Study of GPR81, the lactate receptor, from distant species identifies residues and motifs critical for GPR81 functions

Chester Kuei, Jingxue Yu, Jessica Zhu, Jiejun Wu, Li Zhang, Amy Shih, Taraneh Mirzadegan, Timothy Lovenberg, and Changlu Liu

Johnson & Johnson Pharmaceutical Research & Development, L.L.C., San Diego, California (KC, YJ, ZJ, WJ, ZL, SA, MT, LT, and LC)
Running title: Residues critical for GPR81 function

Address correspondence to:
Changlu Liu
Johnson & Johnson Pharmaceutical Research & Development, L.L.C.
3210 Merryfield Row
San Diego, California 92121
Tel: 858-784-3059
Fax: 858-450-2090
E-mail: cliu9@its.jnj.com

The number of text pages: 34
The number of Tables: 2
The number of Figures: 8
The number of references: 41
Words in Abstract: 242
Words in Introduction: 636
Words in Discussion: 2447

Abbreviations: GPCR, G-protein coupled receptor; 3-OH-Octanoic acid, 3-hydroxy-octanoic acid
Abstract

Receptors from distant species may have conserved functions despite significant differences in protein sequences. While the non-critical residues are often changed in distant species, the amino acids critical in receptor functions are often conserved. Studying the conserved residues between receptors from distant species offers valuable information to probe the roles of residues in receptor function. We identified 2 zebrafish receptors (zGPR81-1 and zGPR81-2) that show about 60% identity to human GPR81, GPR109a, and GPR109b but respond only to L-lactate and not to the GPR109a ligands. Protein sequence comparison among zebrafish GPR81s, mammalian GPR81s, GPR109, and GPR109b identified a common structure (6 Cys residues at the extracellular domains that potentially form three disulfide bonds) in this subfamily of receptors. In addition, a number of residues conserved in all GPR81s but not in GPR109s have been identified. Furthermore, we identified a conserved motif, Cys165-Glu166-Ser167-Phe168, at the 2nd extracellular loop of GPR81. Using site-directed mutagenesis, we showed that Arg71 at the TM2 is very critical for GPR81 function. In addition, we demonstrated that the Cys165-Glu166-Ser167-Phe168 motif at the 2nd ECL is critical for GPR81 function, and the conserved 6 Cys residues at the extracellular regions are necessary for GPR81 function. It is important to mention for those residues important for GPR81 function, the corresponding residues, or motifs in GPR109a are also critical for GPR109a function. These findings help us better understand the interaction between lactate and GPR81 and provide useful information for GPR81 ligand design.
Introduction

Lactate is a metabolite of glucose and also an energy source to many tissues including the central nervous system (Bergersen, 2007; Dienel, 2004). It is essentially produced by all tissues and cell types and at increased levels during intense exercise or under anaerobic conditions (Cheetham et al., 1986; Hughson et al., 1987; Ohkuwa et al., 1984). Elevated concentration of L-lactate has been reported in obesity, and type 2 diabetes (Chen et al., 1993; De Pergola, et al., 1989). Certain tissues and cells such as adipocytes (DiGirolamo, 1992) and astrocytes (Kasischke, 2008) produce higher levels of lactate under normal physiological conditions. Adipose tissues produce lactate particularly after a glucose load or meal ingestion (Hagström et al., 1990; Ahmed et al., 2010). It has been recorded that fat tissues account for about 30% of glucose uptake but a significant amount of the glucose is converted into lactate and redistributed to other tissues (DiGirolamo, 1992). Therefore, lactate serves as an alternative energy venue for glucose. Astrocytes have been known to uptake glucose and convert it to lactate as the preferred energy source for neurons (Dienel, 2004).

Recently, lactate has been identified as a ligand for GPR81 (Liu et al., 2009; Cai et al., 2008). It activates GPR81 with an EC50 value about 5 mM, which is within the physiological concentration range (1-20 mM) of lactate, strongly suggesting that lactate is a physiological ligand for GPR81. Lactate inhibits lipolysis in adipocytes from the wild type mice but not in GPR81-deficient mice (Liu et al., 2009), further indicating that GPR81 is the cognate receptor for lactate and suggesting that GPR81 may be an attractive drug discovery target for metabolic disorders. However the detailed physiological roles of lactate and GPR81 need to be further studied. Since lactate exhibits low affinity and fast turnover rate, it’s very difficult to use lactate as a pharmacological ligand to investigate the in vivo function of this ligand/receptor pair. High affinity GPR81 ligand will be very helpful to facilitate this study. Receptor modeling and mutagenesis studies can provide useful
information for ligand design. In our previous studies, through modeling and mutation studies, we proposed that Arg99, Tyr233, Arg240, and Thr267 of the human GPR81 may play roles in lactate interaction. Since current GPCR modeling is largely based on the homology to rhodopsin and major attention has been focused on the TM3 to TM7, other TMs and particularly the extracellular regions received little attention in GPCR modeling (Benned-Jensen & Rosenkilde, 2009). Accumulating evidence strongly suggest that other TMs (particularly TM2), and the extracellular regions play important roles in ligand/receptor interactions either directly or indirectly (Clark et al., 2009; Benned-Jensen & Rosenkilde, 2009; Zhu et al., 2008; Miura et al., 2003; Ahn et al., 2009; D’Aoust & Tiberi, 2010; Sakai et al., 2010).

In this study, we study GPR81 receptors from distant species (human and fish), which are dramatically different in receptor sequences but still share lactate as the common ligand. Two zebrafish GPR81 receptors have been identified, which share similar identities to human GPR81, GPR109a (the receptor for niacin and β-hydroxybutyrate; Tunaru et al., 2003; Wise et al., 2003), and GPR109b (the receptor for 3-OH-Octanoic acid; Ahmed et al., 2009). Interestingly, the two zebrafish receptors only respond to lactate but not to GPR109a ligands. The functional conservation and the sequence diversity between the human and zebrafish GPR81s offer very useful information to study the role of the residues involved in the specific interactions between lactate and GPR81. In this report, we probed these conserved residues between the human and zebrafish GPR81, but different in GPR109a and GPR109b, using mutagenesis studies. In addition, the 6 Cys residues at the extracellular domain of GPR81 are found to be conserved in GPR81s from all species, GPR109a, and GPR109b. Therefore, in this report, we also investigated the role of these Cys residues.
Materials and Methods

Materials

All materials except where indicated were purchased from Sigma-Aldrich.

