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ACCELERATED COMMUNICATION

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

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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 α-amino acids and was employed to rationally select potential ligands. Measurement of Ca^{2+}-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-α-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 EC_{50} 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 Bräuner-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

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ABBREVIATIONS: GPCR, G-protein-coupled receptor; GPRC6A, G-protein-coupled receptor, family C, group 6, subtype A; h, human; m, murine; mGlu, metabotropic glutamate; ATD, amino-terminal domain; PCR, polymerase chain reaction; BAPTA, 1,2-bis(2-aminophenoxy)-ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid; AM, acetoxymethyl ester; Orn, ornithine; Cit, citrulline; 7TM, seven transmembrane domain.

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 ($mGlu_{1-8}$) receptors (Pin and Duvoisin, 1995), $GABA_{B1-2}$ (Möhler and Fritschy, 1999), and some orphan and pheromone receptors (Cheng and Lotan, 1998; Bräuner-Osborne and Krogsgaard-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 $mGlu_1$ (PDB code 1EWK), we have generated a three-dimensional homology model of GPRC6A, suggesting that the endogenous agonist for GPRC6A is an α-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 L-α-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 $mGlu_1$ receptor (Kunishima et al., 2000), with the aid of the Polish Bioinformatics metaserver (Ginalski et al., 2003) and Easypred (Lambert et al., 2002) including MODELER (Marti-Renom et al., 2000). Based on a high degree of conservation of the region binding the α-amino acid moiety of L-Glu in the $mGlu_1$ receptor and the location of acidic residues, tri-ionized L-Lys was modeled into the binding site by a combination of manual docking and restrained minimization, followed by Monte Carlo conformational searching of ligand and side chains with OPLS-AA in Macromodel 8.1 and Prime 1.1 (Schrödinger Inc., Portland, OR) and analysis of receptor water placement using Glide 2.5 (Schrödinger Inc.). For details, see Supplemental data.

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′-gctcttaataaccctcatgaac-3′ and reverse primer 5′-aaagtaaatacacaatttgcagc-3′ (20 cycles at 50°C annealing). The second PCR was performed with forward primer 5′-catgaactgagcaaatgagac-3′ and reverse primer 5′-gaaacatctcactgggggatc-3′ (30 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 data.

Site-Directed Mutagenesis of hGPRC6A and Chimera h6A/5.24. The 919–921RKR/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 $mGlu_5$ 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 $mGlu_5$, 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′-cactcgacgcgttgtcatacccagatgac-3′ and reverse primer 5′-cttcttctgcggccgcctcctaggaactcaatc-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 Amplex Red horseradish peroxidase-amplified enzyme-linked immunoassay (Molecular 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 (82.5 mM NaCl, 2.0 mM KCl, 1.0 mM $MgCl_2$, 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 $NaHCO_3$, 0.41 mM $CaCl_2$, 0.82 mM $MgSO_4$, 0.3 mM $Ca(NO_3)_2$, 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 μM BAPTA-AM (Sigma-Aldrich).

Electrophysiology. Whole-cell currents were recorded on oocytes 2 to 4 days after injection using two-electrode voltage clamp at −80 mV in normal frog Ringer's solution (115 mM NaCl, 2.5 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, and 10 mM HEPES, pH 7.6). Recording pipettes were filled with 3 M KCl. Recordings were performed at ambient temperatures using OC-725C Oocyte Clamp amplifier (Warner Instruments, Hamden, CT) with a Digidata 1322A interface (Axon Instruments, Union City, CA). The pClamp7 suite of programs (Axon Instruments) was used for data acquisition. All tested compounds were purchased from Sigma-Aldrich.

