Deorphanization of GPRC6A: A Promiscuous L-α-Amino Acid Receptor with Preference for Basic Amino Acids

Petrine Wellendorph, Kasper B. Hansen, Anders Balsgaard, Jeremy R. Greenwood, Jan Egebjerg, and Hans Brüuner-Osborne

Department of Medicinal Chemistry, the Danish University of Pharmaceutical Sciences, Copenhagen, Denmark (P.W., K.B.H., A.B., J.R.G., H.B.-O.); and Department of Molecular Biology, H. Lundbeck A/S, Valby, Denmark (K.B.H., J.E.)

Received September 23, 2004; accepted December 2, 2004

ABSTRACT

One of the most important tasks of molecular pharmacology is the deorphanization of the large number of G-protein-coupled receptors with unidentified endogenous agonists. We recently reported the cloning and analysis of expression of a novel human family C G-protein-coupled receptor, termed hGPRC6A. To identify agonists at this orphan receptor, we faced the challenges of achieving surface expression in mammalian cell lines and establishing an appropriate functional assay. Generating a chimeric receptor construct, h6A/5.24, containing the ligand binding amino-terminal domain (ATD) of hGPRC6A with the signal transducing transmembrane and C terminus of the homologous goldfish 5.24 receptor allowed us to overcome these obstacles. Homology modeling of the hGPRC6A ATD based on the crystal structure of the metabotropic glutamate receptor subtype 1 predicted interaction with L-amino acids and was employed to rationally select potential ligands. Measurement of Ca2+-dependent chloride currents in *Xenopus laevis* oocytes facilitated the deorphanization of h6A/5.24 and identification of L-α-amino acids as agonists. The most active agonists were basic L-amino acids, L-Arg, L-Lys, and L-ornithine, suggesting that these may function as endogenous signaling molecules. Measurement of intracellular calcium in tsA cells expressing h6A/5.24 allowed determination of EC50 values, which confirmed the agonist preferences observed in oocytes. Cloning, cell surface expression and deorphanization of the mouse ortholog further reinforces the assignment of the agonist preferences of hGPRC6A. This study demonstrates the utility of a chimeric receptor approach in combination with molecular modeling, for elucidating agonist interaction with GPRC6A, a novel family C G-protein-coupled receptor.

With the complete human genome sequence accessible, the identification of novel genes has been greatly facilitated, which has led to the prediction of a large number of orphan receptor genes (Venter et al., 2001; Wise et al., 2004). This has sparked intense interest, particularly within the field of G-protein-coupled receptors (GPCRs), which represent about 50% of current drug targets. Deorphanizing receptors (i.e., identifying one or more endogenous agonists) has been difficult, and the physiological relevance of many of these potential drug targets remains unknown (Wise et al., 2004). We identified the human GPCR, family C, subtype 6A (hGPRC6A) receptor by homology searches in sequence databases and subsequently cloned a putative human orphan GPCR (Wellendorph and Brüuner-Osborne, 2004). Cloning revealed the existence of three different splice variant forms, which represent about 50% of current drug targets. Deorphanizing receptors (i.e., identifying one or more endogenous agonists) has been difficult, and the physiological relevance of many of these potential drug targets remains unknown (Wise et al., 2004). We identified the human GPCR, family C, subtype 6A (hGPRC6A) receptor by homology searches in sequence databases and subsequently cloned a putative human orphan GPCR (Wellendorph and Brüuner-Osborne, 2004). Cloning revealed the existence of three different splice variant forms, verified by the exon-intron organization of the gene for hGPRC6A, mapping to chromosome 6q22.31 (GenBank accession numbers AY435125, AY435126, and AY435127). Reverse transcription-PCR analysis showed that the longest isoform of GPRC6A was expressed at the highest level in human tissues, and an ortholog of that form was also found in mice (GenBank accession number AY101365). GPRC6A displays 45% amino acid sequence identity with the goldfish
odorant receptor 5.24 (Speca et al., 1999), 34% identity with the human calcium-sensing receptor (Brown, 1999), and 28% identity with the human taste receptor T1R1 (Nelson et al., 2001; Wellendorph and Bräuner-Osborne, 2004). This places hGPRC6A in family C of GPCRs, which also includes the metabotropic glutamate (mGlu) receptors (Pin and Duvoisin, 1995), GABA\textsubscript{B1,2} (Mohler and Fritschi, 1999), and some orphan and pheromone receptors (Cheng and Lotan, 1998; Bräuner-Osborne and Krosgaard-Larsen, 2000; Robbins et al., 2000; Bräuner-Osborne et al., 2001; Calver et al., 2003). Family C GPCRs are distinguished from other GPCR superfamilies by an unusually large amino-terminal domain (ATD), consisting of a globular ligand binding bi-lobular structure (lobe I and II) connected by a hinge region (Kunishima et al., 2000). Based on the crystal structure of glutamate bound to the ATD of mGlu\textsubscript{U} (PDB code 1EWK), we have generated a three-dimensional homology model of GPRC6A, suggesting that the endogenous agonist for GPRC6A is an \( \alpha \)-amino acid. The lack of cell surface expression of the hGPRC6A protein in mammalian cell lines (Wellendorph and Bräuner-Osborne, 2004) hampered the development of a functional pharmacological assay to examine this prediction. To overcome this obstacle, we have constructed chimeric receptors of hGPRC6A and the close functional 5.24. The chimera composed of the ATD of hGPRC6A, and the 7TM domain and carboxyl terminus of 5.24 (h6A/5.24), confirmed that 1-\( \alpha \)-amino acids can bind to the hGPRC6A ATD and activate the 5.24 dependent signal transduction pathways when expressed in *Xenopus laevis* oocytes or tsA cells, thus supporting our proposed model. We further document these findings by cloning and deorphanization of the native murine homolog of GPRC6A (mGPRC6A) and providing the first steps toward unraveling the function of GPRC6A.

