Cloning, sequencing and tissue distribution of two related G protein-coupled receptor candidates expressed prominently in human lung tissue

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Abstract A novel G protein-coupled receptor, named GPR12A, was cloned by a PCR strategy using degenerate primers designed from sequences conserved among receptors for inflammatory mediators. Screening of a human lung cDNA library with GPR12A as a probe also identified a closely-related cDNA (GPR6C.1) that has been previously reported as GPR4 [13]. GPR12A and GPR6C.1 are 46.1% identical in amino acid sequence, but are less than 33% identical to any other known receptors. Northern analysis revealed that they are expressed prominently in the lung. Although the ligands for GPR12A and GPR6C.1 are unknown, their similarity suggests that they are receptors for ligands of similar or identical chemical nature.

Key words: G protein-coupled receptor; Cloning; Expression; Human lung; Orphan receptor

1. Introduction

G protein-coupled receptors mediate cellular responses elicited by a variety of extracellular stimuli ranging from photons, odorants and ions to neurotransmitters, regulatory peptides and bioactive lipids [1,2]. Molecular cloning of these seven-transmembrane-domain receptors has revealed a large gene family with many common structural features [3–5]. The presence of some partially conserved regions in the sequences of members of this gene family has permitted the use of homologous screening and polymerase chain reaction (PCR) amplification to isolated novel receptors [6–11]. In this paper, we report the cloning and sequencing of two closely-related G protein-coupled receptor candidates that are expressed prominently in human lung tissue.

2. Materials and methods

2.1. Polymerase chain reaction (PCR) amplification

Two degenerate oligonucleotides were designed based on the partially conserved cDNA sequences encoding receptors for interleukin-8, ATP (P_{2U}), thrombin, platelet activating factor (PAF) and prostaglandin E_2. The upstream primer, CISVDRY (5′-TGCATC(T/A)(G/C)(C/T)GTIGACCGCT-3′) corresponds to the sequence of the third transmembrane domain of the receptors. The downstream primer, TMVII (5′-TAIA(C/T)C(C/T)AIGGGTCIAGI(C/A)(A/T)I(C/G)AGTT-3′) corresponds to the sequence of their seventh transmembrane domain. These primers were used in PCR to amplify cDNA fragments encoding novel G protein-coupled receptors from human lung and neutrophil cDNAs. The PCR conditions were as follows: 95°C, 1 min (denaturation); 55°C, 1 min (annealing) and 73°C, 2 min (extension) for 40 cycles. A prominent product of 550 bp was obtained and subcloned into pCR-Script vector (Stratagene) for sequence analysis using the Sequenase 2.0 kit (United States Biochemicals). The sequence of the 550 bp clone (designated GPR12A) was predicted to encode a portion of a novel member of the G protein-coupled receptor family.

2.2. cDNA library screening, subcloning and sequencing

The 550 bp insert of clone GPR12A was ^{32}P-labeled by the random primer method and used as a probe to screen a human lung cDNA library in λgt11 phage (Clontech) under high stringency conditions [12]. Of the 1×10^6 phage recombinants screened, two overlapping clones were isolated that included the full-length coding region of GPR12A. In addition, several overlapping clones with sequences similar to GPR12A were also isolated and designated GPR6C.1. The inserts of these clones were subcloned into pBluescript vector (Stratagene) and subjected to unidirectional deletion using the Erase-a-Base system (Promega). DNA sequences on both strands were determined by using the Sequenase kit. The sequences of GPR12A and GPR6C.1 were assembled and analyzed by the IntelliGenetics Suite computer program.