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 KH_2PO_4, 4.2 mM $NaHCO_3$, 138 mM NaCl, 0.34 mM Na_2HPO_4, 5.6 mM D-Glucose, 20 mM HEPES, 1 mM $CaCl_2$, 1 mM $MgCl_2$ 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 $mGlu_1$ receptor indicated that a number of residues in the $mGlu_1$ 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 $mGlu_1$ 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 L-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 $mGlu_1$ 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 mGlu receptors—an assumption shared by Kuang et al. (2003) in their model of L-Lys bound to the homologous 5.24

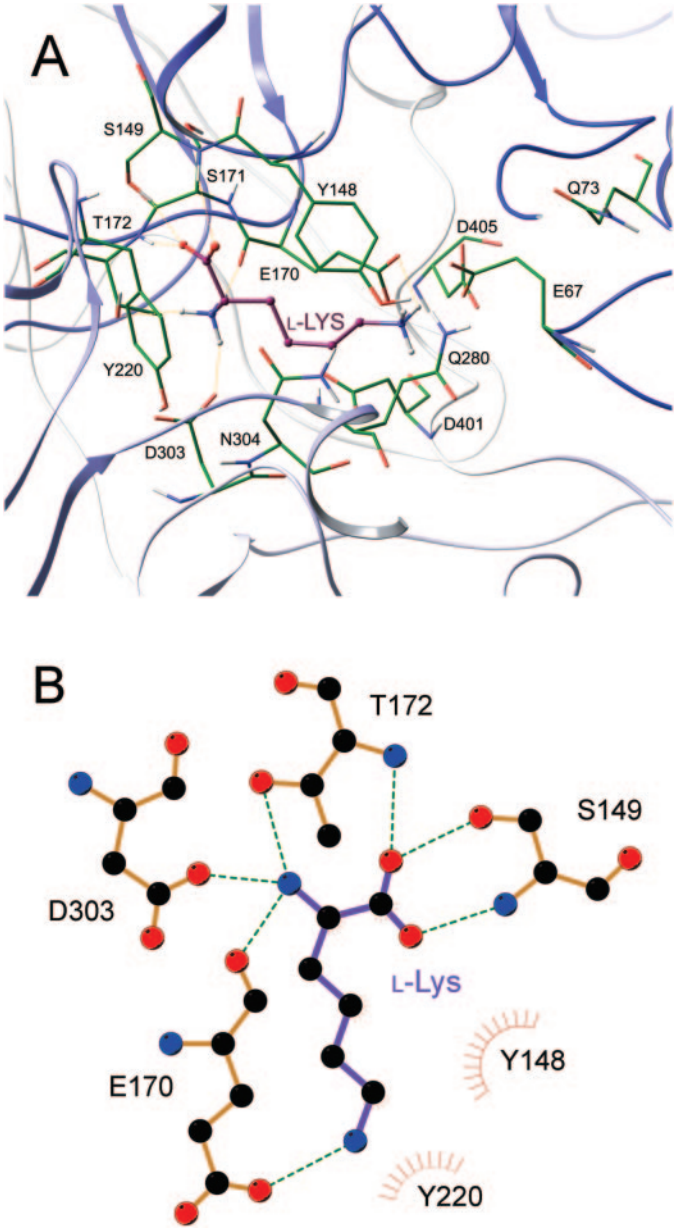

Fig. 1. Details of the three-dimensional model of the hGPRC6A ATD. A, ribbon view of the binding cleft with L-Lys docked into the purported binding pocket. Hydrogen bonds are indicated by yellow lines. B, flat scheme indicating residues that interact directly with the α-amino acid and distal amine moieties of L-Lys. Hydrogen bonds from A are shown with green lines. The hydroxyl group on the side chain of Ser149 interacts with the carboxylic acid group of L-Lys.

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 $mGlu_1$ 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 $mGlu_1$ receptor. The latter tyrosine is also a feature of the $mGlu_2$ 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 $mGlu_1$ 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 $GABA_B$ receptor, a similar motif acts as an endoplasmic retention signal, preventing trafficking of the $GABA_{B(1)}$ receptor subunit to the cell surface. Because mutation of the $GABA_{B(1)}$ 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. Quanti-

