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Study of GPR81, the Lactate Receptor, from Distant Species Identifies Residues and Motifs Critical for GPR81 Functions[S]

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ABSTRACT

Receptors from distant species may have conserved functions despite significant differences in protein sequences. Whereas the noncritical 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 two zebrafish receptors (zGPR81-1 and zGPR81-2) that show approximately 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, GPR109a, and GPR109b identified a common structure (six 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, C165-E166-S167-F168, at the second extracellular loop of GPR81. Using site-directed mutagenesis, we showed that Arg71 at the transmembrane domain 2 is very critical for GPR81 function. In addition, we demonstrated that the C165-E166-S167-F168 motif at the second extracellular loop is critical for GPR81 function, and the conserved six Cys residues at the extracellular regions are necessary for GPR81 function. It is important to mention that 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 (Dienel, 2004; Bergersen, 2007). It is essentially produced by all tissues and cell types and at increased levels during intense exercise or under anaerobic conditions (Ohkuwa et al., 1984; Cheetham et al., 1986; Hughson et al., 1987). Elevated concentrations of L-lactate have been reported in obesity and in type 2 diabetes (Chen et al., 1993). Certain tissues and cells such as adipocytes (DiGirolamo et al., 1992) and astrocytes (Kasischke, 2008) produce higher levels of lactate under normal physiological conditions. Adipose tissues produce lactate in particular after a glucose load or meal ingestion (Hagström et al., 1990; Ahmed et al., 2010). It has been reported that fat tissues account for approximately 30% of glucose uptake but a significant amount of the glucose is converted into lactate and redistributed to other tissues (DiGirolamo et al., 1992). Therefore, lactate serves as an alternative energy venue for glucose. Astrocytes have been known to take up glucose and convert it to lactate as the preferred energy source for neurons (Dienel, 2004).

Lactate has been identified as a ligand for GPR81 (Cai et al., 2008; Liu et al., 2009). It activates GPR81 with an EC_{50} value of approximately 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 wild-type mice but not in those from 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. Because lactate exhibits low affinity and a fast turnover rate, it is 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 li-

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ABBREVIATIONS: GPCR, G protein-coupled receptor; TM, transmembrane; NCBI, National Center for Biotechnology Information; GTPγS, guanosine 5′-*O*-(3-thio)triphosphate; ELISA, enzyme-linked immunosorbent assay; z, zebrafish; ECL, extracellular loop.

gand design. In our previous studies, through modeling and mutation analysis, we proposed that Arg99, Tyr233, Arg240, and Thr267 of the human GPR81 may play roles in lactate interaction. Because 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 the extracellular regions in particular have received little attention in GPCR modeling (Benned-Jensen and Rosenkilde, 2009). Accumulating evidence strongly suggests that other TMs (particularly TM2) and the extracellular regions play important roles in ligand-receptor interactions either directly or indirectly (Miura et al., 2003; Zhu et al., 2008; Ahn et al., 2009; Benned-Jensen and Rosenkilde, 2009; Clark et al., 2010; D'Aoust and Tiberi, 2010; Sakai et al., 2010).

In this study, we analyze 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 that share identities similar 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-hydroxyoctanoic acid) (Ahmed et al., 2009), have been identified. Of interest, 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 for studying 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 six 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 (St. Louis, MO).

Identification and Cloning of Zebrafish *gpr81*-Related Genes. The human GPR81 protein sequence was used as the query to search the NCBI zebrafish genomic DNA database. Four *gpr81*-related sequences were identified from three zebrafish genomic contigs (GenBank accession nos. NW_001877090.1, NW_001877330.1, and NW_001877242.1). The predicted coding regions of the four 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*, and 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 polymerase chain reaction 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 was submitted to GenBank (accession nos: *zgpr81-1*, EU809467; *zgpr81-2*, EU809468; *zgpr81-3*, HM030492; and *zgpr81-4*, HM030493). Specific primers (P9 and P10 for *zgpr81-1*, P11 and P12 for *zgpr81-2*, P13 and P14 for *zgpr81-3*, and 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 Supplemental 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 the pCIneo expression vector (Liu et al., 2009) and served as the template for mutation studies. The resultant 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 served as a positive control and template for GPR109a mutation studies. All GPR109a mutant characterizations were performed in the same way as those 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, coexpressed with the G_{o2} subunit, or coexpressed with G_{o2}, $G\beta_1$, and $G\gamma_2$ together in COS7 cells. The results showed that whereas GPR81 expressed alone offered very low ligand-stimulated [^{35}S]GTPγS binding, coexpression with the G_{o2} subunit increased the ligand-stimulated [^{35}S]GTPγS binding, providing a sufficient signal/noise ratio for the pharmacological characterization of GPR81. Coexpression of GPR81 with G_{o2}, $G\beta_1$, and $G\gamma_2$ together further increased the ligand-stimulated [^{35}S]GTPγS binding without changing the EC_{50} values (Supplemental Fig. 1). In this report, all expression constructs were transiently coexpressed with a human G protein G_{o2} 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 the G_{o2} 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 (Supplemental Fig. 2). To assess the total protein and cell surface protein expression levels, the transfected cells were reseeded in 96-well culture plates in triplicates (4×10^4 cells/well) 1 day after transfection. Two days after transfection, cells were fixed with formalin. The total and cell surface expression of V5-tagged proteins was 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 transfected 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. A GTPγS 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}S]GTPγS (PerkinElmer Life and Analytical Sciences, Waltham, MA) was used as the tracer. [^{35}S]GTPγS binding in the presence of ligand (L-lactate for GPR81 and niacin for GPR109a) was expressed as the percentage of the [^{35}S]GTPγS binding in the absence of ligands. The data were analyzed using GraphPad Prism 5 software (GraphPad Software Inc., San Diego, CA), and the EC_{50} values were calculated as the ligand concentration that stimulated 50% of the maximum ligand-induced response (E_{max}). The EC_{50} and E_{max} values for mutants that did not reach a plateau in the GTPγS binding assay at the highest concentration of ligand used (100 mM L-lactate for GPR81 mutants and 10 mM niacin for GPR109a mutants) were not calculated. For GPR81 mutants, because of the lack of high-affinity radioligand ([^{3}H]lactate

