

The Orphan G Protein-coupled Receptors GPR41 and GPR43 Are Activated by Propionate and Other Short Chain Carboxylic Acids*

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GPR41 and GPR43 are related members of a homologous family of orphan G protein-coupled receptors that are tandemly encoded at a single chromosomal locus in both humans and mice. We identified the acetate anion as an agonist of human GPR43 during routine ligand bank screening in yeast. This activity was confirmed after transient transfection of GPR43 into mammalian cells using Ca²⁺ mobilization and [³⁵S]guanosine 5'-O-(3-thiotriphosphate) binding assays and by coexpression with GIRK G protein-regulated potassium channels in *Xenopus laevis* oocytes. Other short chain carboxylic acid anions such as formate, propionate, butyrate, and pentanoate also had agonist activity. GPR41 is related to GPR43 (52% similarity; 43% identity) and was activated by similar ligands but with differing specificity for carbon chain length, with pentanoate being the most potent agonist. A third family member, GPR42, is most likely a recent gene duplication of GPR41 and may be a pseudogene. GPR41 was expressed primarily in adipose tissue, whereas the highest levels of GPR43 were found in immune cells. The identity of the cognate physiological ligands for these receptors is not clear, although propionate is known to occur *in vivo* at high concentrations under certain pathophysiological conditions.

Within family A of the G protein-coupled receptor (GPCR)¹ gene superfamily (also classified as family 1), there is a phylo-

genetically related group of ~90 receptors that respond to an unusually wide variety of ligand types, considering the relatively close similarity of their primary sequences (1). The group includes receptors that respond to purinergic or pyrimidineric nucleotides (P2Y₁, P2Y₂, P2Y₄, P2Y₆, P2Y₁₁, P2Y₁₂, and P2Y₁₃), modified nucleotides (UDP-glucose), lipids (platelet-activating factor receptor), leukotrienes (BLT₁ and BLT₂ and CysLT₁ and CysLT₂), proteases (protease-activated receptor-1–4), chemoattractants (FPR1), and chemokines. To date, these receptors have no clear homologs in invertebrates, unlike the monoamine or neuropeptide receptors, suggesting a relatively recent evolutionary origin (2, 3). At least 50 GPCRs whose cognate ligands are unknown (orphans) (4) are categorized within this group on the basis of sequence homology. Often, these orphans fall into subsets, being more related to each other than to receptors with known ligands; and this, combined with the ligand diversity noted above, makes it difficult to predict the chemical nature of their ligands. One subset comprises GPR40–43, which were identified as tandemly encoded genes present on cosmids isolated from human chromosomal locus 19q13.1 (5). GPR42 differs from GPR41 at only six amino acid positions; otherwise, the four members of this subfamily share ~30% minimum identity. BLAST searches have identified the next most closely related receptors as the protease-activated receptors. However, the long N-terminal extracellular domains that serve as protease substrates and that are characteristic of protease-activated receptors are absent in the GPR40–43 group, suggesting that they are not activated by a similar mechanism.

By adopting a ligand fishing strategy (4) following heterologous expression of orphan GPCRs in yeast, we found that short chain carboxylic acid anions can activate GPR41 and GPR43 in a dose-dependent and -specific manner. These molecules represent a novel chemical class of GPCR ligand. The known pharmacological and pathological effects of these ligands and the tissue distribution of GPR43 suggest potential roles in immune cell function and hematopoiesis. The relevance of GPR41 is less clear. Furthermore, our data support the hypothesis (5) that GPR42 arose as a tandem duplication of GPR41 in the human lineage and has acquired mutations since duplication that abolish its ability to respond to carboxylate ions. Ligands for GPR40 are described in the accompanying article (6).

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This work is dedicated to the memory of Dr. Richard H. Green.

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¹ The abbreviations used are: GPCR, G protein-coupled receptor; GTPγS, guanosine 5'-O-(3-thiotriphosphate); FLIPR, fluorometric imaging plate reader; PTX, pertussis toxin; hGPR41, human GPR41; rGPR41, rat GPR41; RT, reverse transcriptase; PA, propionic acidemia.

EXPERIMENTAL PROCEDURES

Materials—Carboxylates were obtained from Sigma as sodium salts or free acids and were prepared as 100 mM or 1 M stock solutions of the sodium salt and adjusted to pH 7.0. Peptides for antibody generation were obtained from Severn Biotech Ltd.

Yeast Strain Construction and Reporter Gene Assay—Yeast cells were derived from the dual-reporter gene strain MMY11 (*MATa fus1::FUS1-HIS3 LEU2::FUS1-lacZ far1Δ sst2Δ ste2Δ gpa1::ADE2 his3 ura3 trp1*) (7). To introduce Gα subunits into this strain, expression cassettes comprising the *GPA1* promoter, Gα coding sequence, and the *ADH1* terminator (8) were subcloned into pRS304 and integrated into the *trp1* locus of MMY11. Integrants were selected to have low basal levels of reporter gene activity. This produced an isogenic panel of yeast strains containing a range of functional Gα subunits as follows: MMY12, wild-type Gpa1p; MMY14, Gpa1p/Gα_q; MMY15, Gpa1p/Gα_s; MMY16 Gpa1p/Gα₁₆; MMY19, Gpa1p/Gα₁₂; MMY20, Gpa1p/Gα₁₃; MMY21, Gpa1p/Gα₁₄; MMY22, Gpa1p/Gα_α; MMY23, Gpa1p/Gα₁₁; and MMY24, Gpa1p/Gα₁₃. MMY16 and MMY23 have been described previously (7, 9). Mammalian GPCRs were expressed using the p426GPD vector (10). β-Galactosidase activity was measured in an *in vivo* bioassay during growth in liquid culture using the chromogenic substrate chlorophenolred-β-D-galactopyranoside (7). A library of 80 known or candidate GPCR agonists, similar to those described previously (11), was screened at final concentrations ranging from 0.2 μM to 2.5 mM.

Oocyte Methods—*Xenopus laevis* oocyte isolation and microinjection were performed as described previously (12). Human *GPR43* cRNA was produced using T7 polymerase (Promega) from linearized plasmid pJG3.6-hGPR43 and co-injected with *GIRK1* and *GIRK4* cRNAs into single oocytes in stage V or VI. Potassium current amplitude was measured using dual microelectrode voltage clamp electrophysiology (12).