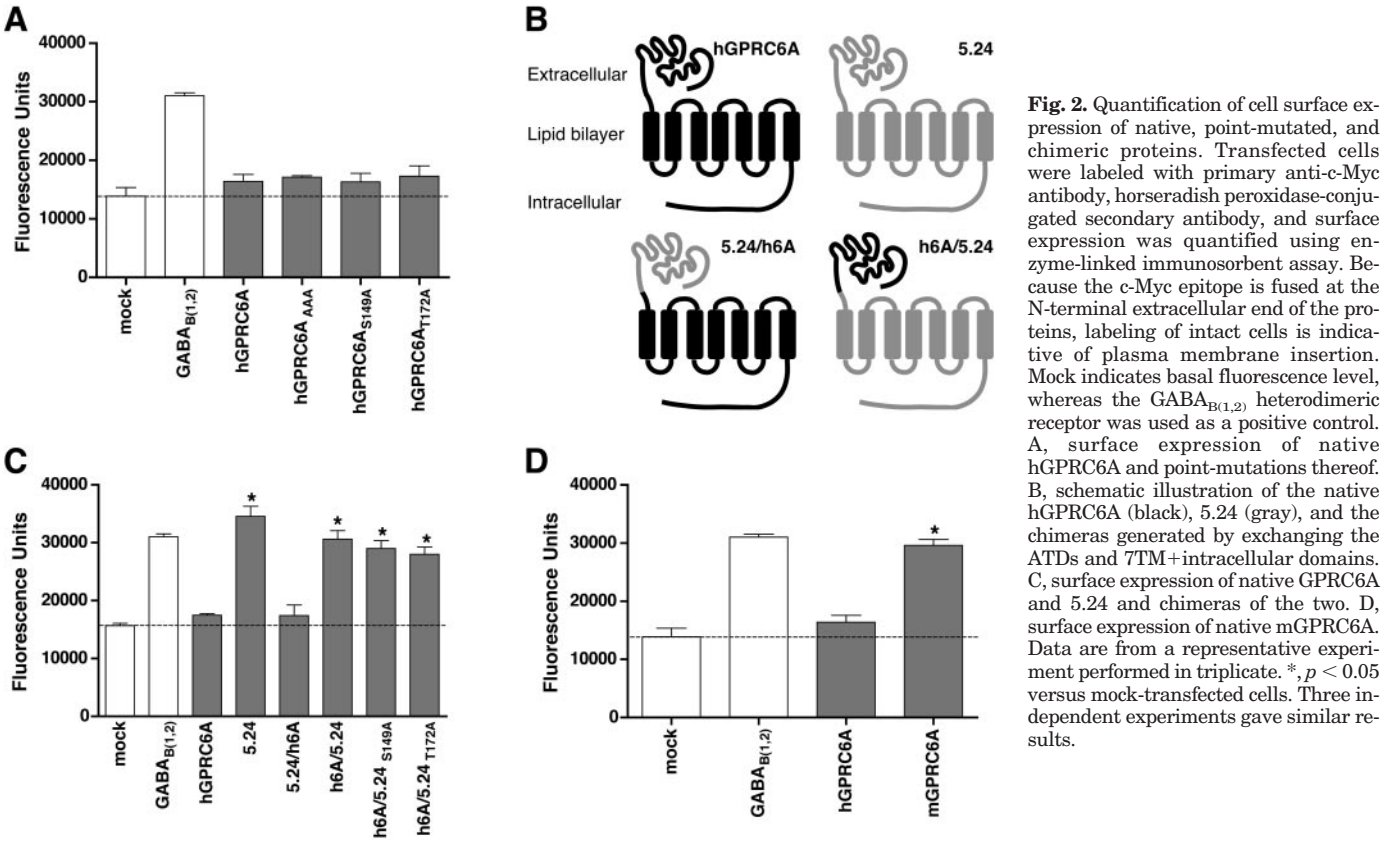

Fig. 2. Quantification of cell surface expression of native, point-mutated, and chimeric proteins. Transfected cells were labeled with primary anti-c-Myc antibody, horseradish peroxidase-conjugated secondary antibody, and surface expression was quantified using enzyme-linked immunosorbent assay. Because the c-Myc epitope is fused at the N-terminal extracellular end of the proteins, labeling of intact cells is indicative of plasma membrane insertion. Mock indicates basal fluorescence level, whereas the $GABA_{B(1,2)}$ heterodimeric receptor was used as a positive control. A, surface expression of native hGPRC6A and point-mutations thereof. B, schematic illustration of the native hGPRC6A (black), 5.24 (gray), and the chimeras generated by exchanging the ATDs and 7TM+intracellular domains. C, surface expression of native GPRC6A and 5.24 and chimeras of the two. D, surface expression of native mGPRC6A. Data are from a representative experiment performed in triplicate. *, $p < 0.05$ versus mock-transfected cells. Three independent experiments gave similar results.