### Materials and Methods

#### Homology Modeling and Ligand Docking

A homology model of the ligand-binding domain of GPRC6A was built from the protein subsequence (1EWK chain A) corresponding to the crystallized extracellular domain of the mGluU receptor (Kunishima et al., 2000), with the aid of the Polish Bioinformatics metaserver (Ginalski et al., 2001; Wellendorph and Bräuner-Osborne, 2004) hampered the development of a functional pharmacological assay to examine this prediction. To overcome this obstacle, we have constructed chimeric receptors of hGPRC6A and the close functional 5.24. The chimera composed of the ATD of hGPRC6A, and the 7TM domain and carboxyl terminus of 5.24 (h6A/5.24), confirmed that 1-\( \alpha \)-amino acids can bind to the hGPRC6A ATD and activate the 5.24 dependent signal transduction pathways when expressed in *Xenopus laevis* oocytes or tsA cells, thus supporting our proposed model. We further document these findings by cloning and deorphanization of the native murine homolog of GPRC6A (mGPRC6A) and providing the first steps toward unraveling the function of GPRC6A.

#### Cloning of GPRC6A

Human GPRC6A was cloned in our laboratory as recently reported (GenBank accession number AY435125) (Wellendorph and Bräuner-Osborne, 2004). Full-length mouse GPRC6A was cloned by nested PCR according to the following protocol (kindly provided by T. M. Strom, Technical University Munich, Germany). The first round of PCR was carried out on cDNA generated from 17-day-old embryos (BD Biosciences, Palo Alto, CA) using Pfu polymerase (Stratagene, La Jolla, CA), forward primer 5'-ctcttctgcggccgcctcctaggaact-3' and reverse primer 5'-aacttctctctctctcctctc-3' (20 cycles at 50°C annealing). The second PCR was performed with forward primer 5'-ctcttctgcggccgcctcctc-3' and reverse primer 5'-aacttctctctctctcctctc-3' (20 cycles at 50°C annealing). A single band of 2900 base pairs was purified (QIAquick Gel Extraction kit; QIAGEN, Hilden, Germany) and TOPO TA cloned into the pCR2.1 vector (Invitrogen, San Diego, CA) according to the manufacturer’s instructions. The obtained cDNA was fully sequenced (MWG Biotech, Ebersberg, Germany) and found to be identical at the protein level with GenBank entry number AY101365.

#### Construction of hGPRC6A and Goldfish 5.24 Chimeras

Chimeras were constructed containing the entire ATD of 5.24 and the 7TM and intracellular domains of hGPRC6A (5.24/h6A) and vice versa (h6A/5.24) by means of overlap extension PCR (Horton et al., 1989). The fusion site in 5.24/h6A was placed between amino acids 16 and 17 upstream of the predicted first transmembrane segment of hGPRC6A (Wellendorph and Bräuner-Osborne, 2004), in accordance with family C chimeras previously generated in our laboratory (Bräuner-Osborne et al., 1999b). The fusion site in h6A/5.24 was similarly constructed upstream of the first TM segment of 5.24 based on an alignment of hGPRC6A and 5.24 (Wellendorph and Bräuner-Osborne, 2004). All PCRs were performed with Pfu polymerase (Stratagene) following the manufacturer’s protocol. Chimeric receptor constructs were confirmed by DNA sequencing (MWG Biotech). Further details on construction of chimeras are available as Supplemental material.

#### Site-Directed Mutagenesis of hGPRC6A and Chimera h6A/5.24

The 919–921KR/AAA mutation in hGPRC6A and point mutations (S149A and T172A) in both hGPRC6A and chimera h6A/5.24 were introduced using the QuikChange mutagenesis kit (Stratagene). Mutations were confirmed by DNA sequencing (MWG Biotech).

#### Epitope Tagging

For cellular expression studies, all receptor constructs were subcloned into a modified pEGFP-N1 vector (BD Biosciences) essentially as described previously (Pagano et al., 2001). In brief, the N-terminal signal peptides were replaced by the mGluU receptor signal peptide, because the latter is known to promote good receptor expression and proper release of the signal peptide. To allow detection of receptor surface expression by immunofluorescence, a c-myc epitope followed by an engineered MluI site was inserted immediately after the signal peptide of mGluU, using a previously generated construct of hGPRC6A in this modified pEGFP-N1 vector (Wellendorph and Bräuner-Osborne, 2004), the cDNA was cut out by the flanking restriction enzymes MluI/NotI and replaced with either the goldfish receptor 5.24 cDNA or chimeric receptor cDNAs. For similar subcloning of mGPRC6A into pEGFP-N1, a MluI/NotI embraced receptor cDNA fragment lacking the native signal peptide was generated by PCR using forward primer 5'-catctgacgggtcatcacccagatgac-3' and reverse primer 5'-ctctctctctctctctctctc-3'. The signal peptide was predicted by the program SignalP with a cleavage site between amino acid positions 20 and 21 (Nielsen et al., 1997).

#### Cell Culture Work and Immunochemistry

Cell culturing of tsA (a transformed HEK 293 cell line) cells and quantification of receptor expression by means of an Amplified peroxidase- and dye-amplified enzyme-linked immunosassay (Probes, Leiden, The Netherlands) was carried out exactly as described previously (Wellendorph and Bräuner-Osborne, 2004). Quantification was accomplished by measuring fluorescence intensity (excitation at 530 nm/emission at 590 nm) on a NOVOSTar microplate reader (BMG Labtechnologies, Offenburg, Germany). All data points were obtained in triplicate and confirmed in three independent experiments. Statistical significance was assessed using student’s t-test.