Cell Biology—Mammalian cells were maintained in Dulbecco's modified Eagle's medium (pH 7.4) containing 10% fetal calf serum and 2 mM glutamine, except where otherwise indicated. HEK293T cells were grown to 60–80% confluency in 90-mm dishes, transfected with cDNA (total DNA = 9 μg) using LipofectAMINE reagent (30 μl; Invitrogen), and collected 48 h after transfection. Immunoblotting was performed on plasma membrane-containing P2 particulate fractions at room temperature in 96-well format as described previously (14). Bound [³⁵S]GTPγS was determined by scintillation counting. Maintenance, transfection, and fluorometric imaging plate reader (FLIPR) (Molecular Devices) assay of internal Ca²⁺ concentration in HEK293 cells were performed as described previously (15). Pertussis toxin (PTX) treatment was carried out by supplementation of the medium at 50 ng/ml for 18 h. Swiss 3T3-L1 and 3T3-L442A fibroblasts (American Type Culture Collection) were grown in medium supplemented with penicillin or streptomycin (100 units/ml or 100 μg/ml, respectively). Cells were differentiated 2 days after confluence by supplementation of the culture medium with 3-isobutyl-1-methylxanthine (500 mM), dexamethasone (0.25 μM), insulin (0.8 μM), and Myoclon for 2 days, followed by insulin (0.8 μM) and Myoclon only for an additional 2 days. RNA was extracted (RNeasy, QIAGEN Inc.) from differentiated cells within eight passages from original stocks.

Antibody Generation—The antigen peptide CEQKGGEEQRADRAERKTSEHSQGC (SB130) corresponds to an internal sequence shared by human GPR41 (hGPR41) and hGPR42 (amino acids 311–335) in the predicted C-terminal tail (5). SB130 was conjugated to the carrier protein tuberculin PPD via the cysteine residues and used to immunize female New Zealand White rabbits. The immune response was monitored by enzyme-linked immunosorbent assay using plates coated with the free peptide, and immunoglobulins from high titer sera were purified using immobilized peptide (Sulfolink, Perbio). Purified antibodies were dissolved in phosphate-buffered saline containing 0.2% bovine serum albumin.

Gene Expression—Primers and probes for TaqMan analysis (PE Applied Biosystems) were designed using Primer Express software (PE Applied Biosystems) and compared with public databases by BLAST searches to confirm specificity (Table I). RNA concentrations were determined using RiboGreen (Molecular Probes, Inc.), and PCRs were performed in an ABI PRISM 7700 sequence detection system (PE Applied Biosystems). For the human tissue samples shown in Fig. 5A, poly(A)⁺ RNA (1 μg) from 20 tissues of four different individuals (two males and two females except prostate) was prepared, reverse-transcribed using Superscript II and random 9-mer priming (Invitrogen), and diluted and plated using Biomek robotics (Beckman Coulter, High Wycombe, UK) to produce 1000 replica plates with each well containing

TABLE I
Oligonucleotides used in TaqMan experiments

Gene	Oligonucleotide sequence
hGPR41	
Forward primer	ACCTGCTGGCCCTGGTG
Reverse primer	GGTCAGGTTGAGCAGGAGCA
Probe	CAAGCTGCAGCGCCGCC
hGPR42	
Forward primer	As hGPR41
Reverse primer	As hGPR41
Probe	CAAGCTGCGGTGCCGCC
hGPR43	
Forward primer	GGCTTTCCTCCGTGCAGTAC
Reverse primer	CCAGAGCTGCAATCCATCCAT
Probe	AGCTACTCCCGCCGCCCTCTG
Mouse Gpr41	
Forward primer	TTCTTGAGCCACACTGCTC
Reverse primer	GCCACCACATGGGACATAT
Probe	TCTTCTTCGTCTGCTTCGGCCCTAT
Adipsin	
Forward primer	TCGAAGGTGTGGTTACGTGG
Reverse primer	GGGTATAGACGCCCGCTT
Probe	TTGCCATTGCCACAGACGCGA
CEBPα^a	
Forward primer	CGCAAGAGCCGAGATAAAGC
Reverse primer	TGGTCAACTCCAGCACCTTCT
Probe	AAACAACGCAACGTGGAGACGCAA
GAPDH^a	
Forward primer	CAAGGTCATCCATGACAACCTTTG
Reverse primer	GGGCCATCCACAGTCTTCTG
Probe	ACCACAGTCCATGCCATCACTGCCAT
β-Actin	
Forward primer	GAGCTATGAGTGCCTGACG
Reverse primer	AGTTTCATGGATGCCACAGGA
Probe	CATCACTATTGGCAACGAGCGGTTCC
RL19	
Forward primer	CAAGCGGATTCTCATGGAACA
Reverse primer	TGGTCAGCCAGGAGCTTCTT
Probe	TCCACAAGCTGAAGGCAGACAAGGC

^a CEBPα, CAAT/enhancer-binding protein-α; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

the cDNA from 1 ng of RNA. TaqMan quantitative PCR analysis of each sample was conducted as described previously (16). For the immune cell samples shown in Fig. 5B, total RNA was extracted from samples derived from up to three individuals using the RNeasy maxiprep procedure (QIAGEN Inc.). Samples were treated with DNase I (Ambion Inc.) and normalized to levels of 18 S ribosomal RNA. Reactions were run in 50 μl containing 5.5 mM MgCl₂, 1× TaqMan buffer A (PE Applied Biosystems), 300 μM dNTP mixture, 20 units of RNase inhibitor, 12.5 units of murine leukemia virus reverse transcriptase, 900 nM each primer, 200 nM probe, 1.25 units of Ampliqaq Gold (PE Applied Biosystems), and 50 ng of total RNA. Cycling conditions were as follows: 30 min at 48 °C, 10 min at 95 °C, and 40 cycles of 15 s at 94 °C, followed by 1 min at 60 °C.

