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Deorphanization of GPRC6A: a promiscuous L- α -amino acid receptor with preference for basic amino acids

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Deorphanization of GPRC6A

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21 text pages

1 table

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42 references

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GPCR, G-protein coupled receptor; ATD, amino-terminal domain; Cit, citrulline; GABA_B, γ -aminobutyric acid type B; GPRC6A, G-protein coupled receptor, family C, group 6, subtype A; mGlu, metabotropic glutamate; Orn, ornithine; 7TM, seven transmembrane domain.

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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 orthologue 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.

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Introduction

With the complete human genome sequence accessible, the identification of novel genes has been greatly facilitated, and this 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 hGPCR6A (human G-protein coupled receptor, family C, subtype 6A) 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 hGPCR6A, mapping to chromosome 6q22.31 (Genbank accession #[AY435125](#), [AY435126](#), and [AY435127](#)). RT-PCR analysis showed that the longest isoform of GPCR6A was expressed at the highest level in human tissues, and an orthologue of that form was also found in mice (Genbank accession #[AY101365](#)), . GPCR6A displays 45% amino acid sequence identity with the goldfish odorant receptor 5.24 (Specia 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 hGPCR6A in family C of GPCRs, which also includes the metabotropic glutamate (mGlu₁₋₈) receptors (Pin and Duvoisin, 1995), γ -aminobutyric acid type B receptors (GABA_{B1-2}) (Möhler and Fritschy, 1999) and some orphan and pheromone receptors (Bräuner-Osborne et al., 2001; Bräuner-Osborne and Krogsgaard-Larsen, 2000; Calver et al., 2003; Cheng and Lotan, 1998; Robbins et al., 2000). 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 mGlu₁ (1EWK.pdb A), we have generated a three-dimensional homology model of GPCR6A, suggesting that the endogenous agonist for GPCR6A is an α -amino acid. The lack of cell surface expression of the hGPCR6A protein in mammalian cell lines (Wellendorph and Bräuner-Osborne, 2004), hampered the development of a functional

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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 carboxy 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* oocytes or tsA cells, thus supporting our proposed model. We further document these findings by cloning and deorphanization of the native murine homologue of GPRC6A (mGPRC6A) and providing the first steps towards 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 corresponding to the crystallized extracellular domain of the mGlu₁ receptor, 1EWK chain A (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 mGlu₁ 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, 2002, 2004) and analysis of receptor water placement using Glide 2.5 (Schrödinger, 2002). *For details, see Supplemental data.*

Cloning of GPRC6A

Human GPRC6A was cloned in our laboratory as recently reported (Genbank accession #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

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Biosciences, Palo Alto, CA, U.S.A.) using *Pfu* polymerase (Stratagene, La Jolla, CA, U.S.A.), forward primer 5'-gctcttaataaccctcatgaac-3', and reverse primer 5'-aaagtaatacacaatttcgac-3' (20 cycles 50°C annealing). The second PCR was performed with forward primer 5'-catgaactgagcaaatgagac-3' and reverse primer 5'-gaaacatctcactggggatc-3' (30 cycles, 50°C annealing). A single band of 2900 bp was purified (QIAquick Gel Extraction kit; Qiagen, Hilden, Germany) and TOPO TA cloned into the pCR2.1 vector (Invitrogen, San Diego, CA, USA) using the manufacturer's instructions. The obtained cDNA was fully sequenced (MWGBiotech, Ebersberg, Germany) and found to be identical with the entry #AY101365 in Genbank at the protein level.

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 previously generated family C chimeras 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 (MWGBiotech). 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, La Jolla, CA, U.S.A.). Mutations were confirmed by DNA sequencing (MWG Biotech).

Epitope tagging

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For cellular expression studies, all receptor constructs were subcloned into a modified pEGFP-N1 vector (BD Biosciences), essentially as previously described (Pagano et al., 2001). Briefly, the N-terminal signal peptides were replaced by the mGlu₅ 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 mGlu₅. Utilizing 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'-cactcgacgctgtgcataccagatgac-3' and reverse primer 5'-cttctctcgccgctcctaggaactcaatc-3'. The signal peptide was predicted by the program SignalP with a cleavage site between amino acid position 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 (HRP) amplified enzyme-linked immunoassay (Molecular Probes, Leiden, The Netherlands) was carried out exactly as earlier described (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 *Xenopus* oocytes, cDNAs were subcloned into the pGEMHE-3Z vector containing an upstream T7 promoter. Linearized plasmids were used to produce cRNAs with mMessage

