Presence of Three Distinct Molecular Species of $G_\alpha$ Protein $\alpha$ Subunit

STRUCTURE OF RAT cDNAs AND HUMAN GENOMIC DNAs*

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We have cloned a new species of rat $G_\alpha$ ($G_3\alpha$) cDNA and genomic DNAs for three distinct human $G_\alpha$ proteins ($G_\alpha1\alpha$, $G_2\alpha$, and $G_3\alpha$). $G_3\alpha$ cDNA codes for a protein of 354 amino acids ($M_r$, 40,522) whose sequence is closely related but distinct from that of the previously isolated rat $G_\alpha$ ($G_2\alpha$). By screening the human genomic libraries with the two rat $G_\alpha$ cDNAs as probes, clones encoding human $G_\alpha1\alpha$, $G_2\alpha$, and $G_3\alpha$ were isolated. The human $G_2\alpha$ and $G_3\alpha$ genes are composed of eight coding exons and seven introns and possess a completely identical exon-intron organization. Southern blot analysis indicates that a single copy of each $G_\alpha$ gene is present per haploid human genome.

In a variety of transmembrane signaling systems, cell surface receptors are linked to effectors which produce second messenger molecules, and $G$ proteins are involved in this system as signal transducers (1). $G$ proteins are a family of guanine nucleotide binding proteins which are structurally homologous and widely distributed in eukaryotic cells.

Well-characterized members of $G$ protein family include $G_\alpha$ and $G_\gamma$, which are involved in stimulation and inhibition, respectively, of hormone-sensitive adenylate cyclase activity, and transducin ($G_\gamma$), which is present in retina and regulates cGMP phosphodiesterase activity in response to light illumination. Another member of $G$ protein, $G_\alpha$, has been purified from mammalian brain, although its precise function has not yet been clarified.

$G$ proteins are composed of three different subunits, $\alpha$, $\beta$, and $\gamma$. The $\alpha$ subunit, which contains the guanine nucleotide binding site, is unique to each $G$ protein, while the $\beta$ and $\gamma$ subunits are similar, if not identical. Recently, cDNA cloning of $G_\alpha$ protein $\alpha$ subunits has been achieved. Two transducin $\alpha$ subunits, $G_\alpha1\alpha$ and $G_\alpha2\alpha$, were coded by two separate mRNAs (3), and their cDNAs were isolated. $G_\alpha$ cDNA was cloned from bovine adrenal (4) and brain (5), and $G_\alpha$ cDNA from bovine brain (6). In a previous paper, we reported cloning and sequence determination of cDNAs coding for $G_\alpha$, $G_\alpha$ (now referred to as $G_2\alpha$), and $G_\alpha$ from rat C6 glioma cells (7). The heterogeneity of $G_\alpha$ species was suggested since the predicted amino acid sequence of rat $G_2\alpha$ differed with the partial amino acid sequence of rat brain $G_\alpha$ protein (41-kDa protein) by 2 amino acid residues.

More recently, clones for $G_\alpha$ cDNA have been obtained from several other sources including mouse macrophages (8), bovine pituitary gland (9), human monocytes (10), human granulocytes (11), human liver (2), and human basal ganglia (12). Comparison of the nucleotide and predicted amino acid sequences of these $G_\alpha$ cDNAs suggests the occurrence of multiple distinct molecular species of $G_\alpha$.

The molecular heterogeneity of $G_\alpha$ had already been indicated from the observation that there are multiple pertussis toxin substrates in mammalian brain (13, 14). Immunological evidence also suggests that the pertussis toxin substrate in human neutrophils is distinct from brain 41-kDa and 39-kDa proteins (15, 16).

In addition to the structural and immunological distinction of multiple $G_\alpha$ species, functional heterogeneity of $G_\alpha$ proteins has been reported. $G$ proteins which are modified by pertussis toxin are found to be involved not only in inhibition of adenylate cyclase (17, 18), but also in activation of phospholipase C (19) and phospholipase A$_2$ (20). Recent evidence suggests that they are also involved in gating of K$^+$ (21–23) and Ca$^{2+}$ channels (24, 25). Furthermore, it is shown that a signal leading to cell proliferation is blocked by the pertussis toxin treatment (26). These observations suggest the presence of multiple molecular species of $G_\alpha$, having discrete individual functions.

In this paper, we describe the sequence of a new $G_\alpha$ ($G_3\alpha$) cDNA clone which we isolated from the same rat C6 glioma cell cDNA library previously used for isolation of $G_2\alpha$ cDNA. We then cloned and characterized three distinct human chromosomal genes (designated as the $G_1\alpha$, $G_2\alpha$, and $G_3\alpha$ genes), which code, respectively, for human homologues of bovine $G_\alpha$ (6), rat $G_2\alpha$ (7), and rat $G_3\alpha$.