#### Oocyte Preparation and Injection

For expression in *X. laevis* oocytes, cDNAs were subcloned into the pGEMHE-3Z vector containing an upstream T7 promoter. Linearized plasmids were used to produce cRNAs with mMessage mMachine kits (Ambion, Austin, TX). Oocytes were surgically removed from mature female *X. laevis* frogs anesthetized in a 0.4% MS-222 (3-aminobenzoic acid ethyl ester) solution (Sigma-Aldrich, St. Louis, MO) for 10 to 15 min. To remove the follicle layer, the oocytes were subsequently placed in OR2 buffer (92.5 mM NaCl, 2.0 mM KCl, 1.0 mM MgCl\textsubscript{2}, and 5.0 mM HEPES, pH 7.5) including 0.5 mg/ml collagenase (type IA) (Sigma-
Aldrich) for 2 to 3 h at room temperature. Healthy-looking stage V–VI oocytes were selected and the following day injected with cRNA (25–75 ng in 50 nl of water) and maintained in Modified Barth’s solution (88 mM NaCl, 1.0 mM KCl, 2.4 mM NaHCO3, 0.41 mM CaCl2, 0.82 mM MgSO4, 0.3 mM Ca(NO3)2, 15 mM HEPES, pH 7.5, 2% sodium pyruvate, 100 IU/ml penicillin, and 100 µg/ml streptomycin) at 18°C. For calcium buffering experiments, oocytes were incubated for 30 min in OR2 buffer containing 100 mM HEPES, pH 7.5, 2% sodium pyruvate, 100 IU/ml penicillin, and 100 µg/ml streptomycin at 18°C. For calcium buffering experiments, oocytes were incubated for 30 min in OR2 buffer containing 100 mM HEPES, pH 7.5, 2% sodium pyruvate, 100 IU/ml penicillin, and 100 µg/ml streptomycin. Finally, cells were incubated for 1 h at room temperature in the dark. 

**Measurement of Intracellular Calcium Levels.** tsA cells were maintained and transfected as described previously (Wellendorph and Bräuner-Osborne, 2004) except that two million cells were plated in a 10-cm dish and transfected the following day with 8 µg of plasmid DNA. The day after transfection, cells were split into poly-D-lysine-coated black 96-well plates with clear bottoms (BD Biosciences). Two days after transfection, pharmacological activity was assessed by measurement of intracellular calcium levels essentially as described previously (Kuang et al., 2003). In brief, cells were washed with assay buffer (5.3 mM KCl, 0.44 mM KH2PO4, 4.2 mM NaHCO3, 138 mM NaCl, 0.34 mM Na2HPO4, 5.6 mM D-Glucose, 20 mM HEPES, 1 mM CaCl2, 1 mM MgCl2, and 1 mg/ml bovine serum albumin, pH 7.4) and preincubated for 2 h at 37°C in 100 µl of assay buffer. The assay buffer was exchanged and cells were preincubated for another 2 h at 37°C. The assay buffer was then replaced with 50 µl of assay buffer containing 6 µM Fluo-4AM (Molecular Probes) and incubated for 1 h at room temperature in the dark. Finally, cells were washed three times with assay buffer without bovine serum albumin and then incubated with 150 µl of assay buffer without bovine serum albumin for 30 min at room temperature in the dark. The cell plate was then transferred to a NOVOstar microplate reader, and responses were recorded at room temperature using excitation/emission wavelengths of 485 and 520 nm, respectively. Responses (Δfluorescence units) were calculated as peak fluorescence after agonist addition subtracted fluorescence before agonist addition. Concentration-response curves were analyzed by nonlinear regression using Prism 4.0 (GraphPad Software, San Diego, CA). Pharmacological experiments were performed in triplicate and repeated in three independent experiments.