did not confer detectable binding because of its low affinity), we did not characterize GPR81 and the GPR81 mutants with a radioligand binding assay. GPR109a mutants were characterized in Scatchard isotherms saturation binding assays using ^{3}H-labeled niacin, as described previously (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, Inc., San Diego, CA). The primary sequence alignment between turkey β_1-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 on the basis of the multiple sequence alignment of family A GPCRs (Mirzadegan et al., 2003). The β_1-adrenergic receptor structure (2VT4) was used as a template, and on the basis of the sequence alignment, a GPR81 homology model was built and loops were refined. The GPR81 homology model was placed into a 30-Å-thick implicit membrane and energy-minimized using the Smart Minimizer algorithm with the 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 300 K, molecular dynamics equilibration, and production runs) with the ligand constrained. The constraints on the ligand were then removed, and the entire system was further minimized. Molecular renderings were done using VMD (Humphrey et al., 1996).

Results

Molecular Cloning and Pharmacological Characterization of Zebrafish GPR81-Like Genes. Using the human GPR81 protein sequence as the query, we blast-searched the zebrafish genomic DNA database using the NCBI Tblastn program and identified four sequence contigs that code for GPR81-like proteins, which share greater than 50% identity with human GPR81 protein. The four putative zebrafish *gpr81*-like genes were designated as *zgpr81-1*, *zgpr81-2*, *zgpr81-3*, and *zgpr81-4*, respectively. The protein sequence alignment among human GPR81, zGPR81-1, zGPR81-2, human GPR109a, and human GPR109b is shown in Fig. 1. The identity between human GPP81, GPR109a, GPR109b, and the putative proteins encoded by zebrafish *gpr81*-like genes is shown in Supplemental 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 although the recombinant zGPR81-1 and zGPR81-2 responded to L-lactate stimulation at EC_{50} values of approximately 1 mM in the GTPγS binding assay (Fig. 2), zGPR81-3 and zGPR81-4 did not respond to lactate stimulation (data not shown). Despite sharing high identity with GPR109a, all four GPR81-like zebrafish receptors did not respond to GPR109a ligands (niacin or β-hydroxybutyrate).

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Human GPR81       MYNGSCCRIEGDTISQVMPPLLIVAFVLGALGNGVALCGFCFHMKTWKPSTVYLFNLA  58
zGPR81-1        MNNSSSVCCAFDAPILDEVLPPVLFSEFVLGLMGNGLALCMFFFHRDSWKPNSIYLAHLA  60
zGPR81-2         MNNSSLCCTYETPLLDAYLPPVLFSEFVLGLMGNGLALCMFFFHRDSWKPNSIYLAHLA  59
Human GPR109a   LEIDKKNCCVFRDDFIVKVLPPVLGLEFIFGLLGNGLALWIFCFHLKSWKSSRIFLFNLA  70
Human GPR109b   LEIDKKNCCVFRDDFIAKVLPPVLGLEFIFGLLGNGLALWIFCFHLKSWKSSRIFLFNLA  70
Consensus               CC      +  ++PP+L   F++G++GNG+AL  F FH  +WK + ++L +LA
                                                  TM1

Human GPR81     VADFLLMICLPFRTDYYLRRRHWAFGDIPCRVGLFTLAMNRAGSIVFLTVVAADRYFKVV  118
zGPR81-1        LADSLVLFCLPFRADYYRRGKHWVYGDAFCRVLLFLLAANRAAGIFFLTAVAVDRYLKIV  120
zGPR81-2        LANSLVLFCLPFRADYYRRGKHWVYGDAFCRVLLFLLAANRAAGIFFLTAVAVDRYLKIV  119
Human GPR109a   VADFLLIICLPFLMDNYVRRWDWKFGDIPCRLMLFMLAMNRQGSIIFLTVVAVDRYFRVV  130
Human GPR109b   VADFLLIICLPFVMDYYVRRSDWNFGDIPCRLVLFMFAMNRQGSIIFLTVVAVDRYFRVV  130
Consensus       +A+ L+++CLPF  D Y R   W +GD+ CR+ LF++A+NR   I+FLT+VA+DRY+++V
                     TM2                                  TM3

Human GPR81     HPHHAVNTISTRVAAGIVCTLWALVILGTVYLLLENHLCVQETAVSCESFIMESAN----  174
zGPR81-1        HPLNRINQMGLRYALWVSVGLWALIIAMTVYLLADKHFYYRNNRTQCESFNICLGHNALS  180
zGPR81-2        HPLNRINQMGLRYALWVSVGLWALIIAMTVYLLADKHFYYLNNHTQCESFNICFGSNSRF  179
Human GRP109a   HPHHALNKISNRTAAIISCLLWGITIGLTVHLLKKKMPIQNGGANLCSSFSICHTF----  186
Human GPR109b   HPHHALNKISNWTAAIISCLLWGITVGLTVHLLKKKLLIQNGPANVCISFSICHTF----  186
Consensus       HP + +N +   A  +  LW + I  TV LL +          C SF +
                          TM4