Identification and cloning of zebrafish gpr81-related genes

Human GPR81 protein sequence was used as the query to search the NCBI zebrafish genomic DNA database. Four gpr81-related sequences were identified from 3 zebrafish genomic contigs (Genbank Accession No. NW_001877090.1; NW_001877330.1; NW_001877242.1). The predicted coding regions of the 4 zebrafish gpr81-related genes were designated zgpr81-1, zgpr81-2, zgpr81-3, and zgpr81-4, respectively. Primers (P1 and P2 for zgpr81-1; P3 and P4 for zgpr81-2; P5 and P6 for zgpr81-3; P7 and P8 for zgpr81-4) flanking the predicted zebrafish gpr81-related gene coding regions were designed to amplify the genes using zebrafish genomic DNA (Biochain, Hayward, CA) as the template. The resulting PCR products were purified and sequenced using internal primers to confirm the sequence identity and open reading frame for each gene. The complete coding region of the zebrafish gene were submitted to Genbank (Accession No: zgpr81-1, EU809467; zgpr81-2, EU809468; zgpr81-3, HM030492; zgpr81-4, HM030493). Specific primers (P9 and P10 for zgpr81-1; P11 and P12 for zgpr81-2; P13 and P14 for zgpr81-3; P15 and P16 for zgpr81-4) were then designed to amplify and clone the coding regions of each gene. The genes were cloned into pCMV-Sport1 (Invitrogen, Carlsbad, CA) and sequence identities were confirmed by DNA sequencing (Eton Biosciences, San Diego, CA). All primer sequences are listed in the Supplementary Table 1.

Mutagenesis of human GPR81 and GPR109a

All mutations were performed using standard mutagenesis protocols. The coding region of human GPR81 with a V5-tag at the N-terminus (V5-GPR81) was cloned into pClneo expression vector (Liu et al., 2009) and served as the template for mutation studies. The resulted mutants were
sequenced by DNA sequencing (Eton Biosciences) to confirm the sequence identities. For GPR109a, the coding region of GPR109a with a V5-tag at the N-terminus was served as positive control and template for GPR109a mutation studies. All GPR109a mutant characterizations were performed in the same way as for GPR81 mutants except where indicated.

Recombinant expression and characterization of GPR81 and GPR109a

We compared the GTPγS binding results of GPR81 and a mutant receptor recombinantly expressed alone, co-expressed with Go2 subunit, or co-expressed with Go2, Gβ1, and Gγ2 together in COS7 cells. The results showed that while GPR81 expressed alone offered very low ligand stimulated 35S-GTPγS binding, co-expression with Go2 subunit increased the ligand-stimulated 35S-GTPγS binding, providing sufficient signal/noise ratio for the pharmacological characterization of GPR81. Co-expression of GPR81 with Go2, Gβ1, and Gγ2 together further increase the ligand stimulated 35S-GTPγS binding without changing the EC50 values (Supplementary Figure 1). In this report, all expression constructs were transiently co-expressed with a human G-protein Go2 expression construct (a human Go2 gene, Genbank Accession No. AF493895, cloned in pcDNA3.1) in COS-7 cells using LipofectAmine (Invitrogen) as the transfection reagent. Cells transfected with Go2 expression construct alone were used as the negative controls. Wild type human GPR81 and GPR109a with a V5 tag at the N-terminus (V5-GPR81 or V5-GPR109a) were used as the positive controls. Two days after transfection, cells were harvested and the cell pellets were stored at -80°C until assay for GTPγS binding or radioligand binding assays. The protein expressions of wild type and all mutants were verified by Western blot analysis using anti-V5 antibody (Invitrogen) to verify the molecular size of the recombinant protein (Supplementary Figure 2). To assess the total protein and cell surface protein expression levels, the transfected cells were re-seeded in 96-well culture plates at triplicates (4 x 10^4 cells/well) one day after transfection. Two day after transfection, cells were then fixed with formalin. The total and cell
surface expression of V5-tagged proteins were quantified by ELISA using Anti-V5 antibodies either in the presence of cell membrane penetrating reagent (1% Triton-x-100, for total protein detection) or absence of cell membrane penetrating reagent (for cell surface protein expression). Mock transfected cells and cells tranfected with a construct expressing GPR81-V5, a GPR81 expression construct with a V5-tag at the C-terminus, were also included as controls. V5-GPR81 expressing cells diluted with mock transfected cells at various ratios were seeded in the same plates to serve as standards for protein expression quantification.

**Pharmacological characterization of zGPR81s, the mutant human GPR81s, and the mutant GPR109a receptors**

GTP\(\gamma\)S assay was used to characterize the pharmacological profile of the zebrafish GPR81, human GPR81 mutant receptors, and GPR109a mutant receptors. The assay was performed as described previously (Liu et al., 2003). \(^{35}\text{S}-\text{GTP}\gamma\)S (PerkinElmer, Los Angeles, CA) was used as the tracer. \(^{35}\text{S}-\text{GTP}\gamma\)S bindings in the presence of ligand (L-lactate for GPR81 and niacin for GPR109a) were expressed as the percentage of the \(^{35}\text{S}-\text{GTP}\gamma\)S binding in the absence of ligands. The data were analyzed using the GraphPad Prism 5 software (GraphPad, San Diego, CA) and the EC\(_{50}\) values were calculated as the ligand concentration that stimulated 50% of maximum ligand-induced response (Emax). The EC\(_{50}\) and Emax values for mutants that did not reach plateau in GTP\(\gamma\)S binding assay at the highest concentration of ligand used (100 mM of L-lactate for GPR81 mutants and 10 mM of niacin for GPR109a mutants) were not calculated. For GPR81 mutants, due to the lack of high affinity radio-ligand (\(^{3}\text{H}-\text{lactate did not confer detectable binding due to its low affinity}), we did not characterize GPR81 and the GPR81 mutants with radioligand binding assay. For GPR109a mutants, they were characterized in Scatchard isotherms saturation binding assays using \(^{3}\text{H}-\text{labeled niacin as described (Liu et al., 2001) as the ligand and the K}_d\) values were calculated.
Molecular Modeling

Molecular modeling was done using Discovery Studio 3.0 (Accelrys Software). The primary sequence alignment between turkey β1 adrenergic receptor, 2VT4 (Protein Data Bank code) and GPR81 was determined using the align123 program implemented in Discovery Studio. The helical alignment was further examined and refined based on the multiple sequence alignment of family A GPCRs (Mirzadegan et al., 2003). The β1 adrenergic receptor structure (2VT4) was used as a template, and based on the sequence alignment, a GPR81 homology model was built and loops refined. The GPR81 homology model was placed into a 30 Å thick implicit membrane and energy-minimized using the Smart Minimizer algorithm with Generalized Born with Implicit Membrane implicit solvent model using the CHARMm forcefield. A manual docking of the lactate ligand was followed by a standard dynamics cascade (which includes steepest decent minimization, conjugate gradient minimization, heating of the system to 300K, molecular dynamics equilibration, and production runs) with the ligand constrained. The constraints on the ligand were then removed and the entire system further minimized. Molecular renderings were done using VMD (Humphrey et al., 1996).