EXPERIMENTAL PROCEDURES

Isolation of cDNA and Genomic Clones—High-density screening of $\lambda$ cDNA and genomic libraries (27) was in 5× SSC (1× SSC: 0.15 M NaCl, 15 mM sodium citrate (pH 7)), 1× Denhardt’s solution (0.02% bovine serum albumin, 0.02% polyvinylpyrrolidone, 0.02% Ficoll), 20 mM sodium phosphate buffer (pH 7.0), 0.1% sodium dodecyl sulfate, 10% dextran sulfate, 100 µg/ml heat-denatured calf thymus DNA, 50% formamide at 42°C for high stringency or at 30°C for low stringency (7). Filters were washed at room temperature in 0.1× SSC at 60°C and 0.1% SDS.
SSC, 0.1% sodium dodecyl sulfate for high stringency or in 1 × SSC, 0.1% sodium dodecyl sulfate for low stringency. 

\( ^{32}P \)-Labeled probes were prepared by random priming (28) or nick translation (29) of DNA fragments purified from agarose gel (Low Gelling Temperature, Bio-Rad).

Rat C6 glioma cell cDNA library was constructed with \( \lambda \)gt10 as

\[ \text{were prepared by random priming (28) or nick translation (29) of} \]

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\[ \text{Rat C6 glioma cell cDNA library was constructed with \( \lambda \)gt10 as} \]

\[ \text{described previously (7). To isolate the full length cDNA of rat Gia,} \]

\[ \text{we constructed the cDNA library using random hexamers as primers.} \]

\[ \text{A clone, } \lambda \text{RG34, was isolated from this cDNA library with rat Gia} \]

\[ \text{cDNA EcoRI-NcoI 161-bp fragment of } \lambda \text{XG14 as a probe.} \]

\[ \text{To isolate genomic clones of Gia similarly, we screened the human} \]

\[ \text{genomic DNA library (20) under low stringency conditions with the} \]

\[ \text{EcoRI fragment from } \lambda \text{XG13 containing the rat Gia coding sequence (7).} \]

\[ \text{Eighteen genomic clones were picked up for restriction analysis} \]

\[ \text{and Southern hybridization at high and low stringency. Two clones,} \]

\[ \text{AHiG11 and AHiG24, were hybridized under high stringency} \]

\[ \text{and identified as the Gia clones. One clone (AHiG67) which} \]

\[ \text{hybridized with the Gia probe under low stringency conditions was analyzed} \]

\[ \text{by restriction mapping and by sequence analysis, and it turned out to} \]

\[ \text{be the Gia clone containing exons 2 and 3. Three clones (AHiG2N-6,} \]

\[ \text{AHiG2N-1, and AHiG2N-16) contained the genomic sequences of the} \]

\[ \text{5′ flanking and exon 1 for human Gia, Gia2, and Gia1, respectively,} \]

\[ \text{were isolated from another human genomic DNA library (31) with the} \]

\[ \text{923-bp EcoRI-PstI fragment of rat Gia cDNA containing most} \]

\[ \text{of exons 3 and all of exons 1 and 2. Human Gia genomic clone} \]

\[ \text{AHiG3-13 and AHiG3-81 were obtained from this library with rat Gia cDNA} \]

\[ \text{(AHiG14) as a probe.} \]

\[ \text{Nucleotide Sequencing—For diexonucleotide sequencing (32) cDNA} \]

\[ \text{and genomic DNA fragments were subcloned into M13mp} \]

\[ \text{to be} \]

\[ \text{The hatched box depicts the amino acid coding region.} \]

\[ \text{The thick lines depict the cDNA inserts of } \lambda \text{XG14 and } \lambda \text{RG34.} \]

\[ \text{The dashed lines indicate the probe for rescanning to isolate } \lambda \text{RG34.} \]

\[ \text{The arrows indicate the direction and extent of DNA sequence that} \]

\[ \text{was determined by the diexonucleotide chain-termination method.} \]

\[ \text{Three Gia Genes} \]

\[ \text{6657} \]
**RESULTS**

*Isolation of Second Gα (G3α) cDNAs from the Rat C6 Glioma Cell Libraries—* In the course of the previous studies (7), we have isolated from a cDNA library of rat C6 glioma cells, a clone, XGX14, whose sequence is highly homologous to but distinct from the previously identified Gα clone (XGX13). We designate the new Gα as G3α and the previous one as G2α. The G3α sequence contained in XGX14, however, lacked the NH2-terminal sequence corresponding to amino acid residues 1–34 of G2α. To obtain a cDNA containing the complete NH2-terminal sequence of G3α, we constructed a new cDNA library from rat C6 glioma cells using random primers and screened this cDNA library with the DNA fragment containing the 5′ region of XGX14. On screening 1 × 106 recombinants, one clone (XRG134) was isolated. This clone was identical with XGX14 in restriction map, but longer in length at both 5′ and 3′ ends (Fig. 1).