Human GPR81     GWHDIMFQLEFFMPLGIILFCSFKIVWSLRRRQQLARQARMKKATRFIMVVAIVFITCYL  234
zGPR81-1        DWHNSFYVIQFFVPTFIVIYCSTCITWQLKGKT-IDKHGKIKRAVRFVLAVALVFIICFF  239
zGPR81-2        TWHNVFYVIQFFVPTFIVIYCSTCITWQLKGKT-IDKHGKIRRAVRFVLAVALVFIICFF  238
Human GPR109a   QWHEAMFLLEFFLPLGIILFCSARIIWSLRQRQ-MDRHAKIKRAITFIMVVAIVFVICFL  245
Human GPR109b   RWHEAMFLLEFLLPLGIILFCSARIIWSLRQRQ-MDRHAKIKRAITFIMVVAIVFVICFL  245
Consensus        WH+  + ++F++P  I+++CS  I+W L+ +  + ++ ++K+A+ F+++VA+VFI C++
                          TM5                                    TM6

Human GPR81     PSVSARLYFLWTVPS---SACD--PSVHGALHITLSFTYMNSMLDPLVYYFSSPSFPKFYN  290
zGPR81-1        PSNVSRIAV-WVLKS-WHNECQYFQDANVAFYITVCFTYFNSVLNPVVYYFSSPAVSRSLR  298
zGPR81-2        PSNISRISM-YVLKL-WYNECQYFSDANDAFKTTVCFTYFNSVLNPVVYYFSSPAVSGSLR  297
Human GPR109a   PSVVVRIRIFWLLHTSGTQNCEVYRSVDLAFFITLSFTYMNSMLDPVVYYFSSPSFPNFFS  306
Human GPR109b   PSVVVRIRIFWLLHTSGTQNCEVYRSVDLAFFITLSFTYMNSMLDPVVYYFSSPSFPNFFS  306
Consensus       PS   R+   + +       C+     + A+ IT++FTY NS+L+P+VYYFSSP
                                                  TM7