**Results**

**Prediction of Agonist Preferences in hGPRC6A by Homology Modeling.** To direct the deorphanization of hGPRC6A, we initially performed structural comparison with homologous family C receptor proteins. The previously published alignment of hGPRC6A and the mGlur1 receptor indicated that a number of residues in the mGlur1 receptor, known to be involved in agonist binding (Kunishima et al., 2000), are conserved between the two receptors (Wellendorph and Bräuner-Osborne, 2004). In fact, all residues directly bound to the α-amino acid moiety of L-Glu in the mGlur1 receptor (Kunishima et al., 2000) are conserved in hGPRC6A, whereas those interacting with the distal carboxylate of L-Glu are not. This suggested that the endogenous agonist(s) for hGPRC6A could be α-amino acid(s) other than L-Glu. A recent study demonstrated the goldfish 5.24 receptor to be responsive to a range of α-amino acids, with Lys and Arg being the most potent (Speca et al., 1999). The fact that hGPRC6A bears highest amino acid identity to the 5.24 receptor suggested that it may also prefer basic amino acids. To expound upon this hypothesis, we chose L-Lys as a medium-length basic amino acid and generated a three-dimensional homology model of the ATD of hGPRC6A using mGlur1 X-ray crystal data as the template, and then docked L-Lys to the putative binding cleft (Fig. 1A). The model was constructed under the assumption that the binding site orientation of L-Lys in hGPRC6A substantially resembles that of L-Glu in the mGlur receptors—an assumption shared by Kuang et al. (2003) in their model of L-Lys bound to the homologous 5.24 receptor suggested that it may also prefer basic amino acids. To expound upon this hypothesis, we chose L-Lys as a medium-length basic amino acid and generated a three-dimensional homology model of the ATD of hGPRC6A using mGlur1 X-ray crystal data as the template, and then docked L-Lys to the putative binding cleft (Fig. 1A). The model was constructed under the assumption that the binding site orientation of L-Lys in hGPRC6A substantially resembles that of L-Glu in the mGlur receptors—an assumption shared by Kuang et al. (2003) in their model of L-Lys bound to the homologous 5.24 receptor.
After a series of refinements, a number of credible complexes were generated with low energies, all characterized by ion pairing of the distal ammonium group of L-Lys with Glu170 of hGPRC6A, although with some differences in ligand and side-chain conformation. After inspection of the results, the best model was chosen as that in which the side-chain carboxylate of Asp303 binds to the α-ammonium group in roughly the same way as the mGlu1 receptor, in which both the agonist and Glu170 side chains were extended and in which Tyr148 occupies a hydrophobic space similar to that of Trp110 in the mGlu1 receptor. The latter tyrosine is also a feature of the mGlu1 receptor binding site, which we and others have previously modeled with high confidence (Malherbe et al., 2001; Clausen et al., 2002). This yields a more polar binding cavity than in 5.24 but framed on two sides by aromatic residues (Fig. 1B), a feature common to a range of amino acid binding sites. Apart from Glu170, the hydrogen bond network in the distal region may be supported by polar residues Gln73, Gln280, and Asn304. Although direct contacts to lobe II in this region have not been established, we found space for water to mediate interdomain binding. Further acidic residues on the weakly conserved loops bearing Tyr74 and Lys409 in mGlu1 are also implicated. Finally, it was apparent that the length and shape of the pocket, and the flexibility of Glu170 and its proximity to the L-α-amino acid recognition site, did not imply specificity for the flexible side chain of L-Lys; rather, it implied that aliphatic side chains possessing strong distal hydrogen bond donors could be accommodated and preferred over amino acids with hydrophobic or acidic side chains. We should note that the precise conformation of L-Lys could not be unequivocally predicted and that we explored alternative binding mode hypotheses, albeit leading to higher energies (see Supplemental data). These are currently being investigated by site-directed mutagenesis to refine the model. However, the central consistent finding was that the agonist would most probably be an α-amino acid, which directed our ligand screening as described below.

Expression Analysis of hGPRC6A and 5.24 Chimeric Receptors. To test the predictions of our homology model and probe the agonist preferences of hGPRC6A, we faced two challenges in developing a suitable pharmacological assay: 1) a poor understanding of the native signaling pathway and 2) the observed intracellular localization of the hGPRC6A protein when expressed in heterologous systems (Wellendorph and Bräuner-Osborne, 2004). Thus, to solve possible trafficking or compartmentalization issues of the hGPRC6A protein, we identified a potential retention motif, RKR, seven amino acids upstream of the C terminus. In the GABA_A receptor, a similar motif acts as an endoplasmic retention signal, preventing trafficking of the GABA_A(1,2) receptor subunit to the cell surface. Because mutation of the GABA_A(1,2) receptor retention signal RSRR to ASAA is able to prevent retention (Pagano et al., 2001), we mutated RKR to AAA in hGPRC6A, but this mutation was unable to improve surface expression (Fig. 2A). We next examined the possibility of agonist-induced internalization of hGPRC6A during cell culturing by mutating residues Ser149 and Thr172 predicted to be involved in agonist binding (Fig. 1B), but we found no effect on surface expression (Fig. 2A). To determine which domain of hGPRC6A might prevent it from being trafficked to the plasma membrane, we made two chimeras by exchanging either the ATD or the 7TM plus carboxyl terminus of hGPRC6A with the corresponding region(s) of 5.24.
tative expression studies in intact tsA cells failed to show any significant cell surface expression of chimera 5.24/h6A (fluorescence not different from that of the mock or hGPRC6A wild-type transfected cells, Fig. 2C). By contrast, cells transfected with chimera h6A/5.24 showed consistently higher intensity fluorescence than hGPRC6A transfected cells, indicating that this chimera was expressed on the cell surface (Fig. 2C). Using equal amounts of DNA for the transfections, native 5.24 was reliably expressed at an even higher level than both h6A/5.24 and the heterodimeric GABA<sub>W1,2</sub> receptor, which served as positive control. This clearly indicates that the 7TM/carboxyl terminus of hGPRC6A is responsible for the lack of surface expression and that this can be alleviated by replacing these domains with the equivalent 7TM/carboxyl terminus of 5.24.

**Deorphanization of hGPRC6A by Functional Analysis of Chimera h6A/5.24.** We have previously used the chimeric receptor approach to document the position of the binding pocket of the calcium-sensing receptor (Bräuner-Osborne et al., 1999b). Thus, because chimera h6A/5.24 contains both the presumed agonist binding domain of hGPRC6A (positioned in the ATD) and the signaling domain of 5.24 and is targeted to the plasma membrane of heterologous expression systems, it represents a valuable pharmacological tool for determining the endogenous agonist(s) for hGPRC6A. Separate reports have indicated that 5.24 couples via the G<sub>a</sub> subfamily of G-proteins, leading to increases in phosphoinositide hydrolysis and in turn causing release of calcium from intracellular stores (Speca et al., 1999; Kuang et al., 2003). Because of the inherent presence of nutrients in cell culture media (particularly L-α-amino acids), and the obvious impracticality of excluding essential amino acids such as L-Arg and L-Lys (Scott et al., 2000), we decided to use the X. laevis expression system, in which the buffer is an amino acid-free Ringer’s solution (see Materials and Methods). In this system, wild-type 5.24 has been shown to produce an inward current upon L-α-amino acid stimulation (Speca et al., 1999) because of the activation of Ca<sup>2+</sup>-activated chloride channels. As expected, we observed a strong inward current in oocytes expressing 5.24 when L-Lys or L-Arg were applied at 10 μM (Fig. 3A). When testing chimera h6A/5.24 in this system, we obtained responses with both L-Lys and L-Arg at 100 μM (Fig. 3C), demonstrating the utility of the system for further pharmacological investigations. Neither uninjected oocytes (Fig. 3B) nor oocytes injected with cRNA encoding hGPRC6A or chimera 5.24/h6A displayed any response to 100 μM L-Arg (data not shown). The possibility of G<sub>i</sub>-coupling was examined in similar experiments with coexpression of hGPRC6A with inwardly rectifying potassium channels (Kir3.1 + Kir3.4) (Schreibmayer et al., 2000).