Immunohistochemistry—Formalin-fixed, paraffin-embedded human tissue sections were deparaffinized and hydrated through graded alcohols. Immunohistochemical staining was performed using a Ventana Nexus automated stainer with Ventana reagents (Ventana Medical Systems, Inc., Tucson, AZ). Antigen retrieval was carried out using protease-1 for 32 min at 37 °C. Rabbit anti-hGPR41 immunoglobulins were applied at 0.2 mg/ml for 32 min at 37 °C, and horseradish peroxidase-conjugated goat anti-rabbit secondary IgG (Ventana Medical Systems, Inc.) was used according to the manufacturer's instructions. An Enhanced diaminobenzidine kit was used for detection, and sections were counterstained with hematoxylin.

RESULTS

The yeast *Saccharomyces cerevisiae* has been established as a useful host for heterologous expression and functional analysis of GPCRs (17). The system is engineered to allow mammalian GPCRs to couple to the endogenous yeast signal transduction pathway that responds to mating pheromone in wild-type cells. The yeast pheromone receptor Ste2p is deleted from the strains commonly used to provide a null background lacking endogenous host receptors. Another advantage, particularly in the analysis of orphan GPCRs for which no ligand is

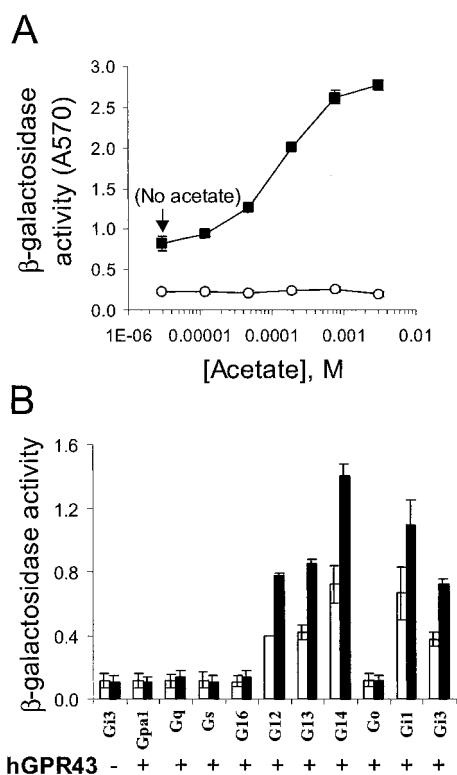


FIG. 1. Activation of the yeast pheromone response pathway by hGPR43. *A*, a yeast strain containing the Gpa1p/G α_{13} chimera and the pheromone pathway-responsive *FUS1-lacZ* reporter gene was transformed with a hGPR43 expression construct. β -Galactosidase activity was assessed in a chromogenic substrate bioassay buffered to pH 7.0. Introduction of hGPR43 into these cells caused significant reporter gene activation in the absence of added acetate (*No acetate*). Exposure to sodium acetate further activated hGPR43 in a concentration-dependent manner (\blacksquare), whereas no activation was detected in control cells expressing the human somatostatin SST2 receptor (\circ). *B*, the G protein coupling specificity of hGPR43 was determined in a panel of yeast strains expressing the yeast wild-type G α subunit Gpa1p or a range of different Gpa1p/G α chimeras. Chimeras contained the five C-terminal amino acids of the indicated mammalian G α subunits fused to the remainder of Gpa1p. *FUS1-lacZ* reporter gene induction due to the constitutive activity of hGPR43 was determined in acetate-free assay medium (*white bars*), and agonist-dependent activity was determined in the presence of 150 μ M acetate (*black bars*). Results from single representative experiments are presented. Data shown are the means \pm S.D. of four independent transformants.

known, is that ligand-independent G protein activation (“constitutive activity”) can frequently be detected, thus confirming functional expression of the receptor (7, 18). We used a set of plasmid constructs encoding orphan GPCRs under the transcriptional control of strong yeast promoters. These were introduced into yeast containing dual reporter genes and a chimeric G α subunit in which the five C-terminal amino acids of mammalian G α_{13} were fused to the remainder of the yeast G α protein Gpa1p (8). In the absence of any added ligand, the orphan hGPR43 caused significant activation of the *FUS1-lacZ* reporter gene, which is under the control of the pheromone response pathway in these cells, indicating that hGPR43 was functionally expressed (Fig. 1, *A* and *B*).

Yeast cells expressing hGPR43 were then used to screen a collection of known GPCR ligands plus compounds with demonstrated biological activity, but no known mechanism of action. Several compounds, all formulated as salts containing acetate counterions (19), activated hGPR43. Further experiments using sodium acetate or ammonium acetate buffered to pH 7.0 indicated that the acetate anion alone was sufficient to cause concentration-dependent reporter gene activation over

and above that attributable to the constitutive activity of hGPR43. The median effective concentration (EC_{50}) of this response was $137 \pm 24 \mu$ M. Control cells expressing the human somatostatin SST2 receptor (8) did not respond to acetate (Fig. 1*A*). To investigate the G protein specificity of this response, hGPR43 was introduced into a series of similar yeast strains containing different yeast/mammalian G α chimeras (8). Elevated reporter levels in the absence of acetate (constitutive activity) and induction in the presence of acetate indicated the activation of the particular chimera. hGPR43 could activate Gpa1p/G α chimeras containing the C termini of mammalian G α_{12} , G α_{13} , G α_{14} , G α_{11} , and G α_{13} (Fig. 1*B*). Because the G protein specificity for chimeras in yeast generally conforms to that observed in mammalian cells (8), this suggests that GPR43 may activate the G β , G γ , and G $\beta\gamma$ families of G proteins.