Human GPR81     KLKICSLKPKQPGHSKTQRPEEMPISNLGRRSCISVANSFQSQSDGQWDPHIVEWH          346
zGPR81-1        KIYMRMSGQKIEDEHEKNNHSSVTVSANVS                                    328
zGPR81-2        KIYMRMLGQKIEE                                                     310
Human GPR109a   TLINRCLQRKMTGEPDNNRSTSVELTGDPNKTRGAPEALMANSGEPWSPSYLGPTSP        363
Human GPR109b   TLINRCLQRKMTGEPDNNRSTSVELTGDPNKTRGAPEALMANSGEPWSPSYLGPTSNNHSKKGHC 387
Consensus        +       K
```

Fig. 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 six 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 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 is underlined.

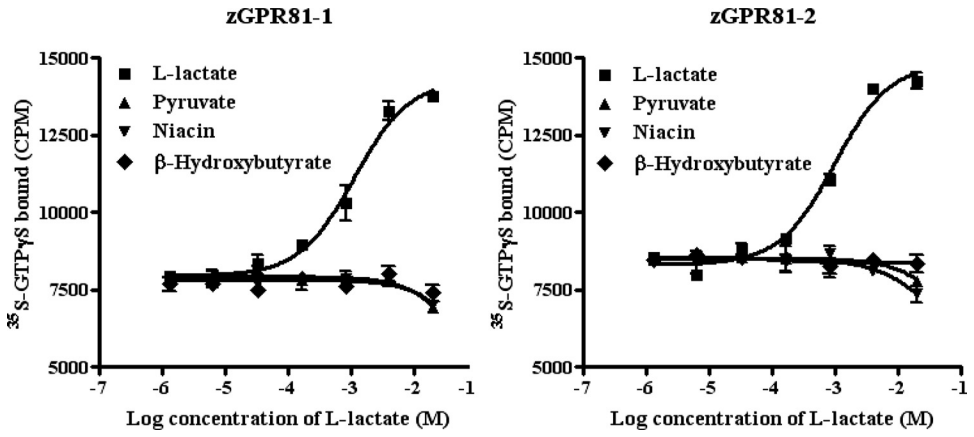

Fig. 2. Pharmacological characterization of zebrafish GPR81s. zGPR81-1zGPR81-2 were coexpressed in COS-7 cells with G_{o2} protein. The receptor-expressing membranes or membranes from control cells expressing G_{o2} were only used in GTPγS binding assays in the presence of different ligands at various concentrations. [^{35}S]GTPγS was used as the tracer. The results were analyzed with GraphPad Prism 5. None of the ligands stimulated any appreciable GTPγS incorporation in the control membranes.

Biochemical Characterization of Recombinant GPR81 and GPR109a Protein Expression. Western blot analysis was used to characterize the size of the wild-type and mutant receptors. Wild-type GPR81 and GPR109a have a predicted molecular mass of 40 and 41 kDa, respectively (without counting post-translational modification). In Western blot analysis of the recombinant protein expression, the wild-type GPR81 and GPR109a and all mutant receptors demonstrated very similar molecular masses at approximately 45 kDa (Supplemental Fig. 2). The total receptor protein expression quantified by ELISA showed that all mutant receptors have expression levels comparable with 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 C88A, C88S, H155A, C165A, and C165S lacked cell surface expression. GPR81 mutants E166A, G166I, S167A, and F168Y have reduced cell surface protein expression (Table 1). For GPR109a mutants, all mutants had cell surface expression levels comparable with 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 six Cys residues. Two of them are located at the N-terminal domains (Cys6 and Cys7), one at the first extracellular loop (ECL) (Cys88), two at the second ECL (Cys157 and Cys165), and one at the third ECL (Cys252). Those Cys residues are conserved among all known mammalian GPR81s, zGPR81-1 and zGPR81-2 from zebrafish, and GPR109a and GPR109b (Fig. 1). We investigated the role of these Cys residues by changing them individually into Ala or Ser residues. As controls, six 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 studied 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. Our initial mutation studies showed that each of R99A (TM3), Y233A (TM6), R240A (TM6), and T267A (TM7) mutations abolish GPR81 receptor activity (Liu et al., 2009). By comparing the sequences between the human and zebrafish GPR81s, we determined that Arg71 in TM2 and Ala100 at TM3 are conserved in all known mammalian GPR81 and in 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 a slight reduction in the E_{max} value (accompanied by a slight reduction of cell surface receptor expression) but not in the EC_{50} value of GPR81 when it was tested using L-lactate as the ligand (Fig. 4). The EC_{50} values and E_{max} values of those mutants are shown in Table 1.

Mutation Studies at the Extracellular Regions of GPR81. His80 at the ECL1 and 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 for 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 whereas mutations at His80 did not produce dramatic changes in GPR81 receptor function, a H155A mutation abolished GPR81 function. Protein expression analysis showed that this mutant lacked cell surface expression (Table 1). A change of Glu166 to Ala, Ser (the corresponding residue in GPR109a), or Ile (the corresponding residue in GPR109b), but not to Asp, abolished GPR81 activity. A S167A mutation reduced the E_{max} value but not the EC_{50} value. This reduction in E_{max} value is probably due to the reduced cell surface expression (Table 1), suggesting that a hydrophilic residue at this position is needed for efficient GPR81 cell surface localization. In support of this hypothesis, a S167T mutation had little effect on receptor expression or receptor functions. An aromatic residue at the 168 position seems important. A Phe168 to Ala mutation abolished GPR81 activity, whereas a F168Y mutation retained activity with a slight E_{max} reduction accompanied by slightly deceased cell surface expression. The EC_{50} and E_{max} values for all mutants are listed in Table 1 and sample results are shown in Fig. 5.