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mMachine kits (Ambion, Austin, TX, U.S.A.). Oocytes were surgically removed from mature female *Xenopus laevis*, anaesthetized in a 0.4% MS-222 (3-aminobenzoic acid ethyl ester) (Sigma-Aldrich, St. Louis, Missouri, U.S.A.) solution for 10-15 min. In order to remove the follicle layer, the oocytes were subsequently placed in OR2 buffer (in mM: 82.5 NaCl, 2.0 KCl, 1.0 MgCl₂, and 5.0 HEPES pH 7.5) including 0.5 mg/ml collagenase (Type IA) (Sigma-Aldrich) for 2-3 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 (in mM: 88 NaCl, 1.0 KCl, 2.4 NaHCO₃, 0.41 CaCl₂, 0.82 MgSO₄, 0.3 Ca(NO₃)₂, 15 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 2-4 days after injection using two-electrode voltage-clamp at -80 mV in Normal Frog Ringer's solution (in mM: 115 NaCl, 2.5 KCl, 1.8 CaCl₂, 1 MgCl₂ and 10 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, U.S.A.) with a Digidata 1322A interface (Axon Instruments, Union City, CA, U.S.A.). 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 previously described (Wellendorph and Bräuner-Osborne, 2004) with the exception that 2 million cells were plated in a 10-cm dish and transfected the following day with 8 µg plasmid DNA. The day after transfection cells were split into poly-D-lysine-coated black 96-well plates with clear bottom (BD Biosciences). Two days post-transfection, pharmacological activity was assessed by measurement of intracellular calcium levels essentially as earlier described (Kuang et al., 2003). Briefly, cells were washed with assay buffer (in mM: 5.3 KCl, 0.44 KH₂PO₄, 4.2 NaHCO₃, 138 NaCl, 0.34 Na₂HPO₄, 5.6 D-Glucose, 20 HEPES, 1 CaCl₂, 1 MgCl₂

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and 1 mg/ml bovine serum albumin, pH 7.4) and pre-incubated for 2 h at 37 °C in 100 µl assay buffer. The assay buffer was exchanged and cells were pre-incubated for another 2 h at 37 °C. The assay buffer was then replaced with 50 µl 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 nm and 520 nm, respectively. Responses (Δ *Fluorescence Units*) were calculated as peak fluorescence after agonist addition subtracted fluorescence before agonist addition. Concentration-response curves were analyzed by non-linear regression using GraphPad Prism 4.0 (GraphPad Software, San Diego, USA). Pharmacological experiments were performed in triplicate and repeated in three independent experiments.

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Results

Prediction of agonist preferences in hGPRC6A by homology modeling

In order 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 mGlu₁ receptor indicated that a number of residues in the mGlu₁ 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 mGlu₁ 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 (Specia 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 mGlu₁ 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.* in their model of L-Lys bound to the homologous 5.24 receptor (Kuang et al., 2003). 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 E170 of hGPRC6A, though 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 D303 binds to the α -ammonium group in roughly the same way as in the mGlu₁ receptor, in which both the agonist and E170 side chains were extended, and in which Y148 occupies a similar hydrophobic space to that of W110 in the mGlu₁ receptor. The latter tyrosine is also a feature of the mGlu₂ receptor binding site, which we and others have previously modeled with high confidence (Clausen et al., 2002; Malherbe et

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al., 2001). This yields a more polar binding cavity than in 5.24, though framed on two sides by aromatic residues (Fig. 1B), a feature common to a range of amino acid binding sites. Apart from E170, the hydrogen bond network in the distal region may be supported by polar residues Q73, Q280 and N304. 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 Y74 and K409 in mGlu₁ are also implicated. Finally, it was apparent that the length and shape of the pocket, and the flexibility of E170 and its proximity to the L- α -amino acid recognition site, did not imply specificity for the flexible side chain of L-Lys, but 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 in order to refine the model. However, the central consistent finding was that the agonist would most likely 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: a poor understanding of the native signaling pathway, and 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. Since 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 desensitization of hGPRC6A during cell culturing, by mutating residues S149 and T172 predicted to be involved in agonist binding (Fig. 1B), but found no effect on surface expression (Fig. 2A). To

