer and inner nuclear layer and ganglion layer). Most cells in liver do not show any expression.

DISCUSSION

We report here the existence of four novel cDNA species for the Gs α signal transduction protein (Fig. 4). These species were isolated from human adult retina and fetal eye cDNA libraries.

Fig. 4. Schematic representation of Gs α cDNA species: I-IV have been reported earlier and VI, VIII, X, XII are identified in this report (see Table II).

and constitute only a minor portion (<5%) of the Gs α mRNA repertoire as judged by the screening of libraries with full length AS175 cDNA and a 5'-region specific probe. Novel exon sequences are found upstream of exon 2 in those species which lack exon 1. Multiple independent cDNA clones (as revealed by end sequences and exon structures) for the novel Gs α species from two different libraries confirm their expression *in vivo*. When these studies were in progress, a canine Gs α cDNA with a similar exon upstream of the exon 1 of known Gs α cDNAs was reported (14). The comparison of the canine genomic sequence with the exon A/B region shows a strong similarity. The sequence conservation from canine to human suggests a possible biological function of these novel exons. However, the tandem direct repeat of GGCA sequence in the exon 1' of canine Gs α cDNA is not present in the exon A/B of human clones. The 5' end of one of our cDNA clones lies 35 bp upstream of the proposed cap site in canine RNAs, suggesting that the true 5'-end of at least some of these RNAs has not yet been identified.

Since only one gene for Gs α has been cloned so far, alternative splicing seems to be the preferred mechanism for generating multiple Gs α mRNA species (12). However, multiple species may arise from very similar yet distinct Gs α genes or by transcription from a different promoter. It may be noted that while alternative splicing of the human dystrophin gene results in multiple isoforms differing at the carboxyl terminus (24), the gene is transcribed from different promoters in neuronal and glial cells (25).

The amino terminal residues in the Gs α protein contain the 'A region' (domain I) which has homology to the corresponding region in bacterial elongation factors and ras proteins, and is highly conserved among distant species from *Dictyostelium* and *Arabidopsis* to human (26–28). The nineteen amino acids of this region (six of which are contributed by exon 1) form the phosphate-binding loop that is thought to interact with guanine nucleotides (20). Point mutations in the Glycine⁴⁹ residue of this GTP-binding pocket affect the GTPase activity and cause elevated cAMP levels (29–31). The loss of amino terminal residues also results in the loss of ability of the α -subunit to bind the $\beta\gamma$ complex and to activate adenylate cyclase (32–34). The 'A region' is interrupted in the putative polypeptides produced from the novel mRNA species reported here. It is, however, interesting to note that *in vitro* the translation of RNA from these species can be initiated by non-AUG codons in the novel 5'-exon A. No obvious homology could be identified between the amino acids deduced from exon 1 and that from exon A+B. The interruption modifies the first six residues of the phosphate binding loop and destroys the consensus G-(X)₄-G-K sequence by replacing the first Gly with a Cys. Presumably, this change will substantially affect their nucleotide-binding capacity and the interaction with $\beta\gamma$ -subunits in the Gs polypeptides and alter their regulatory function.

The high G-C content in the 5'-region has made the reverse transcription of mRNA in this region more difficult. RTPCR and *in situ* hybridization results, however, suggest that the novel Gs α species are differentially expressed in various tissues. The Gs α species reported earlier are expressed in liver and presumably in most tissues and cell types (5). The expression of the novel Gs α species reported here is not detected in liver by two independent methods. It will be interesting to see if these novel Gs α products are able to stimulate adenylate cyclase and are involved in as yet unidentified signal transducing pathways in specific cell types.

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