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# Creation of GPCR-based chemical sensors by directed evolution in yeast

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**G protein-coupled receptors (GPCRs) form a class of biological chemical sensors with an enormous diversity in ligand binding and sensitivity. To explore structural aspects of ligand recognition, we subjected the human UDP-glucose receptor (P2Y14) functionally expressed in the yeast *Saccharomyces* to directed evolution. We sought to generate new receptor subtypes with ligand-binding properties that would be useful in the development of practical biosensors. Mutagenesis of the entire UDP-glucose receptor gene yielded receptors with increased activity but similar ligand specificities, while random mutagenesis of residues in the immediate vicinity of the ligand-binding pocket yielded mutants with altered ligand specificity. By first sensitizing the P2Y14 receptor and then redirecting ligand specificity, we were able to create mutant receptors suitable for a simple biosensor. Our results demonstrate the feasibility of altering receptor ligand-binding properties via a directed evolution strategy, using standard yeast genetic techniques. The novel receptor mutants can be used to detect chemical ligands in complex mixtures and to discriminate among chemically or stereochemically related compounds. Specifically, we demonstrate how engineered receptors can be applied in a pairwise manner to differentiate among several chemical analytes that would be indistinguishable with a single receptor. These experiments demonstrate the feasibility of a combinatorial approach to detector design based on the principles of olfaction.**

*Keywords*: chemical sensing/directed evolution/GPCR/G-protein coupled receptor/olfaction/ligand recognition

## Introduction

G protein-coupled receptors (GPCRs) mediate chemical communications between cells and also function as sensors in an organism's perception of the environment. Receptors involved in intercellular communication are often exquisitely tuned to respond to a predetermined chemical signal while excluding others, for instance responding to serotonin but not tryptophan or melatonin. In contrast, receptors responsible for capturing environmental signals must respond to a broader range of stimuli than would be feasible if each stimulus required its own receptor. Our visual system distinguishes a wide diversity of colors using only three receptors, while the mammalian olfactory system can distinguish tens to hundreds of thousands of compounds using only 350–1200 receptors (Beets, 1970; Polak, 1973; Glusman *et al*., 2001; Zozulya *et al*., 2001; Zhang and Firestein, 2002). The discriminatory powers of the visual and olfactory systems reside in a combinatorial mechanism of perception in which receptors have overlapping specificities. In the olfactory system, a single compound can bind to and activate a number of different receptors and each receptor can respond in varying degrees to a number of related compounds (Schild, 1988; Buck, 1996; Malnic *et al*., 1999).

Following the model of olfactory perception, we have begun to explore the concept of combinatorial recognition by GPCRs as a means of creating broad specificity chemical detectors. We anticipate that an array of receptors with overlapping specificities could sample certain regions of chemical structure space, allowing individual analytes to be identified. With larger arrays it would be possible to detect novel signals via distinctive patterns of receptor activation without having to redesign receptors specifically for individual analytes. One strategy for constructing such arrays would be to exploit the naturally occurring diversity of chemical receptors, including olfactory receptors, as chemical sensors. Such a strategy immediately poses engineering challenges, as olfactory receptors are notoriously difficult to express outside neuronal tissue and non-olfactory receptors are unlikely to have sufficient diversity to function effectively in chemosensory arrays. Ultimately, even sensors based on naturally occurring olfactory receptors will be of limited utility for many analytical applications, as we know that many chemicals are described as 'odorless' and thus presumably fail to stimulate the olfactory system in a distinctive manner. To broaden the potential usefulness of naturally occurring GPCRs as chemical sensors, it will be necessary to explore techniques to optimize a given GPCR for sensing applications. For instance, it could be desirable to enhance receptor sensitivity, to alter responses to the receptor's known ligands or, potentially, to introduce the capacity to bind new sets of pharmacophores that previously did not interact with the receptor.

We have used a yeast system developed for functional expression of heterologous GPCRs as a platform to create novel receptors through directed evolution. Yeast strains that have been utilized for functional analysis of GPCRs and drug screening were constructed by taking advantage of similarities between the yeast mating response pathway and human signal transduction pathways (Silverman *et al*., 1998). In yeast, the α and **a** mating pheromones are ligands for the Ste2 and Ste3 GPCRs, which signal through a heterotrimeric G protein and a MAP kinase pathway to regulate physiological and transcriptional outputs of the mating response (Marsh *et al*., 1991). By replacing the yeast pheromone receptor with a mammalian GPCR, tailoring the G-protein to couple the mammalian

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GPCR to the pheromone response pathway and engineering the output of the pheromone response pathway, we and others have generated strains whose growth depends on functional activation of the inserted mammalian receptor. Such strains have been used for genetic selection to identify receptor ligands, genetic analysis of ligand structure and genetic selection of constitutively active receptors (Manfredi *et al*., 1996; Klein *et al*., 1998; Zhang *et al*., 2002; Arias *et al*., 2003; Sachpatzidis *et al*., 2003; Celic *et al*., 2004). In this paper, we demonstrate that this system can be used to identify receptors with novel ligand recognition properties.