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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. Quantitative 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. 2B). 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. 2B). 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 earlier used the chimeric receptor approach to document the position of the binding pocket of the calcium-sensing receptor (Bräuner-Osborne et al., 1999b). Thus, as 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 (PI) hydrolysis, and in turn causing release of calcium from intracellular stores (Kuang et al., 2003; Speca et al., 1999). Due to 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 *Xenopus laevis* expression system where 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) due to the activation of Ca²⁺-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

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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 co-expression of hGPRC6A with inwardly rectifying potassium channels (Kir3.1+Kir3.4) (Schreibmayer et 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 that not only 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 lowering the concentration 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, as only L-Orn was active at 10 μM in our assay (Fig. 3D). 3 μM concentrations of the three basic amino acids did not elicit any response, and higher concentrations (300 μM) led to decreased currents compared to 100 μM (data not shown) presumably due to 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. Interestingly, 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 appears 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

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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. 1B), 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 appear to be correctly expressed and targeted to the cell surface as shown by quantitative immunolabeling of transfected cells (Fig. 2B). This supports the hypothesis that S149 and T172 are in fact part of the binding site located within the ATD of hGPCR6A, and that the chimeric approach for deorphanization of hGPCR6A 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. As others have previously shown that orthologues of GPCRs may display notable differences in surface expression in heterologous systems (Calver et al., 2003) we therefore isolated the cDNA of the mGPCR6A to obtain a wild-type reference.

Cloning and deorphanization of mGPCR6A

We used the reported full-length sequence of mGPCR6A (Genbank accession # AY101365) to design primers positioned in the 5' and 3' UTR of the gene, and successfully cloned the ORF of mGPCR6A by nested PCR. Alignment of mouse and human GPCR6A revealed that the proteins have 80% overall identity; the ATDs and predicted 7TM regions are 82% identical, while the C-termini of the orthologues 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 Q280 in hGPCR6A which corresponds to K280 in mGPCR6A. The high degree of conservation of residues constituting the binding site suggests very similar ligand preferences. By contrast with its human counterpart, mGPCR6A is trafficked to the cell surface when transiently expressed in intact tsA cells (Fig. 2C). 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 μ M

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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 prior to stimulation, thus demonstrating that mGPRC6A preferentially signals through the G_q pathway (Fig. 3D) (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. 3C and E). 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. 3C and E) and only L-Orn was able to elicit a response at 10 μM, indicating a lower sensitivity of this receptor. This may, however, relate 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

Due to low throughput and variability of responses (Pin et al., 1992), measurement of Ca²⁺-activated chloride currents in oocytes is not an ideal assay for quantitative pharmacology. In order 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 previously mentioned, mammalian cell culture media contain high levels of L-α-amino acids which likely would desensitize the GPRC6A receptors. In agreement with this hypothesis no response was obtained by 1 mM L-Arg from hGPRC6A or mGPRC6A transfected cells and only small responses were observed from h6A/5.24 or 5.24 transfected cells (Fig. 4A). Recently Kuang *et al.* published a modified calcium imaging protocol in which cells expressing 5.24 were pre-incubated with calcium assay buffer for 2 x 2 h before Fluo-4 loading (Kuang et al. 2003). This procedure greatly increased the response from h6A/5.24 or 5.24 transfected cells, presumably due to re-sensitization of the receptors, whereas it did not improve the response from hGPRC6A or mGPRC6A (Fig. 4A). The lack of response from hGPRC6A correlate with the intracellular retention (Fig. 2A) and lack of response in oocytes. As

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shown in Fig. 3F the mGPRC6A receptor elicit a small response in oocytes compared to h6A/5.24 (compare Fig. 3C and 3F and note the difference in scale), which might explain the lack of response in the calcium measurement assay. Alternatively, mGPRC6A might be easier to desensitize or harder to re-sensitize than 5.24 and 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). As can be seen from the EC₅₀ values shown in Table 2, we obtained a rank-order of potency of L-Arg > L-Orn \geq L-Lys = L-Ala \geq Gly = L-Cit > L-Gln = L-Ser \geq 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. In order 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).

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Discussion

We recently reported the cloning and expression analysis of the novel human family C receptor, hGPCR6A. While 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 non-trivial 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 in order to overcome this particular problem. Thus, achieving surface expression of the hGPCR6A binding domain in the *Xenopus* oocyte expression system has enabled us to deorphanize this human receptor by reverse pharmacology. Additional deorphanization of the native murine orthologue of GPCR6A further verifies 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. Collectively, our results demonstrate that GPCR6A is a promiscuous L- α -amino acid receptor with preference for basic amino acids activated in the micromolar range.