The nucleotide and deduced amino acid sequences of rat G3α cDNA are shown in Fig. 2. The sequence contained an open reading frame of 1062 nucleotides coding for 354 amino acids including an initiator methionine with a calculated molecular weight of 40,522. The nucleotide sequence of G3α was about 75% homologous with G2α in the coding region, but their sequences in the 5′ and 3′ flanking regions are entirely different. Although the deduced amino acid sequence of G3α was closer to bovine Gα (6) (94% homology) than rat G2α (85% homology), rat G3α is not the counterpart of bovine Gα. The nucleotide homology between rat G3α cDNA and bovine Gα cDNA was only 80% in the coding region, and there was no homology in their 5′ and 3′ flanking regions (data not shown). As will be described, bovine Gα was found to be a counterpart of human G1α.

*Isolation and Characterization of Human Chromosomal G2α Gene—* Restriction enzyme analysis of the three G2α genomic clones (XHG11, XHG24, and XHG2N-6) isolated as described under "Experimental Procedures," and Southern blot analysis using the fragments of rat G2α cDNA revealed the organization of the human G2α gene as shown in Fig. 3. The coding region of the human G2α splits into 8 exons and 7 introns, and spans 16 kilobases of genomic DNA. In the 3′ flanking region, a short sequence of about 200 bp was found in upstream of the XhoI site (data not shown), which was 80% homologous with the 3′ flanking sequence of rat G2α cDNA (7). This may suggest the presence of an additional exon(s) in the 3′ flanking region of the human G2α gene.

Fig. 4 shows the nucleotide sequence of the 5′ flanking and the coding regions of the human G2α gene. In the coding region, the homology between human and rat G2α was 92 and 99%, in the nucleotide and deduced amino acid sequences, respectively. There were only five amino acid replacements in the total sequence of 355 amino acids; two of them were nonconservative and the three remaining were conservative.

The nucleotide sequence of the 5′ flanking region of human G2α gene was determined up to about 800 bp upstream of the ATG initiator codon. As shown in Fig. 4, the nucleotide sequences of the 5′ flanking region of human and rat G2α is closely related (89% homology). Many genes of higher eukaryotes have several characteristic elements in the promoter region (38). These include "TATA" box, "CAAT" box (39), and "GC" box (40). In this region, there are two typical CCAAT boxes at positions -262 and -411. GC box consensus sequence, GGCCGG, or its complement, CGCCCC, occurs seven times at positions -24, -62, -171, -220, -273, -382, and -455. No TATA-like sequence is found. By computer
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Fig. 4. Nucleotide and predicted amino acid sequences of human \( G_\alpha \) genomic DNA. Numbers indicate the position of nucleotides or amino acid residues starting at the initiator codon. Deduced amino acid sequence of human \( G_\alpha \) is shown above the nucleotide sequence. In rat \( G_\alpha \), amino acids and nucleotides different from human \( G_\alpha \) sequence are shown. Asterisks indicate the gaps introduced to obtain maximal homology. GC boxes are underlined, and CAAT boxes are underlined and overlined. The homologous sequence with the 5' flanking region of human \( c-Ha-ras \) gene was determined up to position 5669.

Isolation and Characterization of Human Chromosomal \( G_\alpha \) Gene—Three genomic clones \( \lambda H G, 2N-1, \) \( \lambda H G, 3-13, \) and \( \lambda H G, 3-81 \) were isolated as described under “Experimental Procedures.” The comparison of the rat \( G_3 \alpha \) cDNA sequence with the sequence of the genomic subclones revealed that \( \lambda H G, 3-13 \) and \( \lambda H G, 3-81 \) overlapped and contained exons 2-4 and exons 4-8 of human \( G_3 \alpha \), respectively. On the other hand, \( \lambda H G, 2N-1 \) contained exon 1 and the 5' flanking region (see Fig. 3). The nucleotide sequence homology of the latter clone with rat \( G_3 \alpha \) cDNA were 80 and 93% in the 5' flanking (94 bp) and the coding (118 bp) sequences, respectively (see Fig. 5). The nucleotide sequence of the coding and 5' flanking region of the human \( G_3 \alpha \) gene is presented in Fig. 5. In the coding region, 92% nucleotide sequence homology was found between the human and rat \( G_3 \alpha \) genes. Human \( G_3 \alpha \) gene differs from rat \( G_3 \alpha \) cDNA at 5 of 354 amino acid residues, and all of the differences are conservative amino acid substitutions. The nucleotide sequence of the 5' flanking region of human \( G_3 \alpha \) gene was determined up to position -860. Homology of the 5' flanking region of the human \( G_3 \alpha \) gene with the rat search.
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Gα cDNA was 80%. In the 5' flanking region of the human Gα gene, TATA-like sequence, TTTAAA, occurs at position -182, and GC boxes are found three times at positions -87, -300, and -410. There is no typical CCAAT box.

