Cloning and characterization of a rabbit ortholog of human Gα16 and mouse Gα15

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Abstract A cDNA was cloned from a rabbit spleen cDNA library which encoded a G-protein α subunit peptide of 374 amino acids, that at the peptide level exhibited 86% and 79% identity with human Gα16 and mouse Gα15, respectively. The rabbit Gα subunit cDNA was subcloned into a mammalian expression vector and transiently co-transfected into HEK-293 cells along with cDNAs encoding the human C3a, C5a, or nociceptin/orphanin FQ receptors. In all three cases the rabbit G α subunit behaved similarly to Gα15 or Gα16 and effectively coupled the transfected receptors to intracellular calcium mobilization pathways. By nucleotide sequence homology and functional activity the rabbit Gα subunit appears to be the ortholog of human Gα16 and mouse Gα15.

Key words: G-protein; Gα16; Gα15; C3a receptor; C5a receptor; Nociceptin receptor; Orphanin FQ receptor

1. Introduction

G-protein coupled receptors (GPCRs) comprise a large family of cell surface receptors with seven hydrophobic membrane spanning domains (reviewed in [1]). GPCRs are activated by a variety of structurally diverse ligands including nucleotides, lipids, small peptides and macromolecules such as chemokines. These receptors transmit signals into the cell via interaction with heterotrimeric G-proteins composed of α, β, and γ subunits. Based on polypeptide sequence similarity, the G-protein α subunits have been grouped into four families, G_s, G_i, G_q, and G_{12}. The α subunit partially dictates the signal transduction pathways utilized by the receptors, those coupled to $G\alpha_s$ stimulate adenyl cyclase, those coupled to $G\alpha_i$, inhibit adenyl cyclase, and those coupled to $G\alpha_{q/11}$ typically are not inhibited by pertussis toxin and couple through phospholipase Cβ.

Gα16 and its mouse ortholog Gα15 are α subunits of the G_q family, which are referred to as 'promiscuous' G-proteins due to their ability to couple a variety of receptors, including receptors normally coupled through G_s (β-adrenergic), $G_{i/o}$ (M2 muscarinic), and G_q/G_{11} (thrombin, vasopressin V_{1A}), to phospholipase C and calcium mobilization pathways [2]. As a result, receptors which in their native environment normally stimulate or inhibit adenyl cyclase through G_s or G_i coupling can, when co-expressed with Gα15 or Gα16, stimulate phospholipase C resulting in an increase in intracellular calcium. Gα15 and Gα16 are useful laboratory tools for manipulating the functional activity of known and orphan GPCRs. In this report we describe the identification of a rabbit G-protein α subunit which shares significant sequence homology with mouse Gα15 and human Gα16. This rabbit Gα16 functions as a promiscuous G-protein, as demonstrated by coupling the C3a, C5a, and nociceptin/orphanin FQ receptors to calcium signaling pathways.

2. Materials and methods

2.1. Materials

Agonists, C3a was obtained from Advanced Research Technologies (San Diego, CA), nociceptin/orphanin FQ was purchased from Bachem (King of Prussia, PA), human rC5a was expressed in *Escherichia coli* and purified to homogeneity. Mammalian expression plasmids encoding C3a and C5a receptors were constructed as described previously [3]. The nocicpetin [4] (orphanin FQ [5]) receptor was cloned from a human whole brain cDNA phage library (Stratagene, La Jolla, CA) which was screened under low stringency hybridization and washing conditions with a probe corresponding to the 5′ portion of a rat novel opioid receptor (ROR-C [6], GenBank accession number D16438). A cDNA, designated 12C, which had an open reading frame that encoded an orphan GPCR of 370 amino acids was identified and subsequently demonstrated by two groups to be the receptor for nociceptin or orphanin FQ [4,5], a heptadecapeptide with pronociceptive activity [7]. The 12C receptor was subcloned into pCDN [8], the same expression plasmid used for the C3a and C5a receptors and for cDNAs encoding Gα16[9] and Gα15[2] (gifts of Mel Simon). Tissue culture and transfection reagents were obtained from Life Technologies (Gaithersburg, MD).