While 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; Bräuner-Osborne et al., 2001; Bräuner-Osborne et al., 1999b), 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 likely 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 hGPCR6A 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 hGPCR6A 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

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the ATD of 5.24 are clearly involved in binding L-Lys (Kuang et al., 2003), any possibility of L-Arg or L-Lys exerting 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 orthologue, 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 demonstrate receptor specificity for L- α -amino acids as the decarboxylated Arg derivative agmatine, 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 (Galvez et al., 2000; Kuang et al., 2003; O'Hara et al., 1993). 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, mGlu₁. Our experimental finding of several amino acid agonists indicates that hGPRC6A is somewhat promiscuous, although there appears to be at least as much preference for basic amino acids as has been shown for the 5.24 receptor (Specia et al., 1999). Curiously, no single acidic residue can be clearly identified in Lobe II corresponding to D388 in 5.24, strongly implicated in its binding of L-Lys (Kuang et al., 2003). Conversely, our model proposes the involvement of E170 (A in 5.24) in Lobe I as a counterion or strong hydrogen bond acceptor, but we are also aware of other acidic residues which could be involved, if for example the conformation of L-Lys in hGPRC6A is to L-Glu in the mGlu₁ receptor, as L-Lys in the bacterial lysine/arginine/ornithine-binding protein (LAOBP) is to L-Glu in the ionotropic

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GluR2 receptor (Oh et al., 1993) (See supplemental data). The minor differences in the agonist profile of mGPRC6A could possibly result from the non-conservation 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 appears 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 it is not known 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). Other family C GPCRs are known to depend on heterodimerization for correct cell surface localization (Möhler and Fritschy, 1999; Nelson et al., 2001). Co-expression with GABA_{B(2)} or T1R3 receptor subunits have 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, 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

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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 calcium-sensing receptor is physiologically significant (Brown, 1999). Finally, as several L- α -amino acids are well-established neurotransmitters, and since 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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Acknowledgements

We thank professor B. Bettler (University of Basel, Switzerland) for providing us with GABA_B receptor constructs, J. Ngai (University of California, Berkeley, CA, U.S.A.) for the goldfish receptor 5.24, and C. S. Zuker (University of California, San Diego, CA, U.S.A.) for the T1R3 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 Ms. Mie V. Kusk, M.Sc. Karen Krzykowski and Dr. Anders A. Jensen for technical assistance.