We next determined whether acetate could activate hGPR43 expressed in mammalian cells. HEK293 cells were transiently transfected with hGPR43 and loaded with the calcium-sensitive fluorescent dye Fluo-4. Intracellular calcium ion concentrations ($[Ca^{2+}]_i$) were measured using the FLIPR. Acetate provoked transient increases in $[Ca^{2+}]_i$ ($EC_{50} = 52 \pm 10 \mu$ M). Control cells, transiently transfected with hGPR40 or the μ -opioid receptor, showed no change in $[Ca^{2+}]_i$ in response to acetate (Fig. 2*A*). To determine which class of G protein was involved in this response, we used the specific inhibitor of G $\beta\gamma$ family proteins, PTX. Treatment with PTX had no significant effect on either the magnitude or EC_{50} of the $[Ca^{2+}]_i$ response to acetate in HEK293 cells expressing GPR43 compared with vehicle-treated cells. In contrast, PTX treatment completely abolished the $[Ca^{2+}]_i$ response of control cells stably expressing the APJ receptor (20) to apelin-13 (Fig. 2*B*). In a separate experiment, we introduced hGPR43 into HEK293T cells by cotransfection in combination with the G $\beta\gamma$ family protein G α_{o1} . Acetate provoked dose-dependent increases in $[^{35}S]GTP\gamma S$ binding in membranes prepared from these cells (Fig. 3). Acetate responses mediated by hGPR43 could also be detected in *X. laevis* oocytes upon coexpression with GIRK G protein-regulated potassium channels. *In vitro* synthesized RNA encoding hGPR43 and the inwardly rectifying potassium channel subunits GIRK1 and GIRK4 (Kir3.1/Kir3.4) were co-microinjected into oocytes, and transmembrane conductance was measured in the presence of a buffer containing a high concentration (90 mM) of potassium ions. Application of acetate resulted in inward shifts in holding current ($EC_{50} = 202 \pm 68 \mu$ M) (Fig. 2*C*). No such shifts occurred in control oocytes injected with GIRK1/GIRK4 alone ($n = 5$) (data not shown). Together, these results confirm the original observation in yeast that hGPR43 is activated by acetate.

Next, we determined whether other carboxylate anions could also activate hGPR43. The C $_3$ carboxylate propionate stimulated $[^{35}S]GTP\gamma S$ binding in HEK293T cell membranes containing hGPR43 and G α_{o1} with a potency similar to that of acetate. Longer chain acid anions were also active. Relative potencies were as follows: acetate (C $_2$) = propionate (C $_3$) = butyrate (C $_4$) > pentanoate (C $_5$) > hexanoate (C $_6$) = formate (C $_1$) (Fig. 3*A* and Table II). A similar rank order of potency for these ligands was found using the FLIPR assay (Table II). Another member of the GPR40–43 receptor subfamily, GPR41, is closely related to GPR43 (43% amino acid identity in human) and is encoded adjacent to *GPR43* at human chromosomal locus 19p13.1 (5). hGPR41 was cotransfected into HEK293T cells with G α_{o1} , and ligand-stimulated $[^{35}S]GTP\gamma S$ binding was assessed in membranes prepared from these cells. As expected from the close sequence similarity, hGPR41 was activated by the same carboxylate ligands, although with a different rank order of potency: propionate = pentanoate = butyrate > acetate > formate (Fig. 3*B*). Thus, hGPR41 and hGPR43 are both

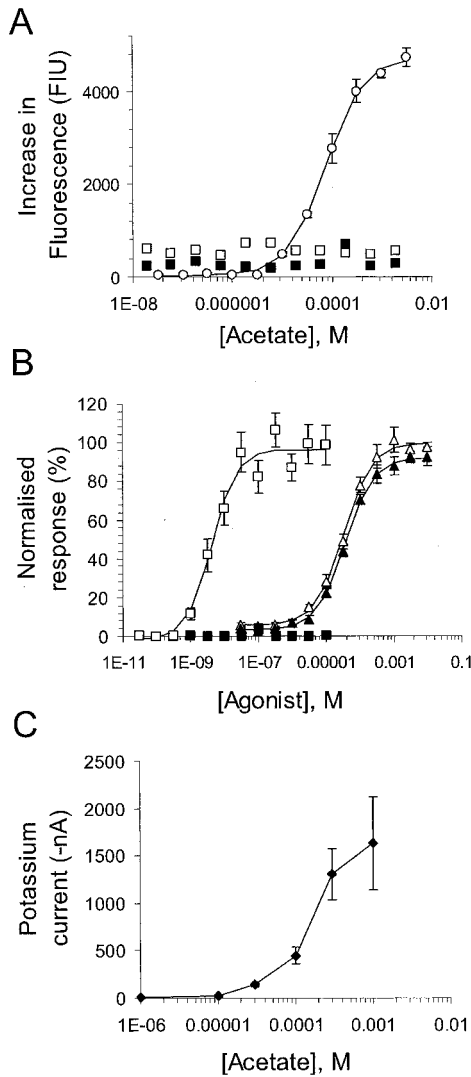


FIG. 2. Functional expression of hGPR43 in mammalian and *Xenopus* cells. *A*, HEK293 cells were transiently transfected with hGPR43 (○), hGPR40 (□), or the μ -opioid receptor (■), and intracellular Ca^{2+} levels were measured using the FLIPR apparatus during challenge with acetate. *B*, HEK293 cells transiently expressing hGPR43 were incubated with PTX (▲) or vehicle (△) prior to challenge with acetate and FLIPR assay. Control rat basophilic leukemia cells stably expressing the APJ receptor were treated in parallel with PTX (■) or vehicle (□) and stimulated with pyroglutamylated (>E) apelin-13 (>ERPRLSHKGPMPF). Data are expressed as a percentage of the maximum fitted response for each receptor in the absence of PTX and are the means \pm S.E. determined from 12 wells. *C*, *Xenopus* oocytes were microinjected with cRNAs encoding hGPR43 and the potassium channel subunits GIRK1 and GIRK4. After 3 days, transmembrane potassium conductance in response to acetate was measured by voltage clamp electrophysiology. Data are the means \pm S.E. of four separate oocytes. Results presented are from single representative experiments. FIU, fluorescence intensity units.

activated by carboxylate anions, but have differing specificities for carbon chain length, with pentanoate (C_5) activating hGPR41 more potently than acetate, but with acetate activating hGPR43 more potently than pentanoate. Formate and several of the longer chain carboxylates were lower efficacy agonists of both receptors relative to propionate. A range of saturated and unsaturated fatty acids containing nine or more carbon atoms were also tested, but no activation was observed for either GPR41 or GPR43 (data not shown). A third member of this family, GPR40, is more distantly related to GPR41 and GPR43 (33% amino acid identity between GPR40 and GPR43 in human). GPR40 is activated by carboxylate anions of longer