Results

Molecular cloning and pharmacological characterization of zebrafish GPR81-like genes

Using human GPR81 protein sequence as the query, we blast-searched the zebrafish genomic DNA database using the NCBI Tblastn program and identified 4 sequence contigs that code for GPR81-like proteins, which share greater than 50% identity to human GPR81 protein. The four putative zebrafish gpr81-like genes were designated as zgpr81-1, zgpr81-2, zgpr81-3, zgpr81-4 respectively. The protein sequence alignment among human GPR81, zGPR81-1, zGPR81-2, human GPR109a, and human GPR109b are shown in Fig. 1.
GPR109a, GPR109b, and the putative proteins encoded by zebrafish gpr81-like genes are shown in Supplementary Table 2. The four putative zebrafish gpr81-like genes were cloned and recombinantly expressed in COS7 cells. Pharmacological characterization of the recombinantly expressed zebrafish receptors indicated that while the recombinant zGPR81-1 and zGPR81-2 responded to L-lactate stimulation at EC$_{50}$ values around 1 mM in GTP$\gamma$S binding assay (Fig. 2), zGPR81-3 and zGPR81-4 did not respond to lactate stimulation (data not shown). Despite sharing high identity to GPR109a, all 4 GPR81-like zebrafish receptors did not respond to GPR109a ligands (niacin or β-hydroxybutyrate).

Biochemical characterization of recombinant GPR81 and GPR109a protein expression

Western blot analysis was used to characterize the size of wild type and mutant receptors. Wild type GPR81 and GPR109a have a predicted molecular weight of 40 kDa and 41 kDa, respectively (without counting posttranslational modification). In Western blot analysis of the recombinant protein expression, the wild type GPR81, GPR109a, and all mutant receptors demonstrated very similar molecular weights at about 45 kDa (Supplementary Figure 2). The total receptor protein expression quantified by ELISA showed that all mutant receptors have comparable expression levels to that of the wild type receptors (Table 1 for GPR81 mutants and Table 2 for GPR109a mutants). Cell surface expression analysis by ELISA showed that GPR81 mutants Cys88Ala, Cys88Ser, His155Ala, Cys165Ala, and Cys165Ser lacked cell surface expression. GPR81 mutants Glu166Ala, Glu166Ile, Ser167Ala, and Phe168Tyr have reduced cell surface protein expression (Table 1). For GPR109a mutants, all mutants had comparable cell surface expression levels to that of the wild type GPR109a (Table 2).
Investigation of Cys residues at the extracellular domain of GPR81

Within the extracellular domains of the human GPR81, there are 6 Cys residues. Two of them are located at the N-terminal domains (Cys6 and Cys7), one at the 1st extracellular loop (ECL, Cys88), two at the 2nd ECL (Cys157, Cys165), and one at the 3rd ECL (Cys252). Those Cys residues are conserved among all known mammalian GPR81s, zGPR81-1, and zGPR81-2 from zebrafish, as well as GPR109a and GPR109b (Fig. 1). We investigated the role of these Cys residues by changing them individually into Ala or Ser residues. As controls, 6 more Cys residues at the transmembrane regions (Cys38 and Cys41 at TM1, Cys67 at TM2, Cys137 at TM3, Cys195 at TM5, and Cys232 at TM6) were studies in parallel. Our results showed that changing any of these Cys residues at the ECL into Ala (Fig. 3) or Ser (Table 1) abolished GPR81 receptor activity. In contrast, mutations of the Cys residues within the transmembrane domain regions have little effect on the receptor activity (Fig. 3, Table 1).

Mutation studies of residues at the transmembrane domains of GPR81

Previously, our initial mutation studies showed that each of Arg99Ala (TM3), Tyr233Ala (TM6), Arg240Ala (TM6), and Thr267Ala (TM7) mutations abolish GPR81 receptor activity (Liu et al., 2009). By comparing the sequences between the human and zebrafish GPR81s, we identified that Arg71 in TM2 and Ala100 at TM3 are conserved in all known mammalian GPR81 as wells as the two zebrafish GPR81 receptors but not in GPR109a and GPR109b. Mutations of Arg71 to Ala, Leu (the corresponding residue in GPR109a), or a conserved residue (Lys) all abolished GPR81 functions. In contrast, changing Ala100 to Gln (the corresponding residue in GPR109a and GPR109b) only produced slight reduction in Emax value (accompanied with slight reduction of cell surface receptor expression) but not EC50 value of GPR81 when tested using L-lactate as the ligand (Fig. 4). The EC50 values and Emax values of those mutants are shown in Table 1.
Mutation studies at the extracellular regions of GPR81

His80 at the ECL1, His155, and Glu166 at the ECL2 are conserved in GPR81 from all species but not in GPR109a and GPR109b. In addition, Cys165, Glu166, Ser167, and Phe168 at the ECL2 form a Cys-Glu-Ser-Phe motif compared with that in GPR109a (Cys-Ser-Ser-Phe) and GPR109b (Cys-Ile-Ser-Phe). In this study, we also assessed the roles of Ser167, and Phe168 (in addition to Cys165 and Glu166) in GPR81. In ECL3, except residue Cys252, other residues are not conserved between the human and zebrafish GPR81s. Therefore no additional residues were selected for mutation studies. Pharmacological characterization of the mutant receptors showed that while mutations at His80 did not produce dramatic changes to GPR81 receptor function, a His155Ala mutation abolished GPR81 function. Protein expression analysis showed that this mutant lacked cell surface expression (Table 1). Change of Glu166 to Ala, Ser (the corresponding residue in GPR109a), or Ile (the corresponding residue in GPR109b), but not Asp, abolished GPR81 activity. A Ser167Ala mutation reduced the Emax value but not the EC$_{50}$ value. This reduction of Emax value is likely due to the reduced cell surface expression (Table 1), suggesting a hydrophilic residue at this position is needed for efficient GPR81 cell surface localization. Supporting this hypothesis, a Ser167Thr mutation had little effect to receptor expression or receptor functions. An aromatic residue at the 168 position seems important. A Phe168 to Ala mutation abolished GPR81 activity while a Phe168Tyr mutation retained activity with slight Emax reduction accompanied by slightly deceased cell surface expression. The EC$_{50}$ values and Emax values for all mutants are listed in Table 1 and example results are shown in Fig 5.