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References

- Armstrong MD and Stave U (1973) A study of plasma free amino acid levels. II. Normal values for children and adults. *Metabolism* **22**:561-569.
- Bräuner-Osborne H, Jensen AA and Krogsgaard-Larsen P (1999a) Interaction of the noncompetitive antagonist CPCCOEt with a chimeric metabotropic glutamate and calcium sensing receptor. *Neuroreport* **10**:3923-3925.
- Bräuner-Osborne H, Jensen AA, Sheppard PO, Brodin B, Krogsgaard-Larsen P and O'Hara P (2001) Cloning and characterization of a human orphan family C G-protein coupled receptor GPRC5D. *Biochim Biophys Acta* **1518**:237-248.
- Bräuner-Osborne H, Jensen AA, Sheppard PO, O'Hara P and Krogsgaard-Larsen P (1999b) The agonist-binding domain of the calcium-sensing receptor is located at the amino-terminal domain. *J Biol Chem* **274**:18382-18386.
- Bräuner-Osborne H and Krogsgaard-Larsen P (2000) Sequence and expression pattern of a novel human orphan G-protein-coupled receptor, GPRC5B, a family C receptor with a short amino-terminal domain. *Genomics* **65**:121-128.
- Brown EM (1999) Physiology and pathophysiology of the extracellular calcium-sensing receptor. *Am J Med* **106**:238-253.
- Bunzow JR, Sonders MS, Arttamangkul S, Harrison LM, Zhang G, Quigley DI, Darland T, Suchland KL, Pasumamula S, Kennedy JL, Olson SB, Magenis RE, Amara SG and Grandy DK (2001) Amphetamine, 3,4-methylenedioxymethamphetamine, lysergic acid diethylamide, and metabolites of the catecholamine neurotransmitters are agonists of a rat trace amine receptor. *Mol Pharmacol* **60**:1181-1188.
- Calver AR, Michalovich D, Testa TT, Robbins MJ, Jaillard C, Hill J, Szekeres PG, Charles KJ, Jourdain S, Holbrook JD, Boyfield I, Patel N, Medhurst AD and Pangalos MN (2003) Molecular cloning and characterisation of a novel GABA_B-related G-protein coupled receptor. *Brain Res Mol Brain Res* **110**:305-317.
- Cheng Y and Lotan R (1998) Molecular cloning and characterization of a novel retinoic acid-inducible gene that encodes a putative G protein-coupled receptor. *J Biol Chem* **273**:35008-35015.
- Clausen RP, Bräuner-Osborne H, Greenwood JR, Hermit MB, Stensbøl TB, Nielsen B and Krogsgaard-Larsen P (2002) Selective agonists at Group II metabotropic glutamate receptors: synthesis, stereochemistry and molecular pharmacology of (S)- and (R)-2-amino-4-(4-hydroxy[1,2,5]thiadiazol-3-yl)butyric acid. *J Med Chem* **45**:4240-4245.
- Closs EI (2002) Expression, regulation and function of carrier proteins for cationic amino acids. *Curr Opin Nephrol Hypertens* **11**:99-107.
- Conklin BR and Bourne HR (1994) Homeostatic signals. Marriage of the flytrap and the serpent. *Nature* **367**:22.
- Galvez T, Prézeau L, Milioti G, Franek M, Joly C, Froestl W, Bettler B, Bertrand HO, Blahos J and Pin JP (2000) Mapping the agonist-binding site of GABA_B type 1 subunit sheds light on the activation process of GABA_B receptors. *J Biol Chem* **275**:41166-41174.
- Ginalski K, Elofsson A, Fischer D and Rychlewski L (2003) "3D-Jury: a simple approach to improve protein structure predictions". *Bioinformatics* **19**:1015-1018.
- He W, Miao FJ-P, Lin C-H, Schwandner RT, Wang Z, Gao J, Chen J-H, Tian H and Ling L (2004) Citric acid cycle intermediates as ligands for orphan G-protein-coupled receptors. *Nature* **429**:188-193.
- Horton RM, Hunt HD, Ho SN, Pullen JK and Pease LR (1989) Engineering hybrid genes without the use of restriction enzymes: gene splicing by overlap extension. *Gene* **77**:61-68.
- Kuang D, Yao Y, Wang M, Pattabiraman N, Kotra LP and Hampson DR (2003) Molecular similarities in the ligand binding pockets of an odorant receptor and the metabotropic glutamate receptors. *J Biol Chem* **278**:42551-42559.
- Kunishima N, Shimada Y, Tsuji Y, Sato T, Yamamoto M, Kumasaka T, Nakanishi S, Jingami H and Morikawa K (2000) Structural basis of glutamate recognition by a dimeric metabotropic glutamate receptor. *Nature* **407**:971-977.