Isolation and Sequencing of Partial Human Gα Gene—Two genomic clones, λH292N-16 and λH2967, were isolated as the clones weakly hybridizing with rat Gα cDNA, as described under "Experimental Procedures." The nucleotide sequence analysis of genomic subclones revealed that these two genomic clones coded for the amino acid sequence more homologous to bovine Gα (6) than G2α or G3α. As can be seen in Fig. 6, homologies of this human gene and bovine Gα cDNA in the coding and the 5' flanking regions are remarkable. In the 5' flanking region, from positions -1 to -115, the homology in nucleotide sequence was 71%. On the other hand, homologies in the partial nucleotide and amino acid sequences of the coding region (exons 1, 2, and 3) were 96 and 100%, respectively. We therefore concluded that this gene is a human homologue of bovine Gα (G1α). Within about 1050 bp upstream of the ATG initiator codon, several transcription signals were noted. Five GC boxes occur at positions -118, -354, -364, and -533, and a consensus hexamer sequence for the steroid receptor binding, TGTTCT (42), at position -784. There was no typical TATA-like sequence nor CCAAT box.

Conservation of the Exon Junctions in All Gα Genes—Table I shows the sequences at the exon-intron boundaries of the human genes for G1α, G2α, and G3α. All introns begin with the sequence GT at the 5' end and at the 3' end with the sequence AG. The positions of the splice junctions on the
tical. Likewise, the junctions of exons

Genes To determine the copy number of the three human

sequence of cDNA for

arrangement in the course of clonings, we carried out the

Southern blot analysis of human genomic DNA digested with

HindIII and BglII. Hybridization was performed under high

stringency conditions, using as probes the genomic sequences

containing the 5' flanking region and exon 1 of each human

G α gene (see the legend to Fig. 3). The discrete bands were

observed, and the sizes of the bands were consistent with

those of the genomic clones (Fig. 7). We conclude that each

of the three G α genes occurs as a single copy per haploid

human genome.
Fig. 7. Southern hybridization analysis of human genomic DNA. Ten micrograms of human DNA was digested with HindIII (lanes 1, 3, and 5) and BglII (lanes 2, 4, and 6) and examined by blot hybridization to human Gi2a (lanes 1 and 2), Gi3a (lanes 3 and 4), and Gila (lanes 5 and 6) genomic DNA fragments containing the exon 1 (see Fig. 3 legend). HindIII-digested DNA size markers (kilobases) are shown.

Discussion

In this paper, we isolated rat G3a cDNA and three distinct human Gα genes, i.e., the G1α, G2α, and G3α genes. Fig. 8 shows the comparison of the deduced amino acid sequences of G1α, G2α, and G3α obtained from different mammalian sources. In addition to the remarkable homologies of the overall structure, the strong conservation of the amino acid sequence in each group of Gα subfamily was striking. More than 98% homologies were maintained among each Gα subfamily. The amino acid sequence of G2α is also strongly conserved between human and rat; only 1 out of 394 amino acids being different (43). The strong conservation of the amino acid sequence of each G protein α subunit among distant mammalian species may reflect the presence of evolutionary pressure to maintain the specific physiological function of each G protein gene product. Each Gα protein may be linked to a specific receptor and thereby involved in a specific signal transducing pathway.

The occurrence of multiple forms of Gα was first noticed when we determined the sequence of G2α cDNA from rat C6 glioma cells (7). In one tryptic peptide obtained from the purified rat brain Gα (41-kDa protein), the amino acid sequence Pro-Asn-Thr-His (in rat brain Gα) was predicted from the nucleotide sequence. However, in rat brain Gα, the sequence was found neither in G2α nor in G3α (Fig. 8), but it was present in bovine (6) and human (12) brain Gα. We consider that Gila may correspond to the 41-kDa protein, one of the two major pertussis toxin substrates in mammalian brain. In rat C6 glioma cells, this molecular species (Gila) does not seem to be a predominant one since Gila cDNA was not isolated from the C6 cell library, and Gila mRNA was...
not detected by Northern blot analysis of C6 cell mRNAs (data not shown).