Pharmacological characterization of GPR109a mutants

To investigate whether residues in GPR109a corresponding to residues critical for GPR81 functions, we evaluated these residues in GPR109a through mutagenesis studies. The GPR109a mutants were characterized by GTP$_{\gamma}$S binding assay for receptor activation and by saturation...
binding to determine the binding affinity using ^3^H-labeled niacin. Previously, through mutagenesis studies, Tunaru et al. (2005) showed that Cys100, Cys177, Cys183, Cys266 are important for GPR109a functions. In this report, we showed that Cys18 and Cys19 at the N-terminus are also critical for GPR109a functions (Fig. 6, Table 2). While changing Cys18 to Ala reduced the sensitivity and affinity of GPR109a to niacin dramatically, changing Cys19 to Ala completely abolished GPR109a functions. The results from receptor cell surface expression analysis demonstrated that both mutants had comparable cell surface expression. The results from this report and previous studies suggest that the 6 Cys residues at the extracellular regions of GPR109a also form three pairs of disulfide bonds. Next, we studied residue Leu83 at the TM2 of GPR109a corresponding to Arg71 in GPR81. Previous study showed that a Leu83Val mutation only slightly reduced the affinity of GPR109a to niacin (Tunaru et al., 2005). In our currently report, Leu83 in GPR109a was changed into Arg (the corresponding residue in GPR81) or Ala and then we analyzed the effects of these mutations to the functions of GPR109a. The results demonstrated that a Leu83Ala mutation reduced the affinity of GPR109a to niacin but did not change the Emax value in the functional GTP\(\gamma\)S binding assay while a Leu83Arg mutation demolished GPR109a activity in both radioligand binding and functional assays (Fig. 6, Table 2).

**Discussion**

The recent finding of L-lactate as the endogenous ligand for GPR81 identifies a new signaling pathway in the human body. L-lactate, a metabolite of glucose, has long been viewed as a byproduct or a waste for anaerobic oxidation of glucose but has gradually gained increased attention as an energy source as well as a signaling molecule (Achten et al., 2004; Kamijo et al., 2000; Lam et al., 2005; Miller et al., 1964; Issekutz et al., 1965). Niacin, a ligand for GPR109a, has been used as an effective medicine for dyslipidemia over the past half century, but it has a side effect of upper trunk flushing due to the activation of Langerhans cells in the skin (Benyó et al.,
The unique expression pattern of GPR81, which is predominantly expressed in the adipose tissues (Ge et al., 2008, Liu et al., 2009) and the link of lactate to glucose metabolism make GPR81 an attractive target for metabolic disorders such as dyslipidemia, obesity, and diabetes. It has been shown that L-lactate can inhibit lipolysis in adipocytes \textit{in vitro} (Boyd et al., 1974; Puhakainen et al., 1993, Liu et al., 2009; Cai et., 2008). However, the exact role of lactate and GPR81 in glucose metabolism and obesity needs to be further studied and will require the development of pharmacological reagents that either activate or block the receptor \textit{in vivo}. The low affinity of lactate for GPR81 coupled with its fast metabolism \textit{in vivo} makes it very impractical for the administration of lactate as a probe agonist. In addition, lactate can be converted to glucose \textit{in vivo} through gluconeogenesis. The administration of lactate will add undesired complications to metabolic studies. The development of small molecule agonists and antagonists for GPR81 will greatly enhance our ability to understand its physiological role. Our previous modeling and mutation studies (Liu et al., 2009) suggested that residues Arg99, Tyr233, Arg240, and Thr267 are critical for GPR81 function and may interact with lactate. In this study, we probed the conserved residues/motifs in GPR81 for their roles in receptor functions and we believe the resulting information will help delineate the molecular mechanism of L-lactate/GPR81 interaction and the design of small molecule agonists and antagonists for GPR81.

The function of GPR81 is conserved in zebrafish

Lactate is a basic metabolite that essentially exists in all animals. If lactate serves as a signaling molecule in the human body as a metabolic feedback signal, it is very likely that it may also play similar roles in many different species. Previously, we have cloned GPR81 from many mammalian species and demonstrated their conservation at both the molecular and the pharmacological levels (Liu et al., 2009). In this study, by searching the zebrafish genomic sequence database, we
identified two zebrafish genes encoding putative receptors that have similar identity to the lactate receptor, GPR81, the niacin receptor, GPR109a (aka HM74A in human and PUMAG in mouse), and GPR109b. Molecular cloning confirms the existence of the two genes and in vitro pharmacology demonstrated that the two zebrafish GPR81-like receptors (namely zGPR81-1 and zGPR81-2) both respond to L-lactate at the physiological levels (1 to 2 mM,) but not to GPR109a agonists such as niacin and β-hydroxybutyrate (Tunaru, et al 2003; Taggart et al., 2005). These results suggest that lactate/GPR81 is an endogenous ligand receptor pair that has been utilized by many species including mammals and fish.