Pharmacological Characterization of GPR109a Mutants. To investigate whether residues in GPR109a corresponded to residues critical for GPR81 functions, we evalu-

TABLE 1

Effects of mutations on GPR81 function

Recombinant mutant receptors were characterized using a GTPγS assay. The EC_{50} values are lactate concentrations that stimulated 50% of E_{max}. The results represent mean ± S.E.M. from two independent experiments. In each experiment, the assays were performed in triplets at each data point. The E_{max} values are calculated as a 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 reagent. The expression of N-terminal tagged V5-GPR81 is arbitrarily set as 100, whereas the cells without GPR81 expression were used as a negative control and the expression level is arbitrarily set as 0. The cells with C-terminal V5-tagged GPR81 (GPR81-V5) were used as a negative control for cell surface staining. The expression levels of mutant receptors are expressed as a percentage of the wild-type V5-GPR81. The results represent the mean of two independent experiments.

Mutations	Region of Residues Changed	Corresponding Residues in		EC_{50} Value for Lactate	E_{max}	Total Protein Expression	Cell Surface Expression
		zGPR81-1/2	GPR109a/b				
				mM		*% WT*	
V5-GPR81				4.26 ± 0.82	100	100	100
GPR81-V5				4.39 ± 0.94	90.9	93.2	4.1
Negative Ct				N.A.	N.A.	0.0	0.0
C6A	N terminus	Cys	Cys	N.A.	N.A.	114.4	179.2
C6S				N.A.	N.A.	104.8	142.6
C7A		Cys	Cys	N.A.	N.A.	131.3	181.4
C7S				N.A.	N.A.	94.7	139.6
C38A	TM1	Cys	Trp	5.61 ± 1.24	106	91.5	96.7
C41A		Phe	Cys	4.85 ± 0.84	94.1	89.8	92.5
C67A	TM2	Cys	Cys	3.18 ± 1.09	85.7	121.5	114.8
R71A		Arg	Leu/Val	N.A.	N.A.	115.6	85.2
R71L				N.A.	N.A.	98.3	143.7
R71K				N.A.	N.A.	109.8	152.5
H80A		His	Asp	4.58 ± 0.71	69.3	91.6	88.3
H80D				4.14 ± 0.89	81.6	114.9	71.4
C88A		Cys	Cys	N.A.	N.A.	135.7	8.3
C88S				N.A.	N.A.	122.4	12.6
A100Q	TM3	Ala	Gln	7.15 ± 1.58	95.8	105.8	85.3
C137A	TM4	Val	Cys	4.27 ± 1.37	80.6	95.9	84.1
H155A	ECL2	His	Met/Leu	N.A.	N.A.	117.6	17.4
C157A		Tyr	Ile	N.A.	N.A.	114.1	125.8
C157S				N.A.	N.A.	94.6	121.8
C165A		Cys	Cys	N.A.	N.A.	91.4	22.7
C165S				N.A.	N.A.	93.8	18.4
E166A		Glu	Ser/Ile	N.A.	N.A.	95.2	61.6
E166S				N.A.	N.A.	112.5	83.9
E166I				N.A.	N.A.	97.8	71.2
E166D				4.46 ± 0.93	87.3	93.2	89.4
S167A		Ser	Ser	5.49 ± 1.46	37.9	108.5	41.7
S167T				5.52 ± 1.19	91.5	104.8	98.3
F168A		Phe	Phe	N.A.	N.A.	117.3	128.9
F168Y				6.14 ± 1.06	81.4	121.1	75.3
C195A	TM5	Cys	Cys	4.17 ± 0.72	73.5	106.9	82.6
C232A	TM6	Cys	Cys	6.18 ± 1.21	84.7	78.4	81.8
C252A	ECL3	Cys	Cys	N.A.	N.A.	85.7	113.3
C252S				N.A.	N.A.	90.5	104.8

WT, wild-type; N.A., no activity (no receptor stimulation by lactate was observed; EC_{50} and E_{max} values were not calculated).

TABLE 2

Effects of mutations on GPR109a function

The mutant GPR109a receptors were characterized using GTPγS binding and radioligand binding assays.EC_{50} values (mean ± S.E.M., $n = 3$) are the concentration of niacin that stimulated 50% of the maximum [^{35}S]GTPγS incorporation in a GTPγS binding assay. The E_{max} values are calculated as a percentage of the maximum response of the wild-type GPR109a stimulated by niacin at concentrations up to 10 mM. K_d values (mean ± S.E.M., $n = 3$) are determined by Scatchard analysis of ^{3}H-labeled niacin binding saturation isotherms. Relative total and cell surface expression was determined by anti-V5-antibody in an ELISA assay in either the presence or absence of 1% Triton X-100 as the penetration reagent. The expression of N-terminal tagged V5-GPR109a is set as 100. Cells without recombinant protein expression served as the negative control and were set at 0. The expression levels of mutant receptors are expressed as a percentage of the wild-type V5-GPR109a.

Mutations	Region of Residues Changed	Corresponding Residue(s) in		EC_{50} Value for Niacin	E_{max} Value	K_d Values	Total Protein Expression	Cell Surface Expression
		zGPR81/2	GPR81					
				μM	*% WT*	*nM*	*% WT*	
V5-GPR109a				1.07 ± 0.15	100	54.6 ± 6.14	100	100
Negative C_t							0.0	0.0
C18A	N terminus	Cys	Cys	65.7 ± 6.4	95.6	N.D.[a]	104.3	95.8
C19A		Cys	Cys	>1000	N.D.[b]	N.D.[a]	122.7	134.6
L83A	TM2s	Arg	Arg	28.3 ± 4.16	97.6	615 ± 87.5	102.4	120.4
L83R				>1000	N.D.[b]	N.D.[a]	96.8	109.5

WT, wild-type; N.D., not determined.