2.3. Eukaryotic expression and functional analysis

cDNA fragments of 1.3 kb and 1.4 kb encoding the predicted full-length proteins of GPR12A and GPR6C.1, respectively, were subcloned into the pRC/CMV expression vector (Invitrogen). These constructs were transiently transfected into human embryonic kidney 293 cells by LipofectAmine (Gibco BRL). To obtain stable transfectants in human myelogenous leukemic K562 cells, the expression constructs were transfected by electroporation at a field strength of 150–200 V/mm and a pulse-length of 2–8 ms using BTX Transfector 300 (BTX Inc.). Cells that survived in the medium containing the neomycin analog G418 (500 μg/ml) were selected 3 weeks after electroporation. The RNA transcripts for GPR12A and GPR6C.1 in K562 stable transfectants were detectable by RT-PCR. The transient 293 transfectants were tested for radioligand-receptor binding with 1 nM ^{3}H-labeled ligands in the presence or absence of 1 μM unlabeled ligands as competitors using a standard filter assay. The intracellular calcium mobilization in the transient and stable transfectants evoked by 1 μM ligands was measured by using Indo-1 as an indicator on an LS50B fluorescence spectrometer (Perkin-Elmer).

2.4. Northern blot analysis

The 1.3 kb and 1.4 kb cDNA inserts that encode full-length coding regions of GPR12A and GPR6C.1 were labeled with ^{32}P and used as a probe in a Northern blot analysis. A human multiple tissue Northern blot containing 2 μg of poly(A)$^+$ RNA in each lane (Clontech) was hybridized and washed under high stringency conditions [12]. Exposure to Kodak XAR film was for 24 h at −70°C with one intensifying screen.

3. Results and discussion

A predominant PCR product of 550 bp was amplified from human lung cDNA using degenerate primers CISVDRY and TMVII, cloned and sequenced. The DNA sequence of clone GPR12A suggested that it may encode a portion of a novel member of the G protein-coupled receptor family. This cDNA

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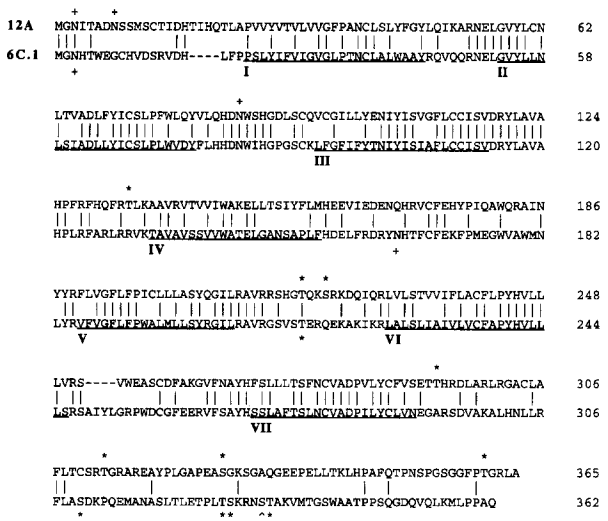

Fig. 1. Alignment of amino acid sequences of GPR12A and GPR6C.1. Gaps, indicated by hyphen, are introduced to obtain maximal homology. Identical residues are aligned by vertical bars. The approximate positions of the putative transmembrane domains are underlined and labeled as I through VII. The + indicates potential N-linked glycosylation sites. The '*' represents potential phosphorylation sites for protein kinase A, whereas the '^' indicates potential phosphorylation site for protein kinase C. The nucleotide sequences of GPR12A and GPR6C.1 have been deposited in the Genbank database under accession numbers U35398 and U35399, respectively.

fragment was used as a probe to screen under high stringency conditions 1×10^6 recombinants of a human lung cDNA library. Two overlapping clones that include DNA sequence identical to that of GPR12A were isolated for further analyses.

The optimal open reading frame of GPR12A has 1095 bp, which encodes a putative receptor protein of 365 amino acids (Fig. 1). In common with all G protein-coupled receptors, GPR12A has seven highly hydrophobic regions of 20–23 residues. It also contains 3 potential N-linked glycosylation sites at Asn^3, Asn^8 and Asn^{86} and seven potential phosphorylation sites for protein kinase A in the second and third intracellular loops and in the carboxyl-terminal cytoplasmic tail.

In the course of screening the human lung cDNA library with the 550 bp fragment of GPR12A as a probe, we also isolated several overlapping clones that have DNA sequences very similar but not identical to the sequence of GPR12A. These overlapping clones, collectively designated GPR6C.1, are more frequently represented in the human lung cDNA library than GPR12A. The overall DNA sequence of GPR6C.1 is 65.3% identical to that of GPR12A. In one continuous 100 bp stretch in the 550 bp probe sequence their identity is 85%, which may explain the isolation of GPR6C.1 by using GPR12A as a probe under high stringency conditions.