2.2. Cloning of rabbit Gα16

Total RNA was prepared from a rabbit spleen using Trizol Reagent (Life Technologies) and first strand cDNA was synthesized using the Superscript II Pre-amplification System (Life Technologies). Oligonucleotide primers corresponding to the 5′ and 3′ ends of the coding sequence of human Gα16 cDNA (GenBank accession number M63904) sequence were synthesized (5′-CTGAGGCCACCGCACCATGGCCCG-3′, reverse 5′-GGGTCACAGCAGGTTGATCTC-3′) and used for RT-PCR (reverse transcriptase polymerase chain reaction) cloning of the rabbit G-protein from spleen cDNA. A 1.14 kb DNA fragment was amplified using the Pfu polymerase (Stratagene) (1 cycle for 2 min at 94°C; 30 cycles at 94°C 30 s, 55°C 30 s, 72°C 3 min, and a 10 min extension at 72°C) and subcloned into the pCR2.1 vector (Invitrogen, Carlsbad, CA). Two independently derived clones were fully sequenced and found to be identical. A second round of PCR was performed to ensure that the sequence derived from the presumptively conserved forward and reverse PCR primers was correct. RACE (Rapid amplification of cDNA ends) reactions were performed using rabbit brain, liver, and lung Marathon-ready cDNA libraries to determine the nucleotides at the 5′ and 3′ ends of the rabbit G-protein cDNA. These libraries were made from poly(A)$^+$ RNA (Clontech, Palo Alto, CA) using the Marathon cDNA amplification kit (Clontech). The 5′ RACE primers used for the first and nested reactions were; 5′-CCTTCTCGTCCTCGCTCAGACACC-3′, 5′-GGAGCAGCC- TGGTGATTTCCTGGTCG-3′, and 5′-CAGC-

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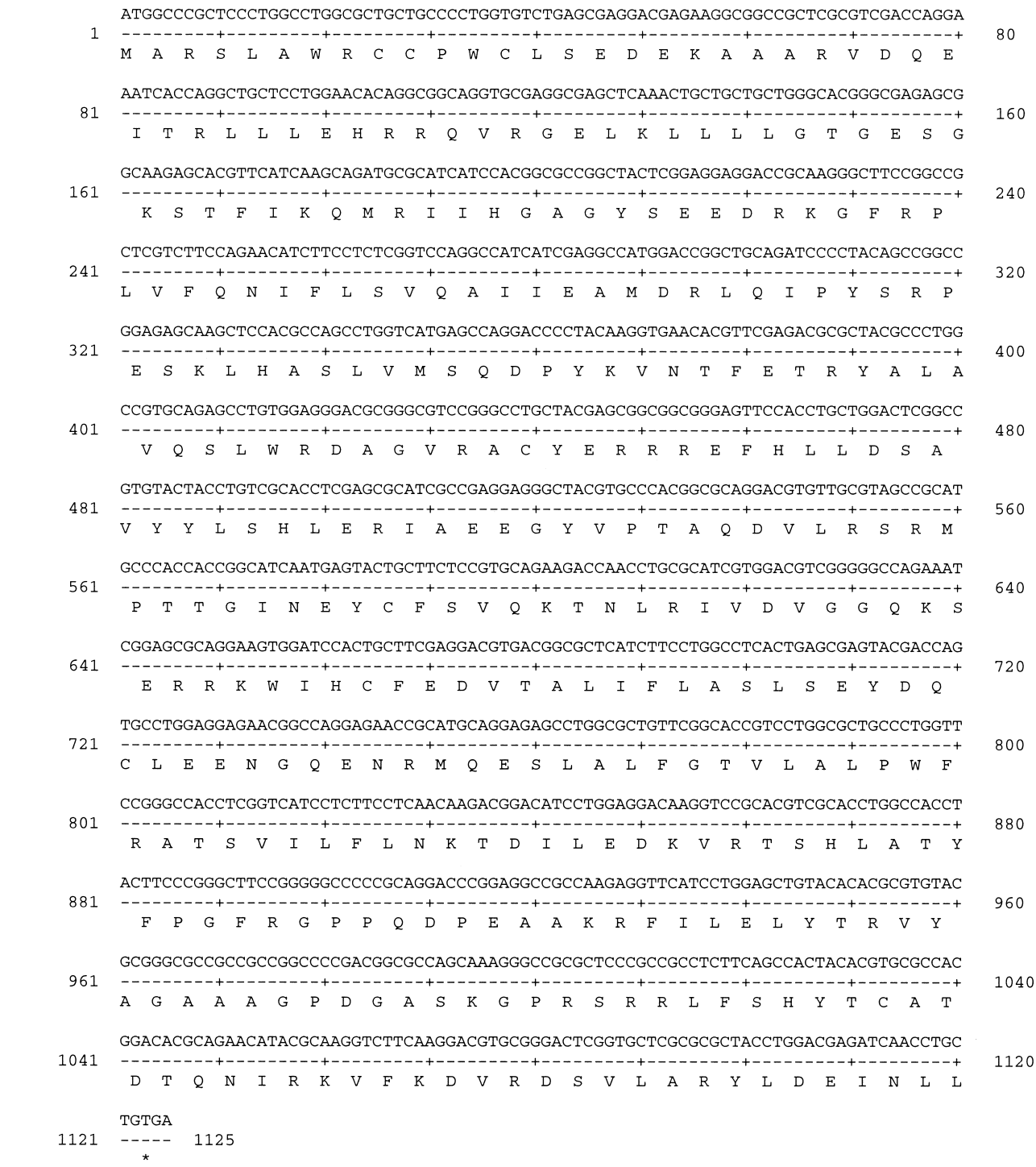