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- Lambert C, Leonard N, De Bolle X and Depiereux E (2002) ESyPred3D: Prediction of proteins 3D structures. *Bioinformatics* **18**:1250-1256.
- Malherbe P, Knoflach F, Broger C, Ohresser S, Kratzeisen C, Adam G, Stadler H, Kemp JA and Mutel V (2001) Identification of essential residues involved in the glutamate binding pocket of the group II metabotropic glutamate receptor. *Mol Pharmacol* **60**:944-954.
- Marti-Renom MA, Stuart A, Fiser A, Sánchez R, Melo F and Sali A (2000) Comparative protein structure modeling of genes and genomes. *Annu Rev Biophys Biomol Struct* **29**:291-325.
- Minakami R, Katsuki F, Yamamoto T, Nakamura K and Sugiyama H (1994) Molecular cloning and the functional expression of two isoforms of human metabotropic glutamate receptor subtype 5. *Biochem Biophys Res Commun* **199**:1136-1143.
- Möhler H and Fritschy JM (1999) GABA_B receptors make it to the top - as dimers. *Trends Pharmacol Sci* **20**:87-89.
- Morris SM, Jr. (2004) Recent advances in arginine metabolism. *Curr Opin Clin Nutr Metab Care* **7**:45-51.
- Nelson G, Hoon MA, Chandrashekar J, Zhang Y, Ryba NJ and Zuker CS (2001) Mammalian sweet taste receptors. *Cell* **106**:381-390.
- Nielsen H, Engelbrecht J, Brunak S and von Heijne G (1997) A neural network method for identification of prokaryotic and eukaryotic signal peptides and prediction of their cleavage sites. *Int J Neural Syst* **8**:581-599.
- O'Hara PJ, Sheppard PO, Thogersen H, Venezia D, Haldeman BA, McGrane V, Houamed KM, Thomsen C, Gilbert TL and Mulvihill ER (1993) The ligand-binding domain in metabotropic glutamate receptors is related to bacterial periplasmic binding proteins. *Neuron* **11**:41-52.
- Oh BH, Pandit J, Kang CH, Nikaido K, Gokcen S, Ames GF and Kim SH (1993) Three-dimensional structures of the periplasmic lysine/arginine/ornithine-binding protein with and without a ligand. *J Biol Chem* **268**:11348-11355.
- Pagano A, Rovelli G, Mosbacher J, Lohmann T, Duthey B, Stauffer D, Ristig D, Schuler V, Meigel I, Lampert C, Stein T, Prézeau L, Blahos J, Pin J, Froestl W, Kuhn R, Heid J, Kaupmann K and Bettler B (2001) C-terminal interaction is essential for surface trafficking but not for heteromeric assembly of GABA_B receptors. *J Neurosci* **21**:1189-1202.
- Pin JP and Duvoisin R (1995) The metabotropic glutamate receptors: structure and functions. *Neuropharmacology* **34**:1-26.
- Pin JP, Waeber C, Prézeau L, Bockaert J and Heinemann SF (1992) Alternative splicing generates metabotropic glutamate receptors inducing different patterns of calcium release in *Xenopus oocytes*. *Proc Natl Acad Sci USA* **89**:10331-10335.
- Robbins MJ, Michalovich D, Hill J, Calver AR, Medhurst AD, Gloger I, Sims M, Middlemiss DN and Pangalos MN (2000) Molecular cloning and characterization of two novel retinoic acid-inducible orphan G-protein-coupled receptors (GPRC5B and GPRC5C). *Genomics* **67**:8-18.
- Quick MW, Lester HA, Davidson N, Simon MI and Aragay AM (1996) Desensitization of inositol 1,4,5-triphosphate/Ca²⁺-induced Cl⁻ currents by prolonged activation of G proteins in *Xenopus oocytes*. *J Biol Chem* **271**:32021-32027.
- Sato T, Shimada Y, Nagasawa N, Nakanishi S and Jingami H (2003) Amino acid mutagenesis of the ligand binding site and the dimer interface of the metabotropic glutamate receptor 1. Identification of crucial residues for setting the activated state. *J Biol Chem* **278**:4314-4321.
- Schreibmayer W, Dessauer CW, Vorobiov D, Gilman AG, Lester HA, Davidson N and Dascal N (1996) Inhibition of an inwardly rectifying K⁺ channel by G-protein alpha-subunits. *Nature* **380**:624-647.
- Schrödinger Inc. (2002, 2004) Macromodel 8.1, First Discovery 2.5, Glide 2.5, Prime 1.1.
- Scott L, Lamb J, Smith S and Wheatley DN (2000) Single amino acid (arginine) deprivation: rapid and selective death of cultured transformed and malignant cells. *Br J Cancer* **83**:800-810.
- Skeberdis VA, Lan J, Opitz T, Zheng X, Bennett MV and Zukin RS (2001) mGluR1-mediated potentiation of NMDA receptors involves a rise in intracellular calcium and activation of protein kinase C. *Neuropharmacology* **40**:856-865.
- Specia DJ, Lin DM, Sorensen PW, Isacoff EY, Ngai J and Dittman AH (1999) Functional identification of a goldfish odorant receptor. *Neuron* **23**:487-498.

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- Venter JC, Adams MD, Myers EW, Li PW and Mural RJ (2001) The sequence of the human genome. *Science* **291**:1304-1351.
- Wellendorph P and Bräuner-Osborne H (2004) Molecular cloning, expression, and sequence analysis of GPRC6A, a novel family C G-protein coupled receptor. *Gene* **335**:37-46.
- Wise A, Jupe SC and Rees S (2004) The identification of ligands at orphan G-protein coupled receptors. *Annu Rev Pharmacol Toxicol* **44**:43-66.

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Footnotes

a)

This work was supported by the Danish Medical Research Council, Apotekerfonden of 1991, the Lundbeck Foundation, and EU grant HPAW-CT-2002-80057 (PW).

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Legends for figures

Figure 1. Details of the three-dimensional model of the hGPCR6A ATD.