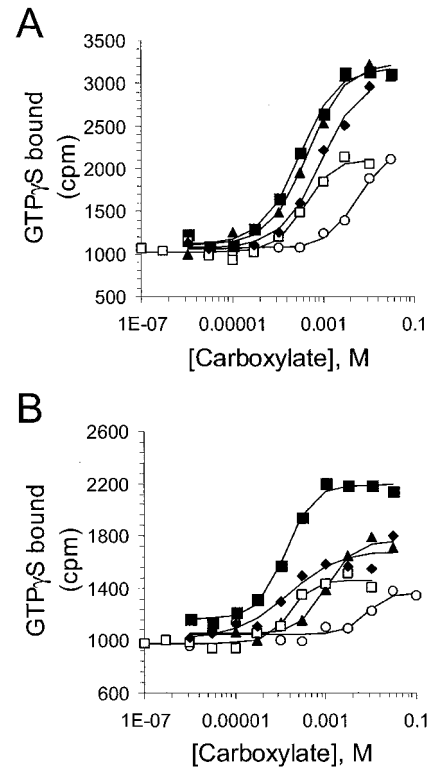


FIG. 3. Short chain carboxylic acids activate hGPR43 and hGPR41. Stimulation of [^{35}S]GTP γ S binding by formate (○), acetate (▲), propionate (■), butyrate (□), or pentanoate (◆) was determined in HEK293T membranes prepared from cells transiently transfected with hGPR43 (*A*) or hGPR41 (*B*) in combination with $\text{G}\alpha_{i1}$. Data from single representative experiments are presented as the means of assays performed in triplicate.

chain length (*i.e.* fatty acids), but does not appear to respond to carboxylate anions of six carbons or less (6).

hGPR42 is also located at 19p13.1 and differs from hGPR41 by only six amino acids (5). We performed similarity searches of public sequence databases to find mammalian orthologs of this pair. Two partially sequenced bacterial artificial chromosomes (GenBankTM/EBI accession numbers AC079472 and AC087143) from the mouse locus that is syntenic with human 19p13.1 contained *GPR40–43* orthologs. In mouse, only one ortholog of the hGPR41/hGPR42 pair was detected (72% amino acid similarity to hGPR41). From the mouse sequence, we designed oligonucleotide primers and amplified a rat hGPR41/hGPR42 ortholog (accession number AX224758). We also identified a *Bos taurus* ortholog from overlapping expressed sequence tags (accession numbers AW632495, AW660795, BE755048, and BI541693). The amino acid sequences of the orthologs were intermediate between hGPR41 and hGPR42, but more similar to hGPR41. Four of the six amino acid positions that differ between hGPR41 and hGPR42 are also conserved among the orthologs. The orthologs match hGPR41 at two or three of these positions, but match hGPR42 at only one (Table III). This suggests that hGPR42 occurred as the result of a gene duplication of hGPR41 that occurred after the human lineage diverged from the rodent and bovine lineages. Furthermore, we found that the rat ortholog is activated by carboxylate anions with similar potencies as hGPR41 (Table II). Because hGPR42 is also reported to occur infrequently in human populations as a polymorphic insert, the mouse, rat, and bovine genes have been named *Gpr41*.²

² The rat orphan GPCR termed GPR41 and described previously by Kimura *et al.* (32) is homologous to hGPR30 and is distinct from the rat ortholog of hGPR41 described here.

TABLE II
EC₅₀ values for carboxylate agonists of GPR41 and GPR43

	EC ₅₀				
	hGPR43		hGPR41	rGPR41	
	FLIPR	GTPγS binding	GTPγS binding	GTPγS binding	Yeast
	<i>μM</i>				
Formate (C ₁)	>1300	5640 ± 1480	7760 ± 3870	1000 ± 370	>10,000
Acetate (C ₂)	52 ± 10	431 ± 85	1020 ± 200	393 ± 91	422 ± 117
Propionate (C ₃)	31 ± 7	290 ± 42	127 ± 14	41 ± 17	7.8 ± 0.7
Butyrate (C ₄)	100 ± 16	371 ± 81	158 ± 35	33 ± 9	64 ± 22
Pentanoate (C ₅)	>50	876 ± 206	142 ± 87	31 ± 8	2.5 ± 0.3
Hexanoate (C ₆)	>300	1300 ± 580	ND ^a	ND	17 ± 2
Heptanoate (C ₇)	Inactive	ND	ND	ND	30 ± 5
Octanoate (C ₈)	Inactive	ND	ND	ND	Inactive

^a ND, not determined.

TABLE III
Amino acid positions differing between hGPR41 and hGPR42

The residue in each position is shown along with the residue at the equivalent position in the rat, mouse, and bovine orthologs and the phenotypes of mutations introduced into rGPR41 (from Fig. 4A). Position 346 in hGPR41 is the extreme C-terminal residue and is outside the region conserved in the orthologs.

Position in hGPR41	Amino acid					Mutant in rGPR41	Phenotype in yeast
	hGPR41	hGPR42	Rat	Mouse	Bovine		
44	Gln	Arg	Arg	Arg	Arg	R40Q	Functional
45	Arg	Cys	Arg	Arg	Arg	R41C	Functional
174	Arg	Trp	Arg	Arg	Arg	R170W	Inactive
227	Leu	Val	Leu	Leu	Ala	L223V	Constitutive activity
256	Ala	Val	TW	Ser	Thr	T252V	Reduced activity
346	Ser	Asn					

To determine the functional relevance of hGPR42, we prepared membranes from HEK293T cells transiently cotransfected with hGPR42 and Gα_{o1}. No stimulation of [³⁵S]GTPγS binding could be detected in response to carboxylate ligands (Fig. 4B). Immunoblotting using affinity-purified anti-SB130 serum, which was raised against a conserved region of the predicted C-terminal tail and which cross-reacts with both hGPR41 and hGPR42, confirmed that hGPR42 was present in the transfected cell membranes at levels equal to or greater than hGPR41 levels in control membranes (data not shown). This suggests that hGPR42 is not activated by carboxylate ligands. We introduced mutations into rat GPR41 (rGPR41) to investigate which of the residues differing between hGPR41 and hGPR42 allow carboxylate responses (Table III). These mutations had various effects on the response to propionate (Fig. 4A), but the most profound phenotype was observed with rGPR41(R170W), which failed to respond. The equivalent position 174 in extracellular loop 2 is positively charged Arg¹⁷⁴ in hGPR41, which could potentially form a salt bridge with the carboxylate ligand, whereas Trp¹⁷⁴ in hGPR42 could not. As expected, this residue is conserved between hGPR41 and mammalian orthologs. We also demonstrated that the single amino acid change W174R in hGPR42 was sufficient to restore responses to propionate (Fig. 4B). The magnitude of the response of hGPR42(W174R) was significantly less than that of wild-type hGPR41, suggesting that the amino acid differences at the other positions also influence receptor function. In conclusion, hGPR42 appears to have arisen by tandem duplication of hGPR41 and to have lost the ability to activate G_i family proteins in response to carboxylate ligands primarily due to an amino acid change at position 174. This does not exclude the possibility that hGPR42 may respond to other ligands.