**Study of zebrafish GPR81 unveils critical motifs/residues for GPR81 function.**

Evolution creates differences between species through millions of years of natural mutations. These differences are certainly reflected at the gene and the protein sequence levels. However, the functions of the proteins are often conserved and the critical residues for a conserved protein are likely retained. Therefore, we hypothesized that by comparing the protein sequences between the human GPR81 and the zebrafish GPR81s, we may learn very useful information that can guide us to study the molecular mechanism of lactate/GPR81 interactions. In this report, we compared residues that are common between human and zebrafish GPR81s but different in GPR109a and GPR109b. Residues conserved between human and zebrafish GPR81s but different from GPR109a and GPR109b may play a role for the specific interaction between lactate and GPR81. While niacin and β-hydroxybutyrate are ligands for GPR109a, L-lactate and α-hydroxybutyrate are ligands for GPR81 (Liu et al., 2009). It seems that the main difference between GPR81 ligands and GPR109a ligands is the position of the hydroxyl group on the side chain. The highly conserved residues in GPR81 but different in GPR109 are likely involved in the interactions between GPR81 and the α-hydroxyl group of L-lactate.
The highly conserved Cys-Glu-Ser-Phe motif in the extracellular loop 2 is essential for GPR81 function

The extracellular loops (ECL), particularly ECL2 and ECL3, of GPR81 are the least conserved regions between the human and zebrafish receptors (Fig. 1). Similarly, these regions are also the least conserved regions between GPR81 and GPR109a. However in ECL2 region there is a Cys165-Glu166-Ser167-Phe168 motif that is conserved in all GPR81 receptors while this motif is Cys177-Ser178-Ser179-Phe180 in GPR109a and Cys177-Ile178-Ser179-Phe180 in GPR109b. Previous mutation studies showed that changing Ser178 to Ile or Phe180 to Ala abolished the receptor function of GPR109a. Hydrogen-bonding between Ser178 in GPR109a and the pyridine nitrogen in niacin has been proposed (Tunaru et al., 2005). Structures comparison of GPR109a ligands, niacin and \( \beta \)-hydroxybutyrate (Fig. 7A), suggests that it’s likely that the \( \beta \)-hydroxyl group in \( \beta \)-hydroxybutyrate also interacts with Ser178 in GPR109a through hydrogen bonding. The functional role of Phe180 in GPR109a is suggested to interact through hydrophobic interactions with other residues in the binding site to increase the rigidity of the binding pocket (Tunaru et al., 2005). In this report, for GPR81, we showed that changes of Glu166 to Ser, Ile, or Ala abolished GPR81 activity, demonstrating the importance of this residue in GPR81 function. A Glu166Asp mutant retained almost full receptor activity, strongly suggesting that an acidic residue is required at this position. The high conservation requirement at this position for GPR81 suggests that Glu166 interacts with a special group in L-lactate that is different from GPR109a ligand, \( \beta \)-hydroxybutyrate. Structural comparison between GPR81 and GPR109a ligands (Fig. 7A) indicates that the \( \alpha \)-hydroxyl group in L-lactate and \( \alpha \)-hydroxybutyrate is a top candidate to interact with Glu166. Phe168 in GPR81 is also critical for GPR81 functions. A Phe168Ala mutation abolished GPR81 function but a Phe168Tyr mutant retained function suggests that an aromatic (or a
A hydrophobic residue is necessary at this position. Computer modeling suggests that Phe168 does not directly interact with L-lactate, but like the role of Phe180 in GPR109a, Phe168 in GPR81 may increase the rigidity of the binding pocket, therefore indirectly affect the interaction between L-lactate and the binding sites in GPR81. Ser167 seems to be slightly less critical. Although Ser167Ala mutation reduced the Emax value, this mutation did not affect the EC50 value. The reduced Emax could be explained by the reduced cell surface expression, which implies that a hydrophilic residue at this position is necessary for efficient cell surface expression of GPR81. A Ser167Thr mutant retained the full function of GPR81 supports this hypothesis.

**Arg71 at TM2 is very critical for GPR81 function**

Transmembrane domains of GPCRs are the focus of ligand receptor interactions. In this study, comparison between GPR81 from all known species, GPR109a, and GPR109b showed that Arg71 in TM2 is conserved in all GPR81 by not in GPR109a, or GPR109b. Mutation of Arg71 to any other amino acid, including a conserved residue, Lys, abolished the receptor activity, strongly suggesting that Arg71 plays an important role for the specific interactions between lactate and GPR81. The role of Arg71 in GPR81 is reminiscent to that of Leu83, the corresponding residue in GPR109a. Previous study (Tunaru et al., 2005) showed that mutation of Leu83 in GPR109a to Val (the corresponding residue in GPR109b), which has a shorter side chain than Leu, slightly reduced the potency of niacin to GPR109a (from 0.7 μM to 3 μM). In this report, we changed Leu83 to Ala, a residue that has a side chain even shorter than that of Val, and found that this mutation reduced the affinity and sensitivity of GPR109a to niacin dramatically. In addition, a Leu83Arg mutation abolished GPR109a function in both ligand binding and receptor activation, suggesting that Leu83 in GPR109a is involved in niacin/GPR109a binding. Leu83 in GPR109a is a hydrophobic residue, suggesting that niacin interacts with Leu83 through hydrophobic interaction. In contrast, Arg71 in GPR81 is a positively charged residue, suggesting that Arg71 interact with L-lactate through ionic
or hydrogen bonding. Structure comparison between GPR81 ligands (L-lactate, α-hydroxybutyrate) and GPR109a ligands (niacin, β-hydroxybutyrate) suggests that the Arg71 residues in GPR81 may interact with the α-hydroxyl group of GPR81 ligands.

Computer modeling suggests that L-lactate directly interact with residues Arg71, Arg99, Glu166, and Arg240 of GPR81.

Previous computer modeling suggested that Arg99 at TM3, Tyr233 and Arg240 at TM6, as well as Thr267 at TM7 may interact with L-lactate (Liu et al., 2009). With the results in the current report, we provided a modified model for the interactions between L-lactate and GPR81 as shown in Fig. 7 (B C). In this current model, we propose that both Arg71 and Glu166 interact with the α-hydroxyl group of L-lactate through hydrogen bonding. Previous mutation studies showed that Arg99 and Arg240 are both critical for GPR81 function (Liu et al., 2009). Receptor modeling of GPR109a suggested that the corresponding residues in GPR109a (Arg111 and Arg251) interact with the carboxyl group of niacin. Consistent with the modeling of GPR109a, our current model for GPR81 suggests that Arg99 and Arg240 of GPR81 form hydrogen bonds with the carboxyl group of L-lactate as well. L-lactate is a very small molecule. In the current model, Tyr 233 and Thr267 seems not be able to interact with lactate directly due to the physical distance. It is possible that those two residues participate in receptor function not directly involved in the interactions with L-lactate. However, we cannot exclude the possibility for their direct interaction with L-lactate. This is because the formation of the extracellular multiple disulfide bonds of GPR81 (as discussed below) may twist the receptor to some degree and bring residues, such as Tyr233 and Thr267 to closer proximities of L-lactate for direct interactions.
Six conserved Cys residues at the extracellular domain of GPR81 may form three pairs of disulfide bonds and create a Cys-knot.