The 1086 bp open reading frame of GPR6C.1 encodes a protein of 362 amino acids that is 46.1% identical to GPR12A. Similar to GPR12A, GPR6C.1 also possesses seven hydrophobic regions with several potential N-linked glycosylation sites and phosphorylation sites (Fig. 1). Recently, we became aware that Heiber and colleagues published the sequence of an orphan receptor, GPR4 [13]. The protein sequence of GPR6C.1 is almost identical to that of GPR4 except 15 amino acids in the carboxyl-terminus. This difference may reflect sequence polymorphism, differential mRNA splicing or sequencing error of the same gene.

A search of the Swiss-Prot database (release 31) showed that GPR12A and GPR6C.1 (GPR4) are unique members of the G protein-coupled receptor family. Less than 33% sequence homology was detected to any other known G protein-coupled receptors. Their closest relatives are P_{2Y} ATP purinoceptor [14], P_{2U} ATP purinoceptor [15,16], somatostatin receptor type 5 [17], thrombin receptor [18] and PAF receptor [19,20]. Compared to most other G protein-coupled receptors, features unique to GPR12A and GPR6C.1 include a phenylalanine instead of a tryptophan in the CFXP sequence of the sixth transmembrane domain, and an aspartate instead of an asparagine in DPXXY of the seventh transmembrane domain. Sharing these features are the receptors for ATP, PAF, thrombin and prostanoids [21]. Heiber et al. suggested that the aspartate in the seventh transmembrane domain may render this unique group of receptors conformations different from most other receptors in binding to their ligands [13].

The distribution of mRNA for GPR12A and GPR6C.1 in human tissues was examined by Northern blot analysis (Fig. 2). Both GPR12A and GPR6C.1 are most abundantly expressed in human lung. The 3.1 kb transcript of GPR12A was also present in the placenta, spleen and testis. Two transcripts (2.4 kb and 3.3 kb) of GPR6C.1 were observed in the lung, kidney, heart, liver, skeletal muscle and ovary, whereas only the 2.4 kb transcript was seen in the placenta. The significance of the presence of two different transcripts for GPR6C.1 is unknown.

In order to identify the ligands for GPR12A and GPR6C.1, transient expression of their full-length coding regions constructed in expression vector pRC/CMV were performed in 293 cells. Stable transfectants were also established in K562 cells for detection of functions that cannot be done in 293 cells due to high levels of background endogenous activities (i.e., intracellular calcium mobilization induced by ATP and lysophosphatidic acid). In our attempts to identify the binding ligands for these putative receptors, [^{3}H]leukotriene D_4 (LTD_4), [^{3}H]-leukotriene B_4 (LTB_4), [^{3}H]PAF, [^{3}H]5-hydroeicosatetraenoic acid (HETE) and [^{3}H]lipoxin A_4 did not specifically bind to the transient transfectants. LTD_4, LTB_4, 5-HETE, ATP and lysophosphatidic acid (LPA) did not significantly increase intracellular calcium concentrations over untransfected control cells in the transient and stable transfectants (Table 1). Since we do not

Table 1
Ligand binding and intracellular calcium mobilization assays of GPR12A and GPR6C.1 transient and stable transfectants

Ligand (1 μM)	Specific [^{3}]ligand binding (total-nonspecific binding)	Increase of $[Ca^{2+}]_i$ (% of un-transfected controls)
Control 1(PGE_2)*	12,900 ± 1,500 dpm	240 ± 40 (293 cell)
LTB_4, LTD_4, PAF 5-HETE, LipoxinA_4	< 10% of total dpm	< 10 (293 cell)
Control 2 (VIP)*	not done	190 ± 20 (K562 cell)
ATP, LPA	not done	< 10 (K562 cell)

*Cells in Control 1 and Control 2 are 293 transient transfectants of human prostaglandin E_2 receptor EP3 subtype [22] and K562 stable transfectants of human vasoactive intestinal polypeptide receptor [23], respectively. 2×10^5 cells and 1×10^4 cells/ml were used in binding and $[Ca^{2+}]$ assays, respectively. Values are mean ± S.E.M. of at least three determinations.