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 $GABA_{B(1,2)}$ 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_q 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^{2+}-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_i-coupling was examined in similar experiments with coexpression of hGPRC6A with inwardly rectifying potassium channels (Kir3.1 + Kir3.4) (Schreibmayer et

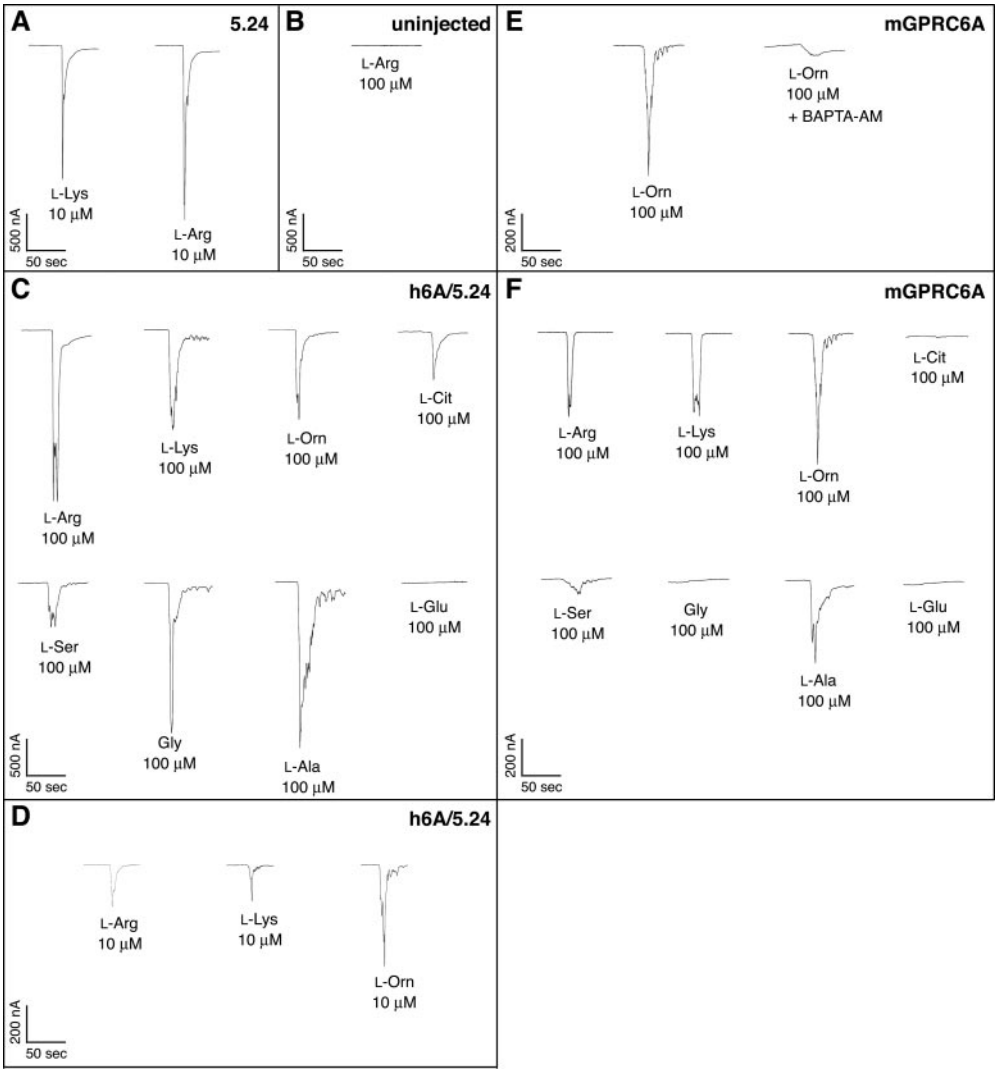

Fig. 3. Deorphanization of GPRC6A in *X. laevis* oocytes. Traces of Ca^{2+}-dependent chloride currents in *X. laevis* were obtained by two-electrode voltage clamping at a holding potential of −80 mV. A, oocytes expressing the goldfish 5.24 receptor stimulated with either 10 μM L-Lys or L-Arg. B, uninjected 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 α-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 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 $GABA_B$ 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.

TABLE 1

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

All compounds were tested at 100 μM except Ca^{2+}, which was tested at 4 mM.