Fig. 1. Nucleotide and deduced polypeptide sequence of rabbit G-protein α subunit. This sequence has been submitted to GenBank, the accession number is AF169627.

AGTTTGAGCTCGCCTCGCACC-3′. The 3′ RACE primers used for the first and nested reactions were; 5′-GGTCTTC-AAGGAC-GTGCGGGACTCG-3′, 5′-GCCACGGACACGCAGAACATACG-C-3′ and 5′-CCGCCTCTTCAGCCACTACACGTG-3′. PCR cycling parameters for RACE were: 95°C 10 min, 5 cycles of 95°C 30 s, 72°C 3 min, then 25 cycles of 95°C 30 s, 70°C 3 min and a 7 min extension at 70°C. The 50 μl RACE reaction contained 0.5 μM primers, 0.2 mM dNTPs, 1.25 units Amplitaq Gold (Perkin Elmer, Foster City, CA), and 2 μl of the original 25 μl PCR reaction as template. Nucleotide sequencing of the RACE amplification products (direct sequencing of the RACE products) verified that the rabbit and human codons at these positions were identical. The full length 1125 bp cDNA was subcloned into pCDN [8] for transient transfection and functional studies.

2.3. Transfection and calcium mobilization assay

Human embryonic kidney 293 cells (HEK-293; ATCC) were grown in Earle's MEM with high glucose and L-glutamine and supplemented with 10% fetal bovine serum. HEK-293 cells were transfected in a T75 culture flask with 5 μg pCDNC5aR, pCDNC3aR, or pCDN12C (no-

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      1                                                    50
 Gα16  MARSLTWRCC PWCLTEDEKA AARVDQEINR ILLEQKKQDR GELKLLLLGP
RGα16  -----A---- ----S----- --------T- L---HRR-V- ---------T
 Gα15  ---------- ------E--T ---I------ --------E- E---------

      51                                                  100
 Gα16  GESGKSTFIK QMRIIHGAGY SEEERKGFRP LVYQNIFVSM RAMIEAMERL
RGα16  ---------- ---------- ---D------ -------L-V Q-I----D--
 Gα15  ---------- -------V-- ---D-RA--- -IY------- Q---D--D--

       101                                                 150
 Gα16  QIPFSRPESK HHASLVMSQD PYKVTTFEKR YAAAMQWLWR DAGIRACYER
RGα16  ---Y------ L--------- ----N---T- --L-V-S--- ---V------
 Gα15  -------D-- Q------T-- ----S----P --V---Y--R ----------

       151                                                 200
 Gα16  RREFHLLDSA VYYLSHLERI TEEGYVPTAQ DVLRSRMPTT GINEYCFSVQ
RGα16  ---------- ---------- A--------- ---------- ----------
 Gα15  ---------- ---------- S-DS-I---- ---------- ---------K

       201                                                 250
 Gα16  KTNLRIVDVG GQKSERKKWI HCFENVIALI YLASLSEYDQ CLEENNQENR
RGα16  ---------- ------R--- ----D-T--- F--------- -----G----
 Gα15  ---------- --R---R--- ---------- ---------- -----D----