TaqMan quantitative reverse transcriptase (RT)-PCR (PE Applied Biosystems) was used to determine mRNA distribution of hGPR41. To distinguish hGPR41 from hGPR42, we used a primer/probe set containing nucleotide differences between the pair. In control experiments, these primers gave large signals from genomic DNA or hGPR41 plasmid, but not from hGPR42

plasmid, confirming their specificity for hGPR41 (data not shown). hGPR41 mRNA was detected in a set of samples from normal human tissues, with the highest level in adipose tissue and lower expression across all tissues tested (Fig. 5A). A second primer set that annealed to sequences common to both hGPR41 and hGPR42 gave the same distribution pattern (data not shown). To confirm expression of GPR41 in adipose tissue, we examined levels of mouse *Gpr41* in 3T3-L1 and 3T3-F442A fibroblasts upon growth factor-induced differentiation into cells with an adipocyte-like morphology and containing cytoplasmic lipid droplets. As expected, CAAAT/enhancer-binding protein-α and adipin, which are characteristic of adipocytes (21, 22), were both induced during *in vitro* adipogenesis, whereas house-keeping genes were either unchanged or mildly repressed (Fig. 6). Mouse *Gpr41* was induced 10–20-fold, although the absolute levels of mouse *Gpr41* mRNA detected even after differentiation were low (3.1 ± 0.5 copies/50 ng of total RNA).

We also performed immunohistochemistry on sections from selected tissues using antisera that cross-react with both hGPR41 and GPR42. Staining of endothelial cells was detected consistently across the sections examined. This was most striking in white adipose tissue, as shown in Fig. 7A, and could be blocked by preincubation with the peptide antigen (Fig. 7B). Positive endothelial cell staining appeared to be mainly in arteries and arterioles rather than in veins and lymphatic vessels. Mesenchymal cells (either fibroblasts or preadipocytes) in the collagen layers of white adipose were also positive. Breast adipocytes were positive, although staining was faint (data not shown). In tonsil, the antibodies stained lymphocytes and plasma cells, primarily in the mucosa and submucosa; and this staining could be competed with the peptide antigen. In the tracheal-bronchial lymph node, there were positive fibroblasts or fibrocytes in the capsule layer, and plasma cells and a subset of lymphocytes were also stained. This is consistent with the RT-PCR data, which also showed hGPR41 in pancreas, spleen, and peripheral blood mononuclear cells (Fig. 5A).

TaqMan RT-PCR for hGPR43 detected mRNA at relatively high levels in lymphatic tissues such as spleen and adenoid as

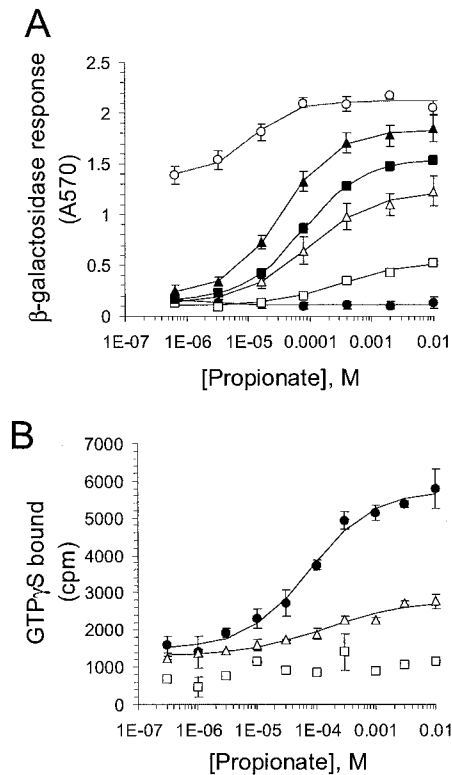


FIG. 4. Phenotypic effects of mutations introduced into rGPR41 and hGPR42. *A*, reporter gene activation was measured in yeast cells expressing wild-type rGPR41 (■) or rGPR41 containing mutations corresponding to the amino acid differences between hGPR41 and hGPR42. Mutations were R40Q (△), R41C (▲), R170W (●), L223V (○), and T252V (□). *B*, HEK293T cells were transiently transfected with hGPR41 (●), hGPR42 (□), or mutant hGPR42(W174R) (△) in combination with $G\alpha_{i1}$. Propionate concentration-response curves were determined in [35 S]GTP γ S binding assays.

well as myometrium and breast (data not shown). A lower level expression of hGPR43 was detected throughout a panel of non-diseased tissues. Consistent with the presence in spleen and adenoid, purified neutrophils and monocytes contained relatively high levels of hGPR43 mRNA, ~50-fold greater than detected in whole spleen (Fig. 5B). hGPR43 was also relatively strongly expressed in peripheral blood mononuclear cells and B-lymphocytes. No signals were detected in glioma, T-cells, or Raji cells. The pattern of widespread, low level expression of hGPR43 may be due to its presence in immune cells, *e.g.* in infiltrating neutrophils.