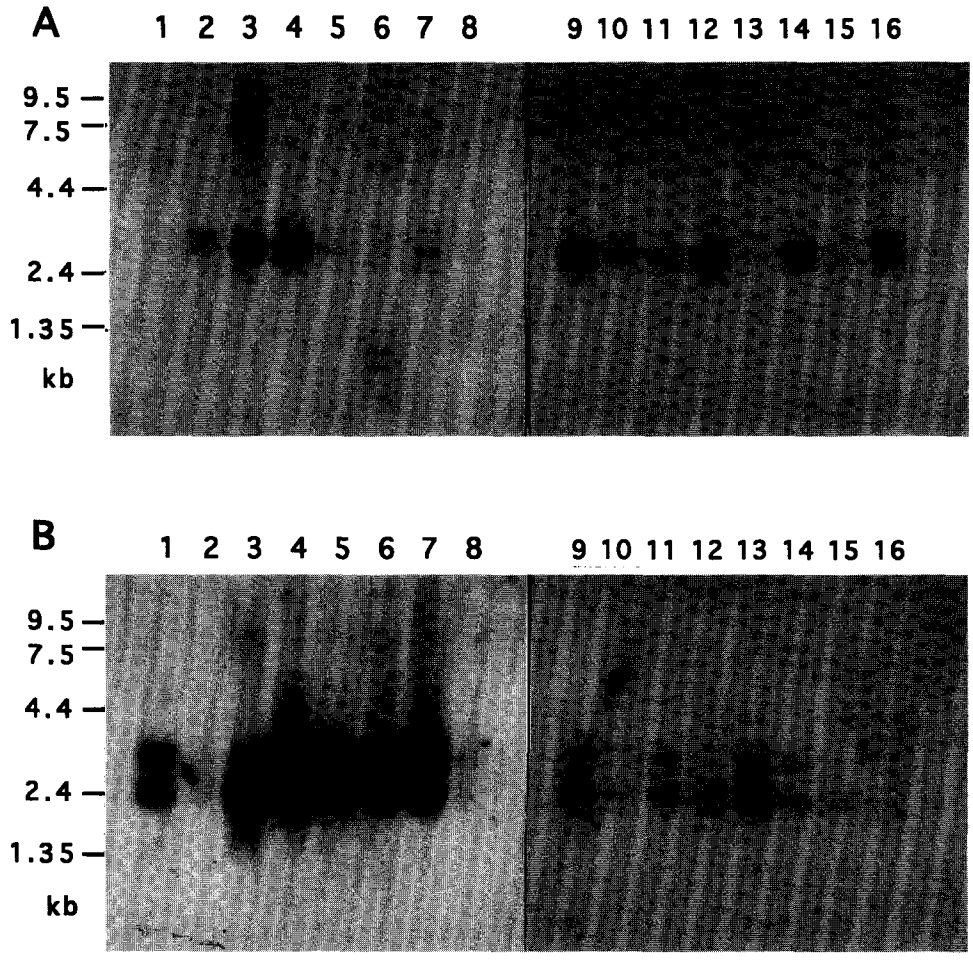

Fig. 2. Northern blot analysis of GPR12A (panel A) and GPR6C.1 (panel B). The positions of RNA molecular weight markers are indicated at the left side. The tissue sources of RNA in lane: 1 = heart, 2 = brain, 3 = placenta, 4 = lung, 5 = liver, 6 = skeletal muscle, 7 = kidney, 8 = pancreas, 9 = spleen, 10 = thymus, 11 = prostate, 12 = testis, 13 = ovary, 14 = small intestine, 15 = colon, 16 = peripheral blood leukocytes.

know whether the functional receptor proteins are properly expressed and coupled to signaling pathways in these heterologous cells, at this point we can not definitively exclude the possibility that the compounds we have tested can indeed interact with GPR12A and GPR6C.1. The sequence similarity of GPR12A and GPR6C.1, however, implies that they are receptors for ligands of similar chemical structure or receptor subtypes for the same ligand.

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