**Fig. 3.** Deorphanization of GPRC6A in X. laevis oocytes. Traces of Ca<sup>2+</sup>-dependent chloride currents in X. laevis were obtained by two-electrode voltage clamping at a holding potential of −80 mV. A, oocytes expressing the goldfish 5.24 receptor stimulated with either 10 μM L-Lys or L-Arg. B, uninjected oocytes show no response when stimulated with 100 μM L-Arg. C, representative traces demonstrating the L-α-amino acid agonist preferences of the h6A/5.24 chimeric receptor using 100 μM concentration. D, of all the tested amino acids, only L-Arg, L-Lys, and L-Orn were able to activate h6A/5.24 at 10 μM concentration (note that the scale is different from that in C). E, oocytes expressing mGPRC6A loose responsiveness to L-Orn when treated with BAPTA-AM. F, representative traces demonstrating the L-α-amino acid agonist preferences of the native mouse ortholog of GPRC6A. All traces are representative of n = 3 for each experiment.
al., 1996) and gave no result (data not shown). Screening the 20 naturally occurring l-α-amino acids at 100 μM concentrations at h6A/5.24 revealed not only that basic l-α-amino acids but also several small neutral aliphatic l-α-amino acids (Ala, Gly, and Ser) are able to activate h6A/5.24, whereas the corresponding d-amino acids are inactive (Table 1). Of this initial panel, only basic amino acids l-Arg and l-Lys showed activity when the concentration was lowered to 10 μM (Fig. 3D). We then examined other endogenous l-amino acids related to these two hits, and found l-ornithine (l-Orn) and l-citrulline (l-Cit) to be agonists at the receptor (Fig. 3C), although with different activities, given that only l-Orn was active at 10 μM in our assay (Fig. 3D). Concentrations of the three basic amino acids at 3 μM elicited no response, and higher concentrations (300 μM) led to decreased currents compared with 100 μM (data not shown) presumably because of desensitization of the receptor and/or the signaling pathway proteins, as reported by others (Minakami et al., 1994; Quick et al., 1996). l-Arg, l-Lys, and l-Orn were thus the most potent agonists tested, showing that the receptor has a preference for basic amino acids. It is interesting that l-Orn and l-Cit are related to in vivo l-Arg metabolism and are both implicated in the urea cycle (Morris, 2004). Other known metabolites of the urea cycle and related pathways, such as agmatine, urea, creatine, creatinine, and spermine, had no known metabolites of the urea cycle and related pathways, such as agmatine, urea, creatine, creatinine, and spermine, had no known metabolites of the urea cycle (Morris, 2004). Other known metabolites of the urea cycle and related pathways, such as agmatine, urea, creatine, creatinine, and spermine, had no effect (Table 1). These related metabolites are characteristically devoid of an α-amino acid moiety, which seems to be a requirement for hGPRC6A affinity. Finally, screening of several established and putative signaling molecules gave no further positive hits (Table 1).

To confirm that activation of the chimeric h6A/5.24 receptor originated from the ATD of hGPRC6A rather than the 7TM domain of 5.24, we performed two point mutations, S149A and T172A, located in the putative binding cleft of the ATD of the receptor. These residues are predicted by our homology model to make interactions with the α-amino acid moiety of the agonist (Fig. 1) and are conserved and proven crucial for ligand binding in mGlu receptors, the calcium-sensing receptor, 5.24, and the GABA<sub>B</sub> receptor (Bräuner-Osborne et al., 1999b; Galvez et al., 2000; Kuang et al., 2003; Sato et al., 2003). When either of these mutants were expressed in oocytes and exposed to l-Lys or l-Arg in concentrations up to 1 mM, no responses were elicited (data not shown), although they seem to be correctly expressed and targeted to the cell surface as shown by quantitative immunolabeling of transfected cells (Fig. 2C). This supports the hypothesis that Ser149 and Thr172 are in fact part of the binding site located within the ATD of hGPRC6A and that the chimeric approach for deorphanization of hGPRC6A is valid. Absolute proof of our findings would require pharmacological verification on the native human receptor, which at this point is impeded by the apparent lack of surface expression. Because others have previously shown that orthologs of GPCRs may display notable differences in surface expression in heterologous systems (Calver et al., 2003), we isolated the cDNA of the mGPRC6A to obtain a wild-type reference.