Recently, Katada et al. (14) purified the 40-kDa Gα species from porcine brain. The partial amino acid sequence analysis of the porcine 40-kDa protein revealed that the protein was different from G1α and G3α, but identical with G2α (44). These results suggest that, of the pertussis toxin substrates present in mammalian brain, the major 41-kDa species and the minor 40-kDa species may correspond to G1α and G2α, respectively. On the other hand, the product of the G3α gene has not yet been isolated, although the gene is expressed in a variety of tissues and cell lines (data not shown). Very recently, Didsbury and Snyderman (11) and Suki et al. (2) isolated a cDNA clone for a new G protein α subunit from the differentiated HL60 cell library and human liver, respectively. The deduced amino acid sequence of their cDNAs were identical with that of our G3α. Identification of protein products and assignment of functions for each molecular species of Gα have to be carried out.

We have determined the entire organization of the human G2α and G3α genes consisting of eight exons and seven introns. An additional exon is also found in the 3′ flanking region of G2α, but this exon does not code for protein. It is remarkable that two genes have exactly the same organization although the lengths of introns are different. The position of the exon junctions in their cDNA sequences are exactly identical. The same splice sites were also conserved in the partial sequence (exons 1, 2, and 3) of the human G1α gene as well as in the human G2α gene. Furthermore, it was found that, in the human G3α gene, 3 out of 12 splice sites were shared with Gα (43). The results suggest that these genes may be evolved from a common ancestral gene.

An evolutionary tree of G protein α subunits can be drawn based on the homologies of the predicted amino acid sequences obtained from various mammalian sources (Fig. 9). It is remarkable that the homologies among three Gα species are higher than that between rod (G1α) and cone (G2α) transducin α subunits (3).

Fig. 10 illustrates the relationship between the exon–intron organization of the human G2α gene and the functional domain structure of its protein product. A domain of NH2-terminal residues 1–39 encoded by exon 1 is hydrophilic and contains the site for limited cryptic digestions. Although this region may be involved in interaction with βγ subunits, its precise function has not yet been shown. Exon 2 encodes a short length region (14 amino acid residues), which is the most conserved among all Gα proteins and responsible for GTP hydrolysis. The most structurally divergent domain is encoded by exons 3 and 4. The amino acid sequences of residues 80–130 is remarkably diverse. Polypeptide secondary structure analysis (45) of this region in G2α is different from other two Gα gene products (data not shown). Probably this region may serve as a site for interaction with an effector. Exon 5 encodes the sequence containing Arg177 which is ADP-ribosylated by cholera toxin in bovine Gα. ADP-ribosylation of Gα by cholera toxin causes a decrease of affinity for βγ subunits (46). The domain encoded by exons 6 and 7 is strongly conserved among all Gα proteins. Presumably, this domain is involved in formation of a core structure for GTP binding together with that coded by exon 2 (49). The sequence, Asn-Lys-X-Asp, consensual to all guanine nucleotide binding proteins, occurs at positions 270–273 of exon 7. The domain encoded by exon 8 may be involved in interaction with a receptor, since the cysteine residue ADP-ribosylated by pertussis toxin is present in this region and also the structure of this region is heterogeneous. Further studies including site-directed mutagenesis and construction of chimeric genes may throw more light on the structure-function relationship of Gα proteins.

The promoter sequence of the human Gα genes has some characteristic features. The regions up to 500 bp upstream of the ATG codon are G + C-rich, and several GC boxes are present in all three Gα genes. Thus, Gα genes may be regarded as "housekeeping genes" which are G + C-rich in their promoter sequences. Both G2α and G3α mRNAs are expressed in all tissues so far examined (data not shown), and the pertussis toxin substrates are ubiquitously present in all tissues. Recently, Murayama and Ui (26) reported the involvement of a pertussis toxin substrate in proliferation of 3T3 cells. It is of interest to note that the sequence homologous with human c-Ha-ras proto-oncogene is present in 5′ flanking region of the human G2α gene. Furthermore, it was found that the promoter of the human Gα gene shares several blocks of sequences homologous with the human c-Ki-ras2 gene (45). A common regulatory mechanism may exist in the gene expression of subunits and ras proteins. If so, it is attractive to speculate that both gene products might function cooperatively in the signal transduction directed toward cell proliferation.

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Addendum—After submission of this manuscript, a paper containing the rat G3α cDNA sequence was published by Jones and Reed (51). The sequence of their G3α was identical to ours.

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