[a] The assays did not reach saturation in the binding assay and the K_d values have not been determined.

[b] The receptor activation did not reach the plateau at the highest concentration tested (10 mM). The E_{max} values have not been determined.

ated these residues in GPR109a through mutagenesis studies. The GPR109a mutants were characterized by a GTPγS binding assay for receptor activation and by saturation binding to determine the binding affinity using ^{3}H-labled niacin. Through mutagenesis studies, Tunaru et al. (2005) showed previously that Cys100, Cys177, Cys183, and Cys266 are important for GPR109a functions. In this report, we showed that Cys18 and Cys19 at the N terminus are also

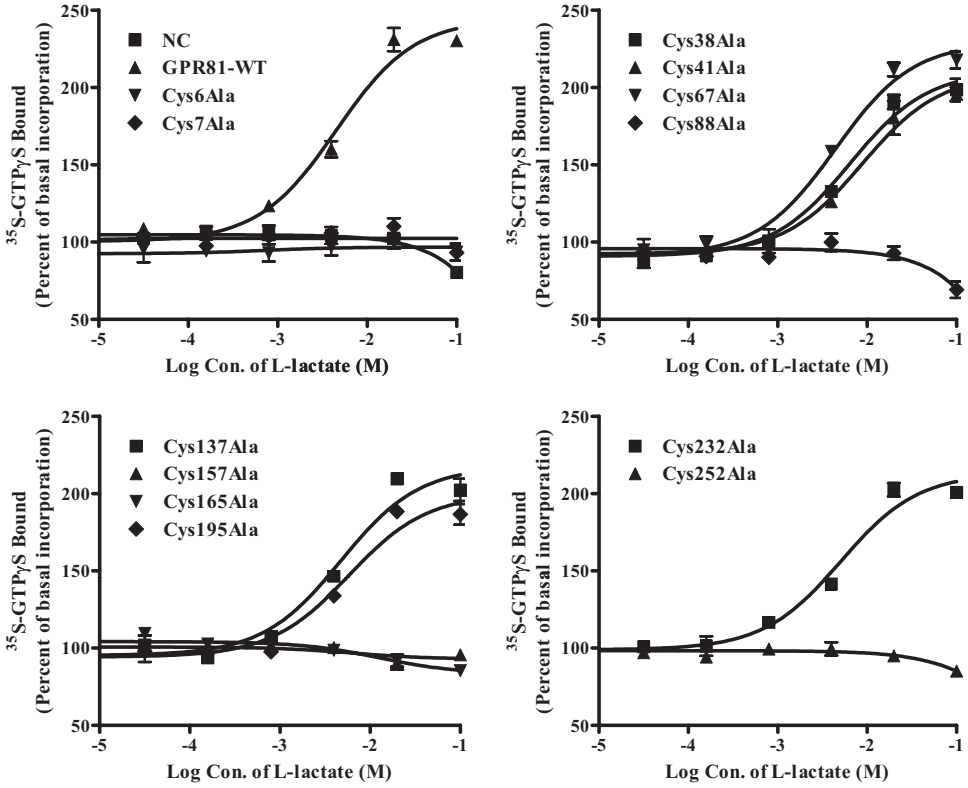

Fig. 3. Characterization of the function of Cys residues at the extracellular domains and transmembrane domains. Cys residues at the extracellular domains and transmembrane domains were changed to Ala residues. The resultant recombinant mutant receptors were characterized by GTPγS binding assay using L-lactate as the ligand. Ligand-stimulated [^{35}S]GTPγS incorporations are shown as a percentage of [^{35}S]GTPγS incorporation in the absence of ligand (basal incorporation). The results were analyzed with GraphPad Prism 5. The EC_{50} values and E_{max} values are listed in Table 1. Cells expressing the wild-type human GPR81 (GPR81-WT) or cells expressing no recombinant GPR81 were used as the positive control and negative control (NC), respectively. Con., concentration.

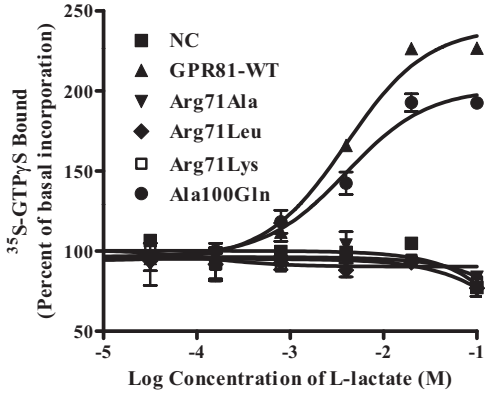

Fig. 4. Mutation studies of residues at the transmembrane domains. Arg71 (TM2) and Ala100 (TM3) were changed into residues as indicated. The resultant mutant receptors were characterized by a GTPγS binding assay using L-lactate as the ligand. Ligand-stimulated [^{35}S]GTPγS incorporations are shown as a percentage of [^{35}S]GTPγS incorporation in the absence of ligand (basal incorporation). The results were analyzed with GraphPad Prism 5. The EC_{50} values and E_{max} values are listed in Table 1. Cells expressing the wild-type human GPR81 (GPR81-WT) or cells expressing no recombinant GPR81 were used as the positive control and negative control (NC), respectively.

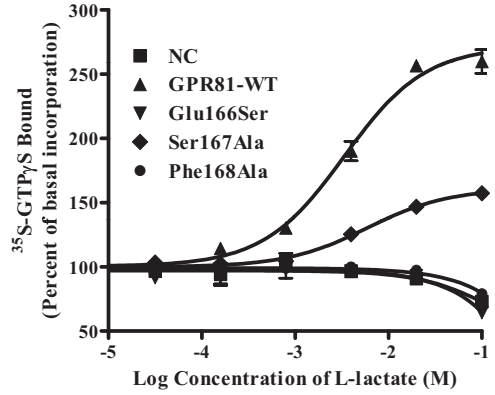

Fig. 5. Function of residues at the extracellular loops. Residues at the extracellular domains were mutated into various residues as indicated. The resultant mutant receptors were characterized by a GTPγS assay using L-lactate as the ligand. Ligand-stimulated [^{35}S]GTPγS incorporations are shown as a percentage of [^{35}S]GTPγS incorporation in the absence of ligand (basal incorporation). The results were analyzed with GraphPad Prism 5. The EC_{50} values and E_{max} values are listed in Table 1. Cells expressing the wild-type human GPR81 (GPR81-WT) or cells expressing no recombinant GPR81 were used as the positive control and negative control (NC), respectively.

critical for GPR109a functions (Fig. 6; Table 2). Whereas 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 six 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. A previous study showed that a L83V 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 on the functions of GPR109a. The results demonstrated that a L83A mutation reduced the affinity of GPR109a to niacin but did not change the E_{max} value in the functional GTPγS binding assay, whereas a L83A mutation demolished GPR109a activity in both radioligand binding and functional assays (Fig. 6; Table 2).

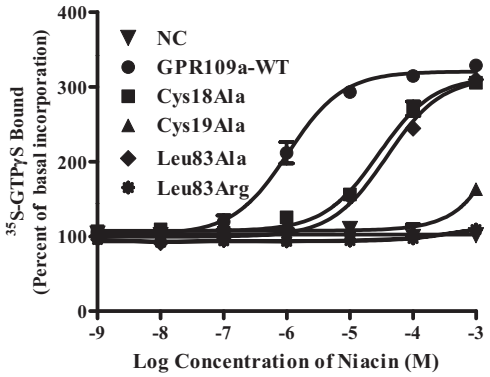

Fig. 6. Mutation studies of GPR109a. Cys18, Cys19, and Leu83 of human GPR109a were mutated into residues as indicated. The resultant recombinant mutant receptors were characterized by a GTPγS assay using niacin as the ligand. Ligand-stimulated [^{35}S]GTPγS incorporations are shown as a percentage of [^{35}S]GTPγS incorporation in the absence of ligand (basal incorporation). The results were analyzed with GraphPad Prism 5. The EC_{50} values and E_{max} values are listed in Table 1. Cells expressing the wild-type human GPR109a (GPR109a-WT) or cells expressing no recombinant GPR109a were used as a positive control and negative control (NC), respectively.