**Cloning and Deorphanization of mGPRC6A.** We used the reported full-length sequence of mGPRC6A (GenBank accession number AY101365) to design primers positioned in the 5’ and 3’ untranslated region of the gene, and we successfully cloned the open reading frame of mGPRC6A by nested PCR. Alignment of mouse and human GPRC6A revealed that the proteins have 80% overall identity; the ATDs and predicted 7TM regions are 82% identical, whereas the C termini of the orthologs are only 50% identical (for sequence alignment, see Supplemental data). Alignment of the two ATDs confirms that all residues in the putative binding pocket (Wellendorph and Bräuner-Osborne, 2004) are conserved, except for Gln280 in hGPRC6A, which corresponds to Lys280 in mGPRC6A. The high degree of conservation of residues constituting the binding site suggests very similar ligand preferences. By contrast with its human counterpart, mGPRC6A is trafficked to the cell surface when transiently expressed in intact tsA cells (Fig. 2D). Subsequent deorphanization experiments were performed on the oocyte expression system, analogous to those performed on h6A/5.24. Initial screening revealed that the basic amino acids l-Arg, l-Lys, and l-Orn at 100 μM were able to elicit agonist responses at mGPRC6A. To seek out potential differences in the G-protein coupling pathway of this native receptor and 5.24, we next examined the specificity of the coupling pathway. As with the h6A/5.24 chimera, responses to agonist could be almost completely obliterated when intracellular calcium was chelated with BAPTA-AM before stimulation, thus demonstrating that mGPRC6A preferentially signals through the G<sub>q</sub> pathway (Fig. 3E) (Skeberdis et al., 2001). Complete screening of l- and d-amino acids at 100 μM established that basic amino acids (l-Arg, l-Lys, and l-Orn) and small aliphatic amino acids (l-Ala and l-Ser) are preferred agonists for mGPRC6A, as was similarly shown for h6A/5.24 (Fig. 3, C and F). The agonist preferences of mGPRC6A differ somewhat from those of hGPRC6A: mGPRC6A does not respond to l-Cit and l-Gly at 100 μM (Fig. 3, C and F), and only l-Orn was able to elicit a response at 10 μM, indicating a lower sensitivity of this receptor. This may relate, however, to other factors such as receptor expression level and batch variation between oocytes. All other screened natural l- and d-amino acids were inactive.

**Quantitative Pharmacological Characterization of h6A/5.24.** Because of low throughput and variability of responses (Pin et al., 1992), measurement of Ca<sup>2+</sup>-activated chloride currents in oocytes is not an ideal assay for quantitative pharmacology. To develop a more robust assay with higher throughput, we thus expressed receptors in tsA cells and measured intracellular calcium levels by use of the fluorescent calcium sensitive dye Fluo-4. As mentioned previously, mammalian cell culture media contain high levels of l-α-amino acids that probably would desensitize the GPRC6A receptors. In agreement with this hypothesis, no response was obtained from 1 mM l-Arg in hGPRC6A- or

**TABLE 1** Compound testing in *X. laevis* oocytes expressing the chimeric receptor h6A/5.24

<table>
<thead>
<tr>
<th>Natural α-amino acids</th>
<th>l-α-amino acids</th>
<th>All other inactive</th>
<th>D-amino acids</th>
<th>All inactive</th>
</tr>
</thead>
<tbody>
<tr>
<td>Active: Arg, Lys, Ala, Gly, Ser (see Fig. 3C)</td>
<td>l-Orn, l-Cit; (see Fig. 3C)</td>
<td>l-Arg, l-Lys, l-Orn</td>
<td>l-Ala, l-Ser</td>
<td>l-Orn, l-Cit</td>
</tr>
</tbody>
</table>

| Metabolic intermediates | Active: l-Orn, l-Cit | Inactive: agmatine, creatine, creatinine, spermine, urea |

All compounds were tested at 100 μM except Ca<sup>2+</sup>, which was tested at 4 mM.
mGPRC6A-transfected cells and only small responses were observed from h6A/5.24- and 5.24-transfected cells (Fig. 4A). Recently, Kuang et al. (2003) published a modified calcium imaging protocol in which cells expressing 5.24 were preincubated with calcium assay buffer for 2 x 2 h before Fluo-4 loading. This procedure greatly increased the response from h6A/5.24- and 5.24-transfected cells, presumably because of resensitization of the receptors, whereas it did not improve the responses from hGRPRC6A or mGPRC6A (Fig. 4A). The lack of response from hGRPRC6A correlates with the intracellular retention (Fig. 2A) and lack of response in oocytes. As shown in Fig. 3F, the mGPRC6A receptor elicits a small response in oocytes compared with h6A/5.24 (Fig. 3, compare C and F and note the difference in scale), which might explain the lack of response in the calcium measurement assay. On the other hand, mGPRC6A might be easier to desensitize or harder to resensitize than 5.24 or h6A/5.24.

Exposure of h6A/5.24-transfected tsA cells to L-Arg elicited a robust concentration-dependent increase in fluorescence intensity (Fig. 4B). We could thus use the modified calcium imaging protocol to determine the potency of all L-α-amino acids previously tested in the electrophysiological experiments (Fig. 4C). Table 2 shows the EC50 values that we obtained; the rank-order of potency was L-Arg > L-Orn ≥ L-Lys = L-Ala ≥ Gly = L-Cit ≥ L-Gln = L-Ser ≥ L-Met, which agrees very well with the results from the electrophysiological experiments, although the weak agonists L-Gln and L-Met were not identified in the latter. The absolute EC50 values also correlate very nicely with the qualitative experiments performed on oocytes. To confirm that the responses obtained originated from the ATD of hGPRC6A, we tested all active compounds at 1 mM on S149A and T172A mutated h6A/5.24. In agreement with the results obtained in oocytes, no responses were detected (data not shown). All active compounds were also inactive when tested on mock-transfected cells at their maximal concentration used for the concentration-response curves (data not shown).