Cys residues at the extracellular domains often form disulfide bridges with other Cys residues either between proteins or within the same molecule. Comparison of GPR81 from all species shows that 6 Cys residues at the extracellular regions are conserved in GPR81 and GPR109a from all species. The conservation of these Cys residues is a clear common feature of this sub-family of receptors and suggests that they play a role in GPR81 and GPR109 functions, likely to form three pairs of disulfide bonds and served as structural components. Mutation studies showed that changing Cys residues (Cys6, 7, 88, 157, 165, and 252) in the extracellular domains to Ala or Ser abolishes the receptor activity strongly suggest that these 6 Cys residues participate the disulfide bond formation. It is likely that these Cys residues form intra-molecular disulfide bonds since if those Cys residues are paired with other molecules they may not have sufficient partner proteins when over expressed in recombinant system. We have expressed GPR81 in many different host cell types, including CHO, 293, SK-N-MC, and COS7 cells, but did not observe significant pharmacological differences, which supports our hypothesis. Another possibility is that those Cys residues may be involved in GPCR dimerization, particularly in homo-dimer, in which disulfide bonds can be formed between molecules without the requirement of other additional proteins. Among those Cys residues, the predicted Cys88-Cys165 bridge (solid line, Fig. 8) is conserved in many GPCRs (Cook et al., 1997; Zhang et al., 1999). Consistent with this statement, mutations of Cys88 or Cys165, coincidently, both reduce GPR81 cell surface expression, indicating this pair of disulfide bond is important for GPR81 cells surface expression. Very similar results have been observed in GPR109a mutation studies, in which mutations at Cys100 and Cys177 (corresponding to Cys88 and Cys 165 in GPR81) resulted mutants lack of cell surface expression (Tunaru et al., 2005). For other 4 Cys residues at the extracellular loop of GPR81, we predict that they form two bridges between Cys6-Cys252 and Cys7-Cys157. Mutations of those Cys residues resulted in
GPR81 mutants devoid of response to lactate despite the comparable cell surface expression to that of the wild type GPR81. It will be very difficult to predict the disulfide-bond assignments without further information. Lactate has a very low affinity to GPR81 ($EC_{50} = 5$ mM). It’s hard to evaluate whether those Cys mutations have equal impact to GPR81 function since we can’t differentiate a reduction of affinity 20 times and a total loss of affinity because we can only use lactate below 100 mM. However, previous study of GPR109a demonstrated that mutations at Cys183 and Cys266 in GPR109a have different impact to GPR109a functions. While mutation at Cys183 abolishes GPR109a function, mutation at Cys266 only reduces the affinity of GPR109a about 30 fold (Tunaru et al., 2005). In this report, we show that mutation at Cys18 in GPR109a resulted in a mutant with reduced affinity to niacin ($EC_{50} = 53$ μM compared with that of 1 μM for the wild type receptor) while mutation at Cys19 led to the complete abolishment of GPR109a function. These results strongly suggest that, in GPR109a, Cys18 pairs with Cys266 while Cys19 pairs with Cys183. Based on the conservation of the Cys residues between GPR109a and GPR81, the disulfide bonds at GPR81 were assigned accordingly (Fig. 8).

Lactate is a very small molecule. Results from the current report and previous studies suggest that residues scattered at different regions, including the extracellular domain and different transmembrane domains, of GPR81 are involved in receptor function. It is difficult to comprehend how lactate can physically interact with residues in GPR81 that may be too far apart. The identification of the Cys-knot may provide some possible explanation to help answering this question. With the close positions between Cys6 and Cys7 as well as Cys157 and Cys165, the formation of three disulfide bonds will bring the extracellular domains (N-terminus, ECL1, ECL2, and ECL3) to a very compact structure through a Cys-knot. In addition, these extracellular bridges may also pull the transmembrane domains to a closer proximity. Furthermore, the tight structure of the extra-cellular domain of GPR81 formed by the Cys-knot may only allow very small molecules
to enter the ligand binding pocket due to the physical space limitation. Therefore, this information may help the ligand design for GPR81 in the future.

**Summary**

At the time of the writing of this manuscript, we are aware of the efforts by IUPHAR to provide nomenclature for the class of receptor encompassing GPR81, GPR109a, GPR109b as hydroxycarboxylic acid receptors with the designation of HCA1, HCA2, and HCA3, respectively. This current study has helped to elucidate the key residues and structural features that are responsible for molecular interactions of the receptors with the individual hydroxycarboxylic acids that activate them. In this report, we characterized two zebrafish GPR81s and demonstrated the functional conservation of the GPR81 from human to fish as a receptor for lactate. Using the sequence information from the zebrafish GPR81, the previous results from mutation studies on niacin receptor GPR109a as the guidance, we characterized residues in GPR81 that might be important for GPR81/lactate interactions, through site-directed mutagenesis. Our results strongly suggest that, besides the previously reported residues, Arg71 at TM2 is a key residue for GPR81 function. In addition, residues in extracellular loops, especially a highly conserved Cys-Glu-Ser-Phe motif in the ECL2 region, are also involved in ligand interactions either directly or indirectly. GPCR dimerization has been proposed for receptor functional regulations. For residues that seem important for receptor function, we can’t exclude that the mutation of these residues disrupted the receptor dimerization therefore led to the loss of receptor functions. One thing that we have to acknowledge is that, due to the low affinity of lactate to GPR81, mutations that led to reduced affinity of the receptor may appeared to be total loss of function since we can only use lactate up to 100 mM. With further higher concentration of lactate, the salt effect will inhibit the receptor function. In addition, the low affinity of lactate to GPR81 makes it impossible to use lactate as a radioligand for receptor characterization. Therefore we were unable to characterize GPR81
mutants using radioligand binding studies. Hopefully in the future a high affinity ligand will be made available for this receptor.