       251                                                 300
 Gα16  MKESLALFGT ILELPWFKST SVILFLNKTD ILEEKIPTSH LATYFPSFQG
RGα16  -Q-------- V-A----RA- ---------- ---D-VR--- ------G-R-
 Gα15  -E------S- -------K-- ---------- ---D-H---- ----------

       301                                                 350
 Gα16  PKQDAEAAKR FILDMYTRMY TGCVDGPEGS KKGARSRRLF SHYTCATDTQ
RGα16  -P-P------ ---EL---V- A-AAA--D-A S--P------ ----------
 Gα15  -RR------S ------A-V- AS-AEPQD-G R--S-A--F- A-F-------

       351                  374
 Gα16  NIRKVFKDVR DSVLARYLDE INLL
RGα16  ---------- ---------- ----
 Gα15  SV-S------ ---------- ----
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Fig. 2. Alignment of polypeptide sequences of human Gα16, rabbit Gα subunit (RGα16) and mouse Gα15, residues identical with top sequence are indicated with a dash (-).

cicpetin/orphanin FQ receptor) in the presence or absence of 1 μg mouse Gα15, human Gα16, or the rabbit G-protein expression vector using Lipofectamine Plus as per the manufacturer's instruction (Life Technologies). A microtiter plate based Ca^{2+} mobilization FLIPR assay [10] (Fluorometric Imaging Plate Reader, Molecular Devices, Sunnyvale, CA) was utilized for the functional characterization of HEK-293 cells transiently expressing various receptor/G-protein combinations. The day following transfection, cells were plated in poly-D-lysine coated 96 well black/clear plates (Becton Dickinson, Bedford, MA). After 18–24 h the media were aspirated and replaced with 1 μM Fluo 3AM (Molecular Probes, Eugene, OR) in Hanks' balanced salts solution with 10 mM HEPES, 200 μM $CaCl_2$, 0.1% bovine serum albumin and 2.5 mM probenecid. After 1 h incubation (37°C, 5% CO_2), the cells were washed three times with the same buffer without dye. The Fluo-3 loaded cells were exposed to various concentration of C3a, nociceptin/orphanin FQ, or rC5a, as appropriate. After initiation of the assay, fluorescence was read every 1 s for 1 min and then every 3 s for the following minute. Agonist was added at 10 s and concentration-response curves were generated by calculating maximal fluorescent counts above background. The EC_{50} is the concentration of agonist producing 50% of the maximal responses.

3. Results and discussion

A cDNA encoding a rabbit G-protein α subunit was cloned from spleen RNA using RT-PCR and oligonucleotide primers derived from the nucleotide sequence of human Gα16. RACE reactions were performed and nucleotide sequencing confirmed that the residues at the 5′ and 3′ ends of the cDNA, encoded by the primers, were conserved between the rabbit and human genes, as well as with the mouse gene. The 1125 bp cDNA encoded a peptide of 374 amino acids (Fig. 1) which, at the peptide level, exhibited 86% and 79% identity with human Gα16 and mouse Gα15, respectively (Fig. 2). This is comparable to the level of amino acid identity between Gα16 and Gα15, which is 85%. The rat ortholog [11] of mouse Gα15 also was recently cloned and it is 94% identical to Gα15. There is a cluster of approximately 25 residues (309–336) where the G-proteins vary the most, apart from these residues the coding regions are quite similar, exhibiting $>90\%$ identity. While studies with the C5a receptor have demonstrated that the carboxy-terminal amino acid residues within this region contribute to receptor coupling, other residues, outside this region, are also important for activation of the receptor [12].

The rabbit cDNA was subcloned into a mammalian expression vector. This plasmid was transiently co-transfected into