**Discussion**

We reported recently the cloning and expression analysis of the novel human family C receptor hGPRC6A. Although the number of genes classified as orphan receptors has steadily climbed in recent years, the identification of endogenous agonists for these has proven to be a nontrivial task. One particular obstacle for pharmacological screening in vitro is the difficulty of obtaining cell surface expression in recombinant systems (Wise et al., 2004). In this work, we present the approach of using chimeric receptors to overcome this particular problem. Thus, achieving surface expression of the hGPRC6A binding domain in the *X. laevis* oocyte expression system has enabled us to deorphanize this human receptor by reverse pharmacology. Additional deorphanization of the native murine ortholog of GPRC6A further verifies the responses from hGRPRC6A or mGPRC6A (Fig. 4A). The lack of response from hGRPRC6A correlates with the intracellular retention (Fig. 2A) and lack of response in oocytes. As shown in Fig. 3F, the mGPRC6A receptor elicits a small response in oocytes compared with h6A/5.24 (Fig. 3, compare C and F and note the difference in scale), which might explain the lack of response in the calcium measurement assay. On the other hand, mGPRC6A might be easier to desensitize or harder to resensitize than 5.24 or h6A/5.24.

Exposure of h6A/5.24-transfected tsA cells to L-Arg elicited a robust concentration-dependent increase in fluorescence intensity (Fig. 4B). We could thus use the modified calcium imaging protocol to determine the potency of all L-α-amino acids previously tested in the electrophysiological experiments (Fig. 4C). Table 2 shows the EC50 values that we obtained; the rank-order of potency was L-Arg > L-Orn ≥ L-Lys = L-Ala ≥ Gly = L-Cit ≥ L-Gln = L-Ser ≥ L-Met, which agrees very well with the results from the electrophysiological experiments, although the weak agonists L-Gln and L-Met were not identified in the latter. The absolute EC50 values also correlate very nicely with the qualitative experiments performed on oocytes. To confirm that the responses obtained originated from the ATD of hGPRC6A, we tested all active compounds at 1 mM on S149A and T172A mutated h6A/5.24. In agreement with the results obtained in oocytes, no responses were detected (data not shown). All active compounds were also inactive when tested on mock-transfected cells at their maximal concentration used for the concentration-response curves (data not shown).

**TABLE 2**

<table>
<thead>
<tr>
<th>Compound</th>
<th>EC50 (pEC50 ± S.E.M.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-Arg</td>
<td>44.1 (4.38 ± 0.11)</td>
</tr>
<tr>
<td>L-Orn</td>
<td>112 (3.96 ± 0.05)</td>
</tr>
<tr>
<td>L-Lys</td>
<td>169 (3.77 ± 0.03)</td>
</tr>
<tr>
<td>L-Ala</td>
<td>173 (3.76 ± 0.02)</td>
</tr>
<tr>
<td>Gly</td>
<td>263 (3.58 ± 0.04)</td>
</tr>
<tr>
<td>L-Cit</td>
<td>287 (3.56 ± 0.09)</td>
</tr>
<tr>
<td>L-Gln</td>
<td>590 (3.23 ± 0.05)</td>
</tr>
<tr>
<td>L-Ser</td>
<td>623 (3.21 ± 0.07)</td>
</tr>
<tr>
<td>L-Met</td>
<td>854 (3.07 ± 0.04)</td>
</tr>
</tbody>
</table>
the utility of the chimeric method. We also demonstrate the usefulness of performing thorough homology-based analysis and molecular modeling of the orphan receptor in question for guiding the selection of potential agonists and targeting residues to be mutated. Together, our results demonstrate that GPRC6A is a promiscuous L-α-amino acid receptor with preference for basic amino acids activated in the micromolar range.

Although chimeric receptors have previously been used for elucidation of the relation between structure and function in both the ligand binding and signaling properties of various receptor domains (Bräuner-Osborne et al., 1999a,b, 2001), this report is to our knowledge the first example of applying such a scheme for deorphanization. Particularly within family C GPCRs, many authors have reported of functional chimera between even vaguely related family members. This can most probably be ascribed to the notion that binding and signaling domains of this receptor class were once two separate entities that merged during evolution (Conklin and Bourne, 1994). The h6A/5.24 chimeric receptor presented in this study is expressed at the plasma membrane of heterologous systems, enabling us to survey the binding domain of the orphan hGPRC6A while exploiting the known signal transduction of a clearly homologous receptor, 5.24. In principle, the observed activity of h6A/5.24 could arise either from agonist activation of the ATD of hGPRC6A or the 7TM/intracellular domains of 5.24. Although it is a reasonable assumption that agonist binding takes place between the lobes of the ATD, and recent mutagenesis studies have shown that residues in the ATD of 5.24 are clearly involved in binding L-Lys (Kuang et al., 2003), any possibility of L-Arg or L-Lys activity at the 7TM domain of 5.24 is ruled out by our observation that the point mutations S149A and T172A within the predicted binding site of the chimeric receptor completely obliterate activity. This implies that hGPRC6A mutated at these positions is in fact binding-deficient and that lack of surface expression of the native receptor is not simply a matter of agonist-induced internalization. Further evidence that we have in fact assayed the binding domain of hGPRC6A is provided by the consistency of results with the agonist profile of the mouse ortholog, mGPRC6A.