**Author contributions:**

*Participated design:* Kuei, Yu, Zhu, Wu, Zhang, Shih, Mirzadegan, Lovenberg, and Liu  
*Conducted experiments:* Kuei, Yu, Zhu, Wu, Shih, and Liu  
*Performed data analysis:* Kuei, Yu, Zhu, Wu, Shih, and Liu  
*Wrote or contributed to the writing of the manuscript:* Kuei, Wu, Shih, Lovenberg, and Liu
References


Puhakainen I and Yki-Järvinen H (1993) Inhibition of lipolysis decreases lipid oxidation and gluconeogenesis from lactate but not fasting hyperglycemia or total hepatic glucose production in NIDDM. *Diabetes* **42**:1694-1699.


Footnote

Reprint request should be addressed to:

Changlu Liu, Johnson & Johnson Pharmaceutical Research & Development. L.L.C., 3210 Merryfield Row, San Diego, CA92121. Email: eliu9@its.jnj.com

Chester Kuei, Jingxue Yu, and Jessica Zhu contributed equally to this manuscript equally.
Figure legends

**Figure 1. Amino acid sequence comparison between the human GPR81, zebrafish GPR81s, human GPR109a, and GPR109b.** The protein sequences of human GPR81, the two putative zebrafish GPR81 proteins (zGPR81-1 and zGPR81-2), and human GPR109a and GPR109b are aligned. The 6 conserved Cys residues located in the predicted extracellular regions are shown in red. Compared with zebrafish GPR81, GPR109a, and GPR109b, the location of one (Cys157) of the two Cys residues at the 2nd extracellular loop (ECL2) of human GPR81 is different. The predicted transmembrane regions are underlined. Residues conserved in GPR81s but different in GPR109a and GPR109b are shaded. The conserved sequence motif (CESF) at ECL2 of GPR81 are underlined.

**Figure 2. Pharmacological characterization zebrafish GPR81.** Zebrafish GPR81-1 (zGPR81) and zebrafish GPR81-2 (zGPR81-2) were co-expressed in COS-7 cells with Go2 protein. The receptor expressing membranes or membranes from control cells expressing Go2 only were used in GTP\(\gamma\)S binding assays in the presence of different ligands at various concentrations. \(^{35}\)S-GTP\(\gamma\)S was used as the tracer. The results were analyzed by Graphpad. None of the ligands stimulated any appreciable GTP\(\gamma\)S incorporation in the control membranes.

**Figure 3. Characterization the function of Cys residues at the extracellular domains and transmembrane domains.** Cys residues at the extracellular domains and transmembrane domains were changed to Ala residues. The resulted recombinant mutant receptors were characterized by GTP\(\gamma\)S binding assay using L-lactate as the ligands. Ligand stimulated \(^{35}\)S-GTP\(\gamma\)S incorporations were shown as percentage of \(^{35}\)S-GTP\(\gamma\)S incorporations in the absence of ligand (basal incorporation). The results were analyzed by Graphpad. The EC\(_{50}\) values and Emax values are
listed in Table 1. Cells expressing the wild type human GPR81 (GPR81-WT) or cells expressing no recombinant GPR81 were used as positive control and negative control (NC) respectively.

**Figure 4. Mutation studies of residues at the transmembrane domains.** Arg71 (TM2) and Ala100 (TM3) were changed into residues as indicated. The resulted mutant receptors were characterized by GTPγS binding assay using L-lactate as the ligand. Ligand stimulated $^{35}$S-GTPγS incorporations were shown as percentage of $^{35}$S-GTPγS incorporations in the absence of ligand (basal incorporation). The results were analyzed by Graphpad. The EC$_{50}$ values and Emax values are listed in Table 1. Cells expressing the wild type human GPR81 (GPR81-WT) or cells expressing no recombinant GPR81 were used as positive control and negative control (NC) respectively.

**Figure 5. Function of residues at the extracellular loops.** Residues at the extracellular domains were mutated into various residues as indicated. The resulted mutant receptors were characterized by GTPγS assay using L-lactate as the ligand. Ligand stimulated $^{35}$S-GTPγS incorporations were shown as percentage of $^{35}$S-GTPγS incorporations in the absence of ligand (basal incorporation). The results were analyzed by Graphpad. The EC$_{50}$ values and Emax values are listed in Table 1. Cells expressing the wild type human GPR81 (GPR81-WT) or cells expressing no recombinant GPR81 were used as positive control and negative control (NC) respectively.

**Figure 6. Mutation studies of GPR109a.** Cys18, Cys19, and Leu83 of human GPR109a were mutated into residues as indicated. The resulted recombinant mutant receptors were characterized by GTPγS assay using niacin as the ligand. Ligand stimulated $^{35}$S-GTPγS incorporations were shown as percentage of $^{35}$S-GTPγS incorporations in the absence of ligand (basal incorporation). The results were analyzed by Graphpad. The EC$_{50}$ values and Emax values are listed in Table 1.
Cells expressing the wild type human GPR109a (GPR109a-WT) or cells expressing no recombinant GPR109a were used as positive control and negative control (NC) respectively.

**Figure 7. Model of GPR81 ligand binding site.**

**A. Structural comparison between GPR81 ligands and GPR109a ligands.** Acid forms of the molecules are shown. **B. Homology modeling of GPR81.** The seven transmembrane helical domains of GPR81 are shown along with the extracellular loops. L-lactate (shown in vdW representation) is docked in the putative binding site formed by residues from TM2, 3, 6, and ECL2 (orange, purple, pink, and brown, respectively). Residues Arg71, Arg99, Glu166 and Arg240 that are important for GPR81 functions are shown in licorice representation. **C. Close up view of the GPR81 binding site.** The carboxylate group of L-lactic acid (in cyan) interacts with the two basic residues Arg99 and Arg240, while the hydroxyl group interacts with basic residue Arg71 and acidic residue Glu166.

**Figure 8. Predicted disulfide bonds among the Cys residues at the extracellular domains of GPR81.** Cys residues that potentially form disulfide bonds are shown in red. The disulfide bond (Cys88-Cys165) conserved in many GPCRs are shown in solid line. Putative disulfide bonds between Cys6 and Cys252 as well as Cys7 and Cys157 are shown in dashed lines. Residues, Arg71, Glu166, Arg99, and Arg240, that potentially interact with L-lactate are shown in green.
Table 1. Effects of mutations on GPR81 function

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Recombinant mutant receptors were characterized using GTP\gammaS assay.

The EC_{50} values are lactate concentrations that stimulated 50% of Emax. The results represent mean ± SEM from two independent experiments. In each experiment, the assays were performed in triplets at each data point.

The Emax values are calculated as percentage of the maximum response of the wild type GPR81. The results represent the mean of two independent experiments.