Natural α-amino acids
L-α-amino acids
Active: Arg, Lys, Ala, Gly, Ser (see Fig. 3C)
All other inactive
D-amino acids
All inactive
Signaling molecules
Inactive Ca^{2+}, γ-hydroxybutyrate, GABA, histamine, serotonin, taurine, vasopressin
Metabolic intermediates
Active: (L-Orn, L-Cit; see Fig. 3C)
Inactive: agmatine, creatine, creatinine, spermine, urea

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_q 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^{2+}-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 hGRPRC6A- or

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 × 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 hGPRC6A 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.

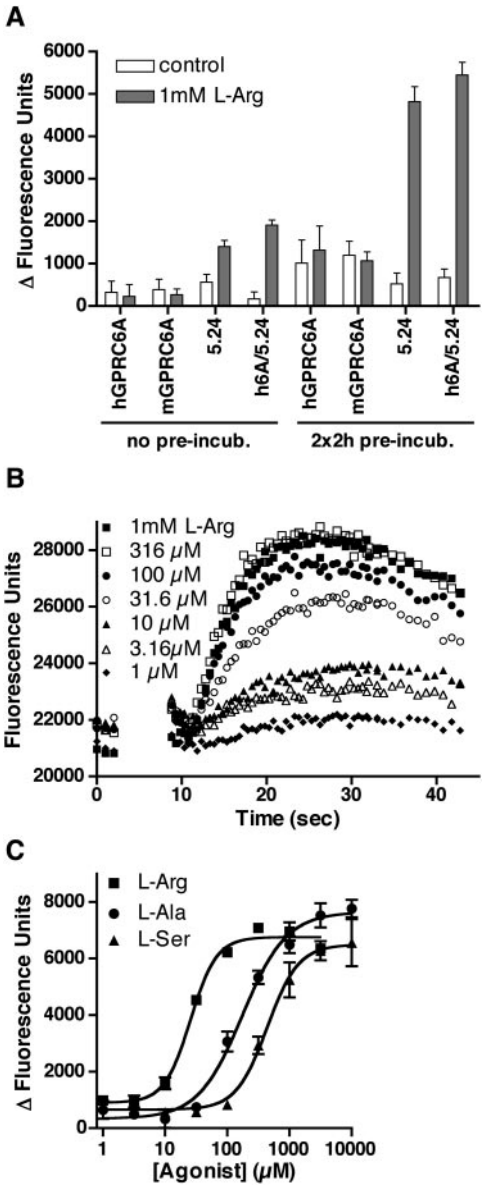

Fig. 4. Pharmacological analysis of GPRC6A by calcium imaging in living tsA cells. tsA cells were transiently transfected and transferred to black 96-well plates with clear bottom. Two days after transfection, cells were loaded with the calcium sensitive dye Fluo-4 and exposed to various concentrations of L-α-amino acids, and responses were measured on a NOVOstar plate reader as described under *Materials and Methods*. A, responses to 1 mM L-Arg by cells transfected with hGPRC6A, mGPRC6A, 5.24, or h6A/5.24 with or without two preincubations of 2 h before loading with Fluo-4. Responses are shown as Δfluorescence units (peak fluorescence after agonist addition subtracted fluorescence before agonist addition). B, representative raw traces of increasing concentrations of L-Arg on tsA cells transfected with h6A/5.24. Compounds were added after 8.8 s. C, representative concentration-response curves of three L-α-amino acids with either basic (Arg) or small (Ala, Ser) side chains. EC_{50} values for all L-α-amino acids are shown in Table 2.

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 EC_{50} 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 EC_{50} 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

TABLE 2

Agonist potencies for L-α-amino acids at tsA cells expressing the chimeric receptor h6A/5.24

EC_{50} values were determined by measurement of intracellular calcium levels by the calcium sensitive dye Fluo-4 as described under *Materials and Methods*. Each experiment was performed three times in triplicate. All remaining natural L-α-amino acids had EC_{50} > 1000 μM. All active compounds listed were full agonists.