(A) Ribbon view of the binding cleft with L-Lys docked into the purported binding pocket. Hydrogen bonds are indicated by dashed lines. (B) Flat scheme indicating residues that interact directly with the α -amino acid and distal amine moieties of L-Lys. Hydrogen bonds from Fig. 1A are shown with green lines. It is the hydroxyl group on the side chain of S149 that interact with the carboxylic acid group of L-Lys.

Figure 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 ELISA. 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 hGPCR6A and point-mutations thereof. (B) Schematic illustration of the native hGPCR6A (black), 5.24 (grey), and the chimeras generated by exchanging the ATDs and 7TM+intracellular domains. (C) Surface expression of native GPCR6A and 5.24 and chimeras of the two. (D) Surface expression of native mGPCR6A. Data are from a representative experiment performed in triplicate. Asterices indicate $p < 0.05$ versus mock-transfected cells. Three independent experiments gave similar results.

Figure 3. Deorphanization of GPCR6A in *Xenopus* oocytes.

Traces of Ca²⁺-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 μ M L-Lys or L-Arg. (B) Uninjected oocytes show no response when stimulated with 100 μ M L-Arg. (C) Representative traces demonstrating the L- α -amino acid agonist preferences of the h6A/5.24 chimeric receptor using 100 μ M concentration. (D) Of all the tested amino acids, only L-Arg, L-Lys, and L-Orn are able to activate h6A/5.24 at 10 μ M concentration (note that

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the scale is different from Fig. 3C) (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 orthologue of GPRC6A. All traces are representative of n=3 for each experiment.

Figure 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, exposed to various concentrations of L- α -amino acids and responses were measured on a NOVOstar plate reader as described in *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 pre-incubations of two hours before loading with Fluo-4. Responses are shown as Δ *Fluorescence 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 seconds. (C) Representative concentration-response curves of three L- α -amino acids with either basic (Arg) or small (Ala, Ser) side chains. EC₅₀ values for all L- α -amino acids are shown in Table 2.

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TABLE 1

Compound testing in *Xenopus laevis* oocytes expressing the
chimeric receptor h6A/5.24.^a

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: Ca²⁺, γ -hydroxybutyrate, GABA, histamine,
serotonin, taurine, vasopressin

Metabolic intermediates

Active: (L-Orn, L-Cit; see Fig. 3C)

Inactive: agmatine, creatine, creatinine, spermine, urea

^a All compounds were tested at 100 μ M except Ca²⁺ which was tested in 4 mM

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TABLE 2

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

EC₅₀ 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.

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

All remaining natural L- α -amino acids had EC₅₀ > 1000 μ M

All active compounds listed above were full agonists.

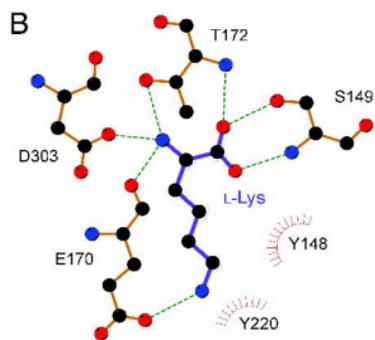
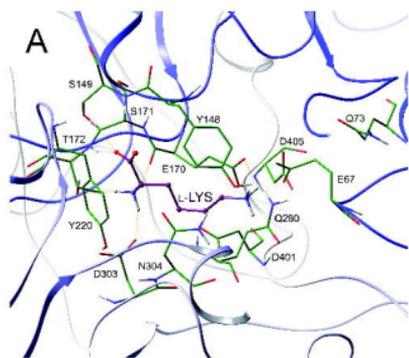