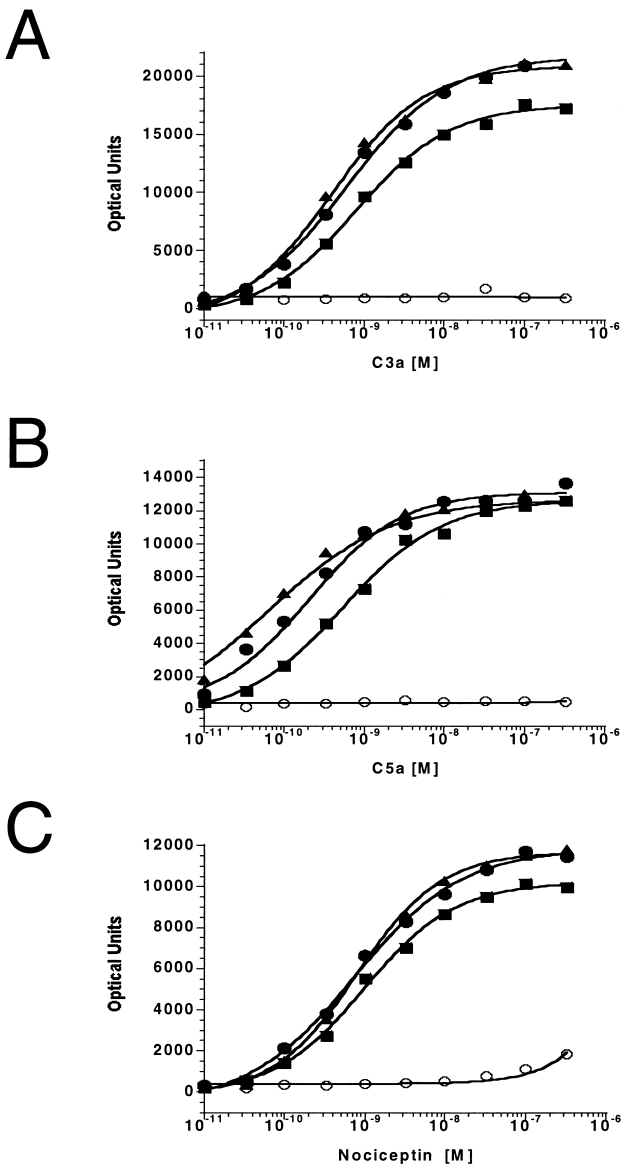

Fig. 3. Calcium mobilization dose response curves obtained on FLIPR with HEK-293 cells transiently expressing human C3a receptor (A), C5a receptor (B), or nociceptin/orphanin FQ receptor (C) when cotransfected with Gα16 (●), rabbit G α subunit (▲), mouse Gα15 (■), or no additional G-protein (○). Values presented are the mean of three concentration-response curves run on three individual plates.

HEK-293 cells along with the cDNAs encoding the human C3a, C5a, or nociceptin/orphanin FQ receptors. These receptors were chosen because it is known that they will couple to Gα16 [13] and induce a Ca^{2+} mobilization response while the receptors expressed alone are ineffective [14,15]. Similarly, HEK-293 cells were cotransfected individually with the same receptors and either Gα16 or Gα15. The transfected cells were utilized in a functional assay monitoring mobilization of intracellular calcium following challenge with varying concentrations of the appropriate ligand, either C3a, rC5a, or nociceptin/orphanin FQ. The rabbit G-protein α subunit behaved similarly to Gα15 or Gα16, i.e. functioning as a promiscuous coupler of the C3a, C5a or nociceptin receptors to intracellular calcium mobilization pathways. The maximal responses and the EC_{50} values obtained with cells transfected with the C3a, C5a, or nociceptin/orphanin FQ receptors in response to challenge with the cognate ligands were similar whether the receptors were transfected with Gα15, Gα16, or with the rabbit G-protein α subunit (Fig. 3). Functional coupling of receptors to Ca^{2+} in HEK-293 cells was absolutely dependent on cotransfection of exogenous G-protein. Cells transfected solely with the receptor expression plasmids induced no Ca^{2+} response to concentrations of agonist up to 500 nM (Fig. 3, open circles). Similarly, cells transfected with the G-proteins alone did not respond to the agonists (data not shown).

The rabbit G-protein α subunit cDNA presented herein shares sequence identity with both Gα15 and Gα16 and, based on the high degree of sequence similarity, it is likely that all three are orthologs. In addition to their structural similarity these three G-proteins behave similarly in coupling the C3a, C5a, and nocicpetin/orphanin FQ receptors to intracellular calcium pathways, most likely mediated via phospholipase C. Based on the functional and sequence similarity of the rabbit cDNA to the human and mouse G-proteins we would propose that this cDNA represents rabbit Gα15. While the G-protein α subunits tested in this study functioned similarly with the three test receptors, it is possible that other receptors may preferentially couple to one of the these promiscuous G-proteins, as has been observed with chimeric G-proteins [15]. Rabbit Gα15 may prove useful to facilitate the functional expression of orphan GPCRs whose cognate ligand and normal signal transduction pathways are unknown.

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