Relative total and cell surface expression were determined by anti-V5-antibody in ELISA assays either in the presence or absence of 1% Triton-X-100 as the penetration reagents. The expression of N-terminally tagged V5-GPR81 is arbitrarily set as 100 while the cells without GPR81 expression were used as a negative control and the expression level is arbitrarily set as 0. The cells with C-terminally V5-tagged GPR81 (GPR81-V5) were used as a negative control for cell surface staining. The expression levels of mutant receptors were expressed as percentage of the wild type V5-GPR81. The results represent the mean of two independent experiments.

^No activity, no receptor stimulation by lactate was observed. The EC_{50} and Emax values are not calculated.
Table 2. Effects of mutations on GPR109a function

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<th>Corresponding residue(s) in gGPR81</th>
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The mutant GPR109a receptors were characterized using GTPγS binding and radioligand binding assays.

EC<sub>50</sub> values (mean ± SEM, n = 3) are the concentration of niacin that stimulated 50% of maximum 35S-GTPγS incorporation in GTPγS binding assay.

The Emax values are calculated as percentage of the maximum response of the wild type GPR109a stimulated by niacin at concentrations up to 10 mM.

K<sub>d</sub> values (mean ± SEM, n = 3) are determined by Scatchard analysis of 3H-labeled niacin binding saturation isotherms.

Relative total and cell surface expression were determined by anti-V5-antibody in ELISA assay either in the presence or absence of 1% Triton-X-100 as the penetration reagents. The expression of N-terminally tagged V5-GPR109a is set as 100. Cells without recombinant protein expression were served as the negative control and set as 0. The expression levels of mutant receptors were expressed as percentage of the wild type V5-GPR109a.

<sup>a</sup>ND, the receptor activation did not reach the plateau at the highest concentration tested (10 mM). The Emax values have not been determined.

<sup>b</sup>ND, the assays did not reach saturation in the binding assay and the K<sub>d</sub> values have not been determined.
Figure 1

<table>
<thead>
<tr>
<th>Protein</th>
<th>Sequence</th>
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<tbody>
<tr>
<td>Human GPR81</td>
<td>MYNGSCCRIBGDTISQVMFLLLIVAFVLGALGNGVALCPFHMKTKPSTVLYNLALA 58</td>
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<tr>
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<td>Human GPR109b</td>
<td>LEIDKMNCCVFRRDFDIACKLFALSIFGLGgLNLWIFCHLKSWKSSRIFLNLAL 70</td>
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<tr>
<td>Consensus</td>
<td>CC       + ++P+S+L F++G++GN+AL F FH ++K + ++L +LA</td>
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<td>zGPR81-1</td>
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<td>zGPR81-2</td>
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<tr>
<td>Human GPR109a</td>
<td>VADFLLMICLPFRTDYYLRRRHWAFGDIP</td>
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<tr>
<td>Human GPR109b</td>
<td>VADFLLMICLPFRTDYYLRRRHWAFGDIP</td>
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<tr>
<td>Consensus</td>
<td>+A+ L+++CLPF  D Y   R    W +GD+ CR+ LF++A+NR   I+FLT+VA+DRI+++V</td>
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<tr>
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<td>Human GPR109b</td>
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<tr>
<td>Consensus</td>
<td>HP+ +N +    A  +   LW + I  TV LL</td>
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<td>Human GPR109b</td>
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<tr>
<td>Consensus</td>
<td>WH+ + ++F+S+P I+++CS  I+X L+ + ++ ++K+A+ F+++VA+VFI C++</td>
</tr>
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<tr>
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<tr>
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<tr>
<td>Human GPR109a</td>
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<tr>
<td>Human GPR109a</td>
<td>PSVVRIRIFWLHITGTCQENVYRSVDALPILITTSFYMNSLPLVYFFSSPFPFNFFS 306</td>
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<tr>
<td>Consensus</td>
<td>PS     R+ +     C+ + A+ IT+++FTY NS+L+P+VYFSSSF</td>
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<td>Human GPR81</td>
<td>KLKICSLKPQHSGKQTQRFEPMPSLNLGRNSCVANPSQDSQSQDPDMHIVEW 346</td>
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<tr>
<td>zGPR81-1</td>
<td>KIYMMKSGKIEDHEDNNNSSVTVSANS 328</td>
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<tr>
<td>zGPR81-2</td>
<td>KIYMMKSGKIEDHEDNNNSSVTVSANS 328</td>
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<td>Human GPR109a</td>
<td>TLINRCRQMTGFRNDRNRTSTVELGDMPKTRGAPRALMNAGPSFWSPSYGPTSP 363</td>
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<tr>
<td>Human GPR109b</td>
<td>TLINRCRQMTGFRNDRNRTSTVELGDMPKTRGAPRALMNAGPSFWSPSYGPTSP 363</td>
</tr>
<tr>
<td>Consensus</td>
<td>+ K</td>
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Figure 2

Graphs showing the binding of $^35$S-GTPγS to zGPR81-1 and zGPR81-2 as a function of the log concentration of L-lactate. The graph includes data points for L-lactate, Pyruvate, Niacin, and $\beta$-Hydroxybutyrate. The x-axis represents the log concentration of L-lactate, while the y-axis represents the $^35$S-GTPγS bound (CPM).
Figure 3
Figure 4

![Graph showing log concentration of L-lactate (M) vs. %S-bound (percent of basal incorporation). The graph compares the incorporation of labeled L-lactate with different variants of GPR81.]
Figure 5

[Graph showing the Log Concentration of L-lactate (M) against the percentage of basal incorporation of $^{35}$S-GTPyS bound for different mutants: NC, GPR81-WT, Glu166Ser, Ser167Ala, and Phe168Ala.]
Figure 6

![Graph showing the log concentration of niacin (M) on the x-axis and % 35S-GTPγS bound on the y-axis. The graph compares various mutations: NC, GPR109a-WT, Cys18Ala, Cys19Ala, Leu83Ala, and Leu83Arg. Each mutation is represented by a different symbol, and the graph shows the percentage of basal incorporation at different niacin concentrations.](image-url)
Figure 7

A  

GPR81 Ligands  

- L-lactic acid  
- α-Hydroxybutyric acid

GPR109a Ligands  

- Niacin  
- β-Hydroxybutyric acid

B  

C  

Arg99  
Arg240  
Arg71  
Glu166
Figure 8