Figure 1

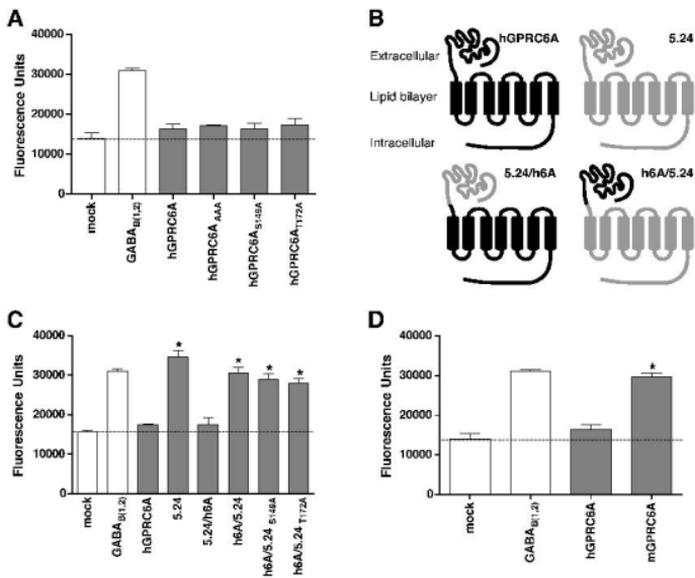


Figure 2

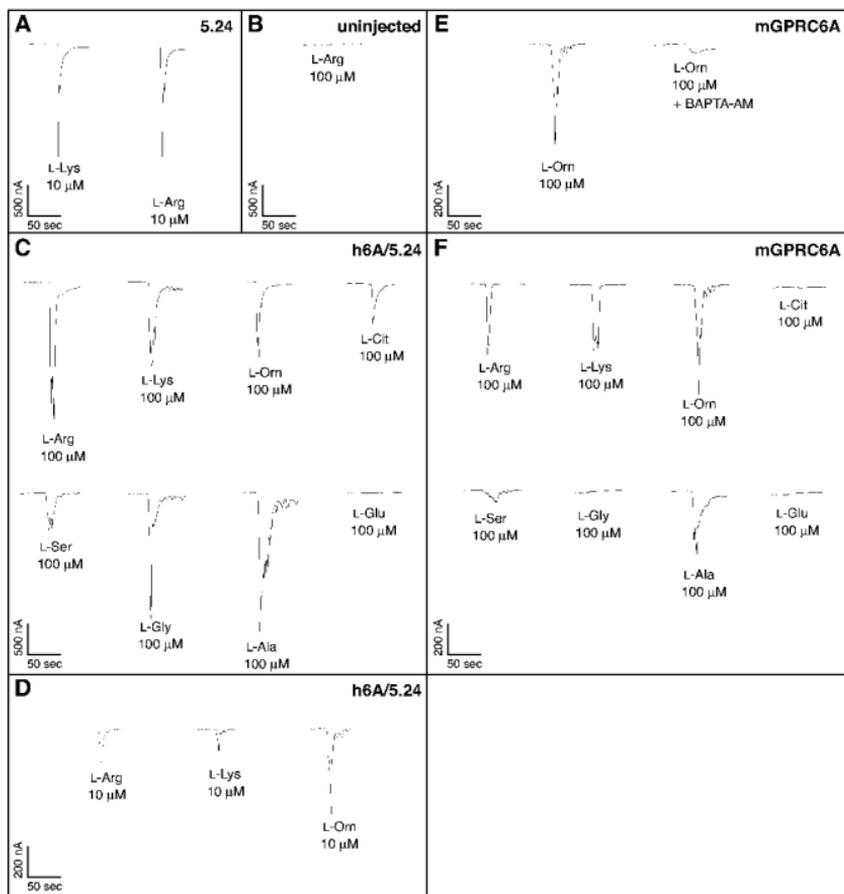


Figure 3

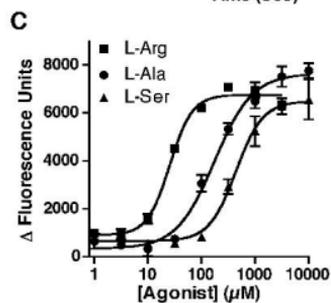
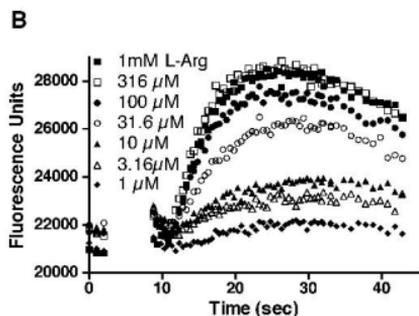
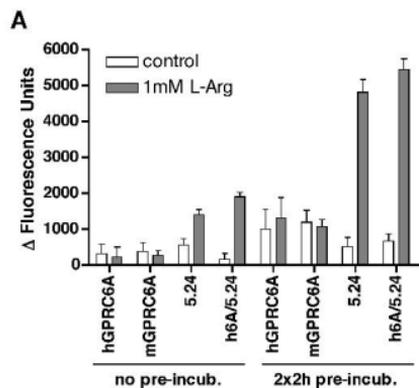


Figure 4