DISCUSSION

We have described the identification of ligands of GPR41 and GPR43. These orphan GPCRs are two members of a family of four (along with GPR40 and GPR42) that are related on the basis of primary sequence and colocalization at the same chromosomal locus (5). We originally identified acetate, present in our screening set as a counterion of several basic peptides, as an agonist of hGPR43. We then demonstrated that other short chain carboxylic acids, including propionate, also had agonist activity and that GPR41 could be activated by similar ligands. Together with evidence that GPR40 is activated by longer chain carboxylates, *i.e.* fatty acids (6), this identifies GPR40, GPR41, and GPR43 as a family of receptors activated by carboxylic acid anions. These findings were unexpected because this group of receptors is most closely related to the family of protease-activated receptors that are activated by an internal motif within their primary sequences following protease cleavage. There is, however, significant homology between GPR40

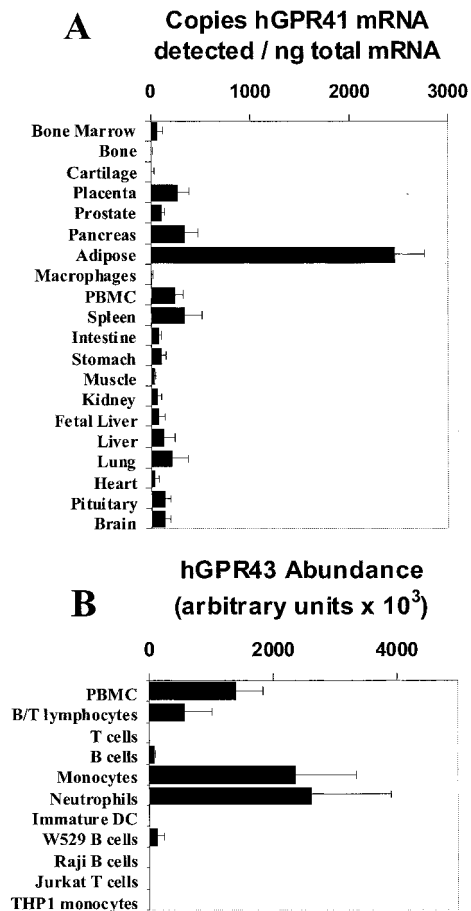


FIG. 5. Tissue localization of hGPR41 and hGPR43 using TaqMan methodology. *A*, hGPR41 mRNA levels were determined using poly(A)⁺ RNA from 20 tissues and GPR41-specific primers. Bars show the means \pm S.D. for samples from four individuals per tissue. The levels of β -actin in these samples varied within a normal range and have been described previously (16). *B*, hGPR43 mRNA levels were determined in immune cell samples. Relative levels were determined by calculating the threshold cycle (C_t), which is the cycle number at which reporter fluorescence passes a fixed threshold above the base line during the exponential phase of PCR, when none of the reaction components are limiting. An arbitrary cutoff of 40 cycles is used as a reference, and relative abundance is expressed as $2^{\Delta\Delta C_t}$, where $\Delta\Delta C_t = 40 \text{ cycles} - C_t$. Bars show the means \pm S.D. determined in duplicate. PBMC, peripheral blood mononuclear cells.

family receptors and the leukotriene receptor family; and because leukotrienes also contain a carboxylic acid moiety, this may reflect an evolutionary relationship and/or similar mode of ligand binding.

GPR42, which differs from hGPR41 at only six amino acid positions, is most likely the result of a recent gene duplication event. This is supported by the finding of single orthologs of hGPR41/hGPR42 in other mammalian species and by the greater sequence similarity of these orthologs to hGPR41. Furthermore, the rat ortholog responded to carboxylates with similar potencies as hGPR41, whereas GPR42 failed to respond to carboxylates. The lack of response appears to be largely due to the amino acid at position 174, which is arginine in hGPR41 and mammalian orthologs, but tryptophan in GPR42. Switching this residue by mutagenesis abolished the function of rGPR41 or partially restored function to GPR42. Thus, GPR42 appears to have acquired loss-of-function mutations since duplication. Conceivably, GPR42 may be a functional GPCR that responds to other as yet uncharacterized ligands, although this is unlikely considering that GPR42 differs from hGPR41 at so few residues. TaqMan RT-PCR using GPR42-specific primers

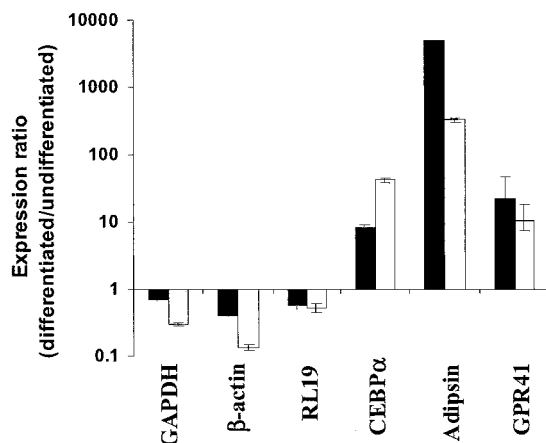


FIG. 6. Induction of mouse *Gpr41* mRNA in cultured Swiss 3T3 fibroblasts upon insulin/3-isobutyl-1-methylxanthine/dexamethasone-induced differentiation into adipocytes. Black bars, 3T3-L1 cells; white bars, 3T3-F442A cells. Total RNA (1 μ g) was reverse-transcribed using Superscript II according to the manufacturer's instructions with random priming. mRNA levels of three housekeeping genes (glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), β -actin, and ribosomal protein RL19), two adipose-specific genes known to be induced upon differentiation of 3T3 cells (CAAT/enhancer-binding protein- α (*CEBP α*) and adipsin), and mouse *Gpr41* were determined by TaqMan RT-PCR and comparison with genomic DNA standards. Bars show the means \pm range of experiments performed in duplicate.

detected no signal for *GPR42* mRNA in samples from normal human tissues (data not shown), although definitive determination of whether *GPR42* is transcribed may require sample genotyping because the *GPR42* gene is reportedly present in only a subset of individuals as a polymorphic insert (5).