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 (Miller et al., 1964; Issekutz et al., 1965; Kamijo et al., 2000; Achten and Jeukendrup, 2004; Lam et al., 2005). 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., 2005, 2006; Cheng et al., 2006; Maciejewski-Lenoir et al., 2006). 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 in vitro (Boyd et al., 1974; Puhakainen and Yki-Järvinen, 1993; Cai et al., 2008; Liu et al., 2009). 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 in vivo. The low affinity of lactate for GPR81 coupled with its fast metabolism in vivo makes the administration of lactate as a probe agonist very impractical. In addition, lactate can be converted to glucose 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.

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. In a previous study, we 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 identity similar to the lactate receptor, GPR81, the niacin receptor, GPR109a (as known as 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 level (1–2 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 used 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 probably 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 are different in GPR109a and GPR109b. Residues conserved between human and zebrafish GPR81s but different from those in GPR109a and GPR109b may play a role for the specific interaction between lactate and GPR81. Whereas 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 probably 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 ECLs, particularly ECL2 and ECL3, of GPR81 are the least conserved regions between the human and zebrafish receptors (Fig. 1). Likewise, these regions are also the least conserved regions between GPR81 and GPR109a. However in the ECL2 region, there is a C165-E166-S167-F168 motif that is conserved in all GPR81 receptors, whereas this motif is C177-S178-S179-F180 in GPR109a and C177-I178-S179-F180 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). Structural comparison of

GPR109a ligands, niacin, and β-hydroxybutyrate (Fig. 7A) suggests that it is likely that the β-hydroxyl group in β-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 E166D 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 the GPR109a ligand, β-hydroxybutyrate. Structural comparison between GPR81 and GPR109a ligands (Fig. 7A) indicates that the α-hydroxyl group in L-lactate and α-hydroxybutyrate is a top candidate to interact with Glu166. Phe168 in GPR81 is also critical for GPR81 functions. A F168A mutation abolished GPR81 function but a F168Y mutant retained function, suggesting that an aromatic (or 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 and therefore indirectly affect the interaction between L-lactate and the binding sites in GPR81. Ser167 seems to be slightly less critical. Although the S167A mutation reduced the E_{max} value, this mutation did not affect the EC_{50} value. The reduced E_{max} 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 S167T mutant retained the full function of GPR81, supporting this hypothesis.

Arg71at 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 GPR81s but 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 of that of Leu83, the corresponding residue in GPR109a. A 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 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 L83R 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 interacts with L-lactate through ionic or hydrogen bonding. Structure comparison between GPR81 ligands (L-lactate and α-hydroxybutyrate) and GPR109a ligands (niacin and 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 Interacts with Residues Arg71, Arg99, Glu166, and Arg240 of GPR81. Previous computer modeling suggested that Arg99 at TM3, Tyr233 and Arg240 at TM6, and

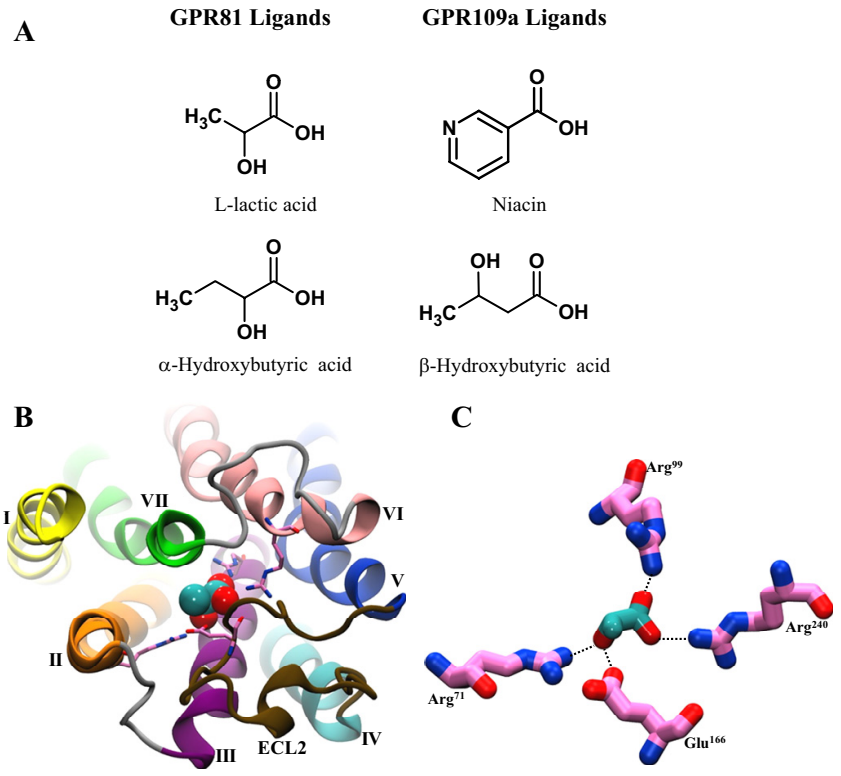

Fig. 7. Model of the GPR81 ligand binding site. A, structural comparison between GPR81 ligands and GPR109a ligands. Acid forms of the molecules are shown. B, homology modeling of GPR81. The seven-transmembrane helical domains of GPR81 are shown along with the extracellular loops. L-Lactate (shown in van der Waals representation) is docked in the putative binding site formed by residues from TM2, TM3, TM6, 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, and the hydroxyl group interacts with basic residue Arg71 and acidic residue Glu166.