Compound	EC_{50} (pEC_{50} ± S.E.M.)
	μM
L-Arg	44.1 (4.38 ± 0.11)
L-Orn	112 (3.96 ± 0.05)
L-Lys	169 (3.77 ± 0.03)
L-Ala	173 (3.76 ± 0.02)
Gly	263 (3.58 ± 0.04)
L-Cit	287 (3.56 ± 0.09)
L-Gln	590 (3.23 ± 0.05)
L-Ser	623 (3.21 ± 0.07)
L-Met	854 (3.07 ± 0.04)

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 chimeras 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 flexible hydrogen bond donors rather than having bulky, hydrophobic or acidic side chains. We find a dramatic effect of the S149A and T172A h6A/5.24 mutants on receptor activity, validating the modeled L-α-amino acid recognition site. Similar mutation studies on other family C GPCRs have unequivocally demonstrated that corresponding residues are crucial for maintaining ligand affinity and/or receptor function (O'Hara et al., 1993; Galvez et al., 2000; Kuang et al., 2003). The precise environment of the distal end of the hGPRC6A binding pocket is less clear on the basis of the available homology to the only family member yet characterized by X-ray crystallography, $mGlu_1$. Our experimental finding of several amino acid agonists indicates that hGPRC6A is somewhat promiscuous, although there seems to be at least as much preference for basic amino acids as has been shown for the 5.24 receptor (Speca et al., 1999). It is curious that no single acidic residue can be clearly identified in lobe II corresponding to Asp388 in 5.24, strongly implicated in its binding of L-Lys (Kuang et al., 2003). On the other hand, our model proposes the involvement of Glu170 (Ala in 5.24) in lobe I as a counter-ion or strong hydrogen bond acceptor, but we are also aware of other acidic residues that could be involved if, for example, the conformation of L-Lys in hGPRC6A is related to L-Glu in the $mGlu_1$ receptor as L-Lys in the bacterial lysine/arginine/ornithine-binding protein is related to L-Glu in the ionotropic GluR2 receptor (Oh et al., 1993) (See supplemental data). The minor differences in the agonist profile of mGPRC6A could possibly result from the nonconservation of amino acid residue 280 of the binding pockets, although this remains speculative. Work is currently underway in our laboratories to establish conclusively which residues are involved in binding of the distal end of basic amino acids.

Having characterized the agonist preferences of both human and mouse GPRC6A, it seems plausible that the physiological relevance of this novel receptor will subsequently be found. Data on the signal transduction of 5.24 and mGPRC6A imply that hGPRC6A will also prove to be G_q-coupled once sufficient surface expression is obtained. So far, whether the native GPRC6A in vivo is expressed at the cell surface or serves its purpose at an intracellular membrane, as suggested for other recently deorphanized GPCRs (Bunzow et al., 2001), is not known. Other family C GPCRs are known to depend on heterodimerization for correct cell surface localization (Möhler and Fritschy, 1999; Nelson et al., 2001). Coexpression with $GABA_{B(2)}$ or T1R3 receptor subunits has proven insufficient to alleviate intracellular retention of hGPRC6A (data not shown), which does not, however, rule out the need for another interacting protein. The clear sequence divergence in the C termini of human and mouse GPRC6A (only 50% amino acid identity) indicate that this region may dictate the differences in cell surface expression.

In goldfish, the 5.24 receptor is expressed specifically in the olfactory epithelium, and functions as an odorant receptor, enabling the organism to sense amino acids in the environment. Sequence identity and the agonist preferences revealed in this work would argue for GPRC6A performing an analogous role to the goldfish 5.24 receptor. The more widespread presence of hGPRC6A in human tissues (Wellendorph and Bräuner-Osborne, 2004), however, points to a different physiological function. Prominent expression in kidney, liver and brain, in conjunction with the fact that the identified agonists L-Arg, L-Orn, and L-Cit (and indirectly L-Lys) are intermediates of the urea cycle (Morris, 2004) lead us to the proposition that GPRC6A might be a regulatory component of this important metabolic pathway. By analogy, a recently published study found GPCRs to respond to specific intermediates of the citric acid cycle (He et al., 2004), implying that such metabolites, well known for their biochemistry, may also function as signaling molecules. Given that free plasma concentrations of the nine agonists identified in this study are in the micromolar range in fasting adults (Armstrong and Stave, 1973) and thus correspond to the range of potencies found in our study (Fig. 3 and Table 2), sensing the free amino acid concentrations in blood would seem a plausible role for the receptor, just as sensing of calcium by the calci-

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.

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