GPR41 and GPR43 activate the $G_{i/o}$ family proteins, as indicated by agonist-stimulated [35 S]GTP γ S binding in membranes from HEK293T cells transfected with either receptor. Similarly, activation of GIRK channels in *Xenopus* oocytes by hGPR43 is a characteristic G_i -mediated response (23). However, the [Ca^{2+}] $_i$ response to acetate in HEK293 cells expressing hGPR43 was resistant to blockade by PTX, suggesting that hGPR43 may activate G_q in addition to G_i family proteins. The $G\alpha$ specificity in a panel of $G\alpha$ chimeras in yeast was largely consistent with that observed in mammalian cells. rGPR41 was constitutively active in yeast when coexpressed with Gpa1p/ $G\alpha_{11}$ and Gpa1p/ $G\alpha_{13}$ chimeras (data not shown) and responded to carboxylates when coexpressed in yeast with the Gpa1p/ $G\alpha_o$ chimera (Table II). Other yeast $G\alpha$ chimeras did not support rGPR41 coupling or constitutive activity.

GPR43 is expressed in immune cells, whereas GPR41 appears to be expressed in blood vessel endothelial cells, particularly in adipose tissue, with significant expression also in immune cells and endothelial cells of other tissues. GPR41 levels in adipocytes appear to be low because only weak staining was observed in adipocytes of human adipose tissue sections and because 3T3 cells contained very low levels of mouse *Gpr41* mRNA after *in vitro* adipogenesis. Consistent with this, we have been unable to demonstrate reproducible inhibition of isoprenaline-stimulated lipolysis by carboxylate ligands in rat primary adipocytes (data not shown). This is in contrast to known adipose G_i -coupled receptors such as EP $_3$ (24, 25) and suggests that GPR41 does not directly regulate lipolysis.

At least two of the GPR41 and GPR43 agonists identified here occur in humans in tissue fluid under certain physiological or pathophysiological conditions. Acetate is produced in the liver as a metabolite of ethanol, but this is rapidly absorbed from the circulation by tissues and converted to acetyl-CoA, so concentrations of acetate from this source may not reach levels high enough to activate GPR41 or GPR43. Accumulation of

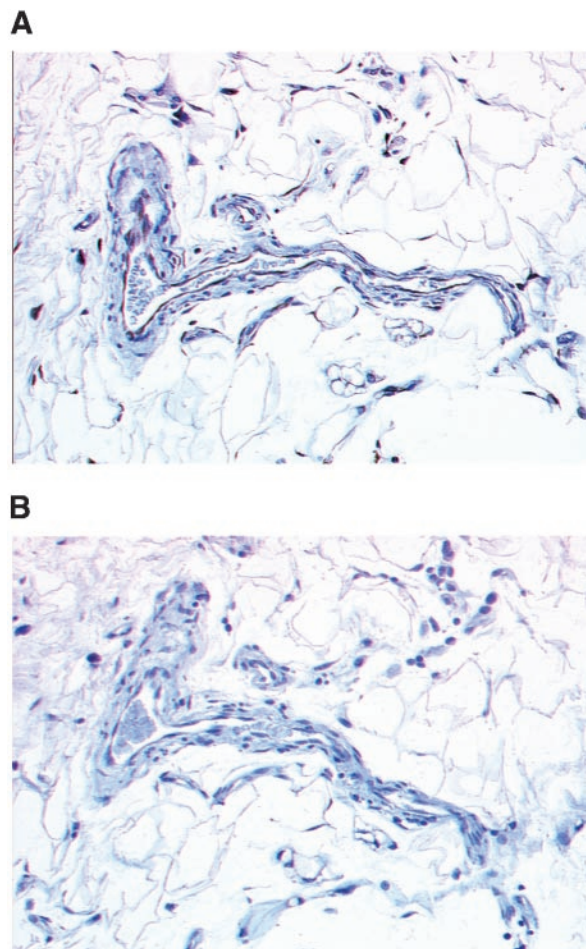


FIG. 7. Immunolocalization of hGPR41 and hGPR42. A, adipose tissue section stained with affinity-purified rabbit immunoglobulins. The peptide antigen used in antibody generation is conserved in the C-terminal tails of both hGPR41 and hGPR42, and the antibodies reacted with both proteins upon immunoblotting. B, adjacent tissue section stained after preincubation of immunoglobulins with the peptide antigen.

propionate in the blood is a characteristic feature of the disease propionic acidemia (PA) (26). This rare inherited disorder is caused by deficient activity of propionyl-CoA carboxylase (EC 6.4.1.3). This results in an inability to convert propionyl-CoA, formed through catabolism of essential amino acids and fatty acids containing odd numbers of carbon atoms, to methylmalonyl-CoA. PA causes neonatal or infantile symptoms of ketoacidosis resulting from lowering of blood pH. Another clinical feature is the impairment of immune function and frequent infection, often due to opportunistic pathogens (26). Low serum IgG and IgM, leukopenia, lymphopenia, granulopenia, and deficiency of peripheral B-cells have variously been associated with PA (27). Immunodeficiency is common to several other diseases that also result in propionate accumulation, including inherited defects in methylmalonyl-CoA mutase (26). In contrast, ketoacidoses that result from accumulation of organic acids other than propionate are not reported to have profound effects on immune function.

Propionate itself has been implicated as the immunosuppressive factor in PA serum (28). Propionate inhibits the proliferation of granulocyte/macrophage progenitor cells and colony formation of T-lymphocytes in culture (29, 30). It also suppresses proliferation and maturation of hematopoietic progenitor cells (31) and inhibits lymphocyte activation (28). These properties are apparently not due to cytotoxicity and are specific to pro-

propionate over other organic acids such as methylmalonate (29). Hence, there is a striking overlap between the tissue distribution of GPR43, a receptor activated by propionate, and the cell types in which propionate has reported pharmacological effects *in vivo*. The concentrations of propionate that elicit immunosuppressive effects *in vitro* are high (1–10 mM), but similarly high levels occur in the sera of PA patients (26), and these levels are consistent with the concentrations required to activate GPR43 in recombinant assays. An involvement in PA can also be postulated for GPR41 because this receptor is also expressed in immune cells. Although other mechanisms for the immunosuppressive effects of propionate have been proposed (28), the possible role of GPR40 family receptors in this disease process should now be considered. To our knowledge, no abnormality of other tissues expressing GPR41, such as adipose or vascular tissue, has been described in PA, although the severity of acidosis may mask this. For both GPR41 and GPR43, full description of their functional roles will await the development of specific high affinity agonist and antagonist ligands and the evaluation of null alleles.

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