From the three-dimensional model of the ATD of hGPRC6A we predicted that the binding site would favor α-amino acids. Deorphanization now allows us to confirm this. The identified set of agonists demonstrates receptor specificity for L-α-amino acids in that agmatine (the decarboxylated Arg derivative), urea, and spermine show no activity. Furthermore, the agonists identified are either small or hydrophobic or acidic side chains. We find a dramatic effect of flexible hydrogen bond donors rather than having bulky, hydrophobic or acidic side chains. We find a dramatic effect of flexible hydrogen bond donors rather than having bulky, hydrophobic or acidic side chains. We find a dramatic effect of flexible hydrogen bond donors rather than having bulky, hydrophobic or acidic side chains. We find a dramatic effect of flexible hydrogen bond donors rather than having bulky, hydrophobic or acidic side chains. We find a dramatic effect of flexible hydrogen bond donors rather than having bulky, hydrophobic or acidic side chains. We find a dramatic effect of flexible hydrogen bond donors rather than having bulky, hydrophobic or acidic side chains. We find a dramatic effect of flexible hydrogen bond donors rather than having bulky, hydrophobic or acidic side chains. We find a dramatic effect of flexible hydrogen bond donors rather than having bulky, hydrophobic or acidic side chains. We find a dramatic effect of flexible hydrogen bond donors rather than having bulky, hydrophobic or acidic side chains. We find a dramatic effect of flexible hydrogen bond donors rather than having bulky, hydrophobic or acidic side chains. We find a dramatic effect of flexible hydrogen bond donors rather than having bulky, hydrophobic or acidic side chains. We find a dramatic effect of flexible hydrogen bond donors rather than having bulky, hydrophobic or acidic side chains. We find a dramatic effect of flexible hydrogen bond donors rather than having bulky, hydrophobic or acidic side chains. We find a dramatic effect of flexible hydrogen bond donors rather than having bulky, hydrophobic or acidic side chains. We find a dramatic effect of flexible hydrogen bond donors rather than having bulky, hydrophobic or acidic side chains. We find a dramatic effect of flexible hydrogen bond donors rather than having bulky, hydrophobic or acidic side chains. We find a dramatic effect of flexible hydrogen bond donors rather than having bulky, hydrophobic or acidic side chains. We find a dramatic effect of flexible hydrogen bond donors rather than having bulky, hydrophobic or acidic side chains. We find a dramatic effect of flexible hydrogen bond donors rather than having bulky, hydrophobic or acidic side chains. We find a dramatic effect of flexible hydrogen bond donors rather than having bulky, hydrophobic or acidic side chains. We find a dramatic effect of flexible hydrogen bond donors rather than having bulky, hydrophobic or acidic side chains. We find a dramatic effect of flexible hydrogen bond donors rather than having bulky, hydrophobic or acidic side chains. We find a dramatic effect of flexible hydrogen bond donors rather than having bulky, hydrophobic or acidic side chains. We find a dramatic effect of flexible hydrogen bond donors rather than having bulky, hydrophobic or acidic side chains. We find a dramatic effect of flexible hydrogen bond donors rather than having bulky, hydrophobic or acidic side chains. We find a dramatic effect of flexible hydrogen bond donors rather than having bulky, hydrophobic or acidic side chains. We find a dramatic effect of flexible hydrogen bond donors rather than having bulky, hydrophobic or acidic side chains. We find a dramatic effect of flexible hydrogen bond donors rather than having bulky, hydrophobic or acidic side chains. We find a dramatic effect of flexible hydrogen bond donors rather than having bulky, hydrophobic or acidic side chains. We find a dramatic effect of flexible hydrogen bond donors rather than having bulky, hydrophobic or acidic side chains. We find a dramatic effect of flexible hydrogen bond donors rather than having bulky, hydrophobic or acidic side chains. We find a dramatic effect of flexible hydrogen bond donors rather than having bulky, hydrophobic or acidic side chains. We find a dramatic effect of flexible hydrogen bond donors rather than having bulky, hydrophobic or acidic side chains. We find a dramatic effect of flexible hydrogen bond donors rather than having bulky, hydrophobic or acidic side chains. We find a dramatic effect of flexible hydrogen bond donors rather than having bulky, hydrophobic or acidic side chains. We find a dramatic effect of flexible hydrogen bond donors rather than having bulky, hydrophobic or acidic side chains. We find a dramatic effect of flexible hydrogen bond donors rather than having bulky, hydrophobic or acidic side chains. We find a dramatic effect of flexible hydrogen bond donors rather than having bulky, hydrophobic or acidic side chains. We find a dramatic effect of flexible hydrogen bond donors rather than having bulky, hydrophobic or acidic side chains. We find a dramatic effect of flexible hydrogen bond donors rather than having bulky, hydrophobic or acidic side chains. We find a dramatic effect of flexible hydrogen bond donors rather than having bulky, hydrophobic or acidic side chains. We find a dramatic effect of flexible hydrogen bond donors rather than having bulky, hydrophobic or acidic side chains. We find a dramatic effect of flexible hydrogen bond donors rather than having bulky, hydrophobic or acidic side chains. We find a dramatic effect of flexible hydrogen bond donors rather than having bulky, hydrophobic or acidic side chains. We find a dramatic effect of flexible hydrogen bond donors rather than having bulky, hydrophobic or acidic side chains. We find a dramatic effect of flexible hydrogen bond donors rather than having bulky, hydrophobic or acidic side chains. We find a dramatic effect of flexible hydrogen bond donors rather than having bulky, hydrophobic or acidic side chains. We find a dramatic effect of flexible hydrogen bond donors rather than having bulky, hydrophobic or acidic side chains. We find a dramatic effect of flexible hydro
um-sensing receptor is physiologically significant (Brown, 1999). Finally, because several L-α-amino acids are well-established neurotransmitters, and because specific transporters of cationic amino acids exist in the brain (Closs, 2002), GPRC6A could well represent a novel receptor relevant to the nervous system. At this point, further clarification of the physiological role of GPRC6A should be sought by identifying selective and potent agonists and antagonists as well as potential interacting proteins and by loss-of-function studies in vivo.

Acknowledgments

We thank professor B. Bettler (University of Basel, Switzerland) for providing us with GABA_B receptor constructs, J. Ngiu (University of California, Berkeley, CA) for the goldfish receptor 5.24, and C. S. Zuker (University of California, San Diego, CA) for the TiR3 receptor. We are grateful for the computational support of the Danish Center for Supercomputing and the Australian Centre for Advanced Computing and Communications. Finally, we thank Mie Kusk, Karen Krzywykowski, and Dr. Anders L. Jensen for technical assistance.

References


Deorphanization of GPRC6A 597