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 and 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 seem not be able to interact with lactate directly because of 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 of their direct interaction with L-lactate 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, in closer proximity to 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 six 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 subfamily of receptors and suggests that they play a role in GPR81 and GPR109 functions, probably 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 suggesting that these six Cys residues participate in the disulfide bond formation. It is likely that these Cys residues form intramolecular disulfide bonds because if those Cys residues are paired with other molecules they may not have sufficient partner proteins when they are overexpressed in the recombinant system. We have expressed GPR81 in many different host cell types, including Chinese hamster ovary, 293, SK-N-MC, and COS7 cells, but did not observe significant pharmacological differences, which supports our hypothesis. Another pos-

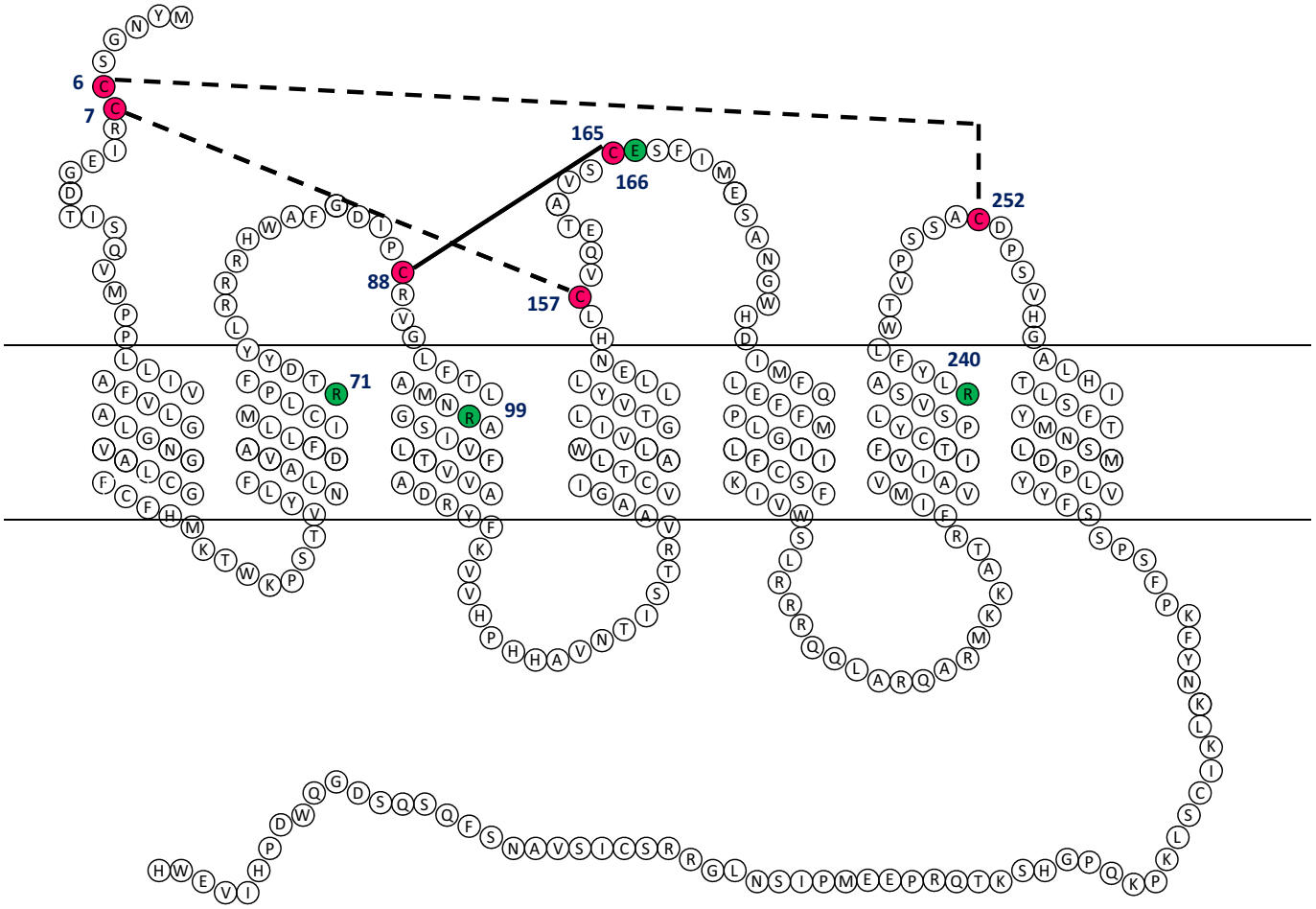

Fig. 8. Predicted disulfide bonds among the Cys residues at the extracellular domains of GPR81. Cys residues that potentially form disulfide bonds are shown in red. The disulfide bond (Cys88–Cys165) conserved in many GPCRs is shown as a solid line. Putative disulfide bonds between Cys6 and Cys252 and between Cys7 and Cys157 are shown as dashed lines. Residues, Arg71, Glu166, Arg99, and Arg240, which potentially interact with L-lactate, are shown in green.

sibility is that those Cys residues may be involved in GPCR dimerization, particularly in a homodimer, in which disulfide bonds can be formed between molecules without the requirement of additional proteins. Among those Cys residues, the predicted C88–C165 bridge (solid line, Fig. 8) is conserved in many GPCRs (Cook and Eidne, 1997; Zhang et al., 1999). Consistent with this statement, mutations of Cys88 or Cys165 coincidentally both reduce GPR81 cell surface expression, indicating that this pair of disulfide bonds is important for GPR81 cell surface expression. 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 in mutants with lack of cell surface expression (Tunaru et al., 2005). For the other four Cys residues at the extracellular loop of GPR81, we predict that they form two bridges between C6 and C252 and C7 and C157. Mutations of those Cys residues resulted in GPR81 mutants devoid of response to lactate despite the cell surface expression comparable with 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 is hard to evaluate whether those Cys mutations have equal impact on GPR81 function as we cannot differentiate a reduction of affinity 20 times and a total loss of affinity because we can only use lactate at concentrations less than 100 mM. However, a previous study of GPR109a demonstrated that mutations at Cys183 and Cys266 in GPR109a have different impacts on GPR109a functions. Whereas mutation at Cys183 abolishes GPR109a function, mutation at Cys266 only reduces the affinity of GPR109a approximately 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), whereas mutation at Cys19 led to the complete abolishment of GPR109a function. These results strongly suggest that, in GPR109a, Cys18 pairs with Cys266, whereas Cys19 pairs with Cys183. On the basis of 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 a possible explanation to help answer this question. With the close positions between Cys6 and Cys7 and between 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 extracellular domain of GPR81 formed by the Cys knot may only allow very small molecules to enter the ligand-binding pocket because of 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 article, we are aware of the efforts by International Union of Basic and Clinical Pharmacology to provide nomenclature for the class of receptor encompassing GPR81, GPR109a, and 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 and the previous results from mutation studies on niacin receptor GPR109a as 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 cannot exclude the possibility that the mutation of these residues disrupted receptor dimerization and therefore led to the loss of receptor functions. One thing that we have to acknowledge is that, because of the low affinity of lactate to GPR81, mutations that led to reduced affinity of the receptor may appear to be total loss of function because we can only use lactate up to 100 mM. With a 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. We hope that in the future a high-affinity ligand will be made available for this receptor.

Authorship Contributions

Participated in research 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.

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