To initiate our study of the directed evolution of GPCRs, we sought to isolate mutants of the human UDP-glucose receptor (KIAA0001, P2Y14) with altered ligand specificity. The UDP-glucose (UDPG) receptor is part of a large family of nucleotide receptors, some of which have affinity to sugar nucleotides (Abbracchio *et al*., 2003). Sugar nucleotides are key reagents in the biological or chemoenzymatic synthesis of carbohydrates. Sugar nucleotides are structurally diverse, with similar physicochemical properties, and therefore represent a challenging target for inexpensive, high-throughput chemical analysis. We reasoned that a sugar nucleotide sensor such as the human UDPG receptor would present a good starting point for the development of chemosensors that could be used to assay sugar nucleotides and their derivatives. Here we report the successful use of directed evolution to create a family of UDPG receptors. By random mutagenesis of the entire receptor gene and genetic selection for growth in the presence of ligand, we identified receptors sensitized to all ligands, but with essentially unaltered ligand preference. Subsequently, by targeting mutagenesis to motifs anticipated to interact with ligand, we identified receptor mutants with changes in ligand specificity and efficacy. Here, changing ligand specificity refers to changing the relative sensitivity of the receptor to existing receptor ligands and not necessarily changing the ensemble of chemical ligands that can physically bind/activate a receptor. Among the receptors generated by targeted mutagenesis were a receptor with 'inverted' stereochemical preference for UDP-galactose (UDP-Gal) versus UDPG and a receptor that is more robustly activated by a partial agonist, UDP. Finally, as an example of how engineered receptors can be utilized in a combinatorial manner, we show how pairwise application of engineered receptors can be used to identify uniquely an unknown ligand with a single pair of measurements. This demonstrates the feasibility of a combinatorial approach to detector design using engineered receptors.

## Materials and methods

### Materials

UDPG, UDP-galactose (UDP-Gal), UDP-*N*-acetylglucosamine (UDP-glcNAC), UDP-*N*-acetylgaltactosamine (UDP-galNAC), uridine triphosphate (UTP), uridine diphosphate (UDP), glucose-1-phosphate (G-1-P), glucose-6-phosphate (G-6-P), UDP-glucose-pyrophosphorylase (UGPase), glycogen synthase (GS), pyrophosphatase (PPase) and fluorescein (FDG) were purchased from Sigma-Aldrich (St. Louis, MO). Mutazyme was purchased from Stratagene.

### Strains and plasmids

Mutagenesis and selection were performed in yeast strain CY10560 ($P_{FUS1}$-*HIS3 ade2Δ3447 ade8Δ3457 can1-100 far1Δ1442 his3Δ200 leu2-3,112 lys2 sst2Δ1056 ste14*::*trp1*::*LYS2 ste18γ6–3841 ste3Δ1156 trp1-1 ura3-52*). β-Galactosidase assays were performed using yeast strain CY10981 ($P_{FUS1}$-*HIS3 can1-100 far1Δ1442 his3Δ200 leu2-3,112 lys2 sst2Δ2 ste14*::*trp1*::*LYS2 ste3Δ1156 trp1-1 ura3-52*) carrying plasmid Cp1021 ($P_{FUS1}$-LacZ 2 μm *URA3*). The UDP-glucose receptor was cloned into plasmid Cp1651 to yield plasmid pAH1 ($P_{PGK1}$-hP2Y14 2 μm *LEU2*) for expression in the host strains.

### Mutagenesis and selection of sensitized receptor mutants

The entire UDPG receptor gene was mutagenized via error-prone mutagenesis to an estimated frequency of ~2–5 mutations/kb following the Mutazyme protocol. A library of mutants was generated by gap repair cloning and plated to near confluence on selective media. A total of $(1–2)\times10^5$ colonies were screened by replica plating to SC–His medium (Kaiser *et al*., 1994) with or without ligand. Yeast growth media was supplemented by 1 mM 3AT, a competitive inhibitor of the *HIS3* reporter gene product, which sets the threshold for reporter gene activation.

### Targeted mutagenesis and selection of functional receptor mutants

To generate targeted mutants, oligonucleotides with randomized sequences corresponding to the codons to be mutagenized were utilized to generate overlapping PCR products. The HIAR motif corresponds to P2Y14 amino acids 250–253 in TM6, the KExT motif corresponds to amino acids 277–280 in TM7, the NMY motif corresponds to amino acids 104–106 in TM3 and the AxxFY motif corresponds to amino acids 98–102 in TM3. Mutant libraries were generated by gap repair using overlapping PCR products and transforming to media selective for recombined plasmids. To select for functional mutants, libraries were replica plated to selective media containing one of six ligands: UDP-Gal, UDPG, UDP-galNAc, UDP-glcNAc, UDP or dTDP-glucose (50 μl of 1 mM concentration spread on 30 ml of SC–Leu-His agar medium in 8.5 cm Petri plates).

### β-Galactosidase assays

β-Galactosidase assays were carried out as described previously (Chambers *et al*., 2000), with the exception that cultures were incubated with ligand in 500 μl cultures in 48-well culture blocks rather than in microtiter plates as described. Schild plot analysis was carried out as described, using visual interpolation to read inhibitor concentrations corresponding to the $EC_{20}$ of each plot (Limbird, 1996).

## Results

### Genetic selection of sensitized receptor mutants

We initially sought to redirect the ligand specificity of the human UDPG receptor by random mutagenesis of the complete gene, followed by selection for mutants responsive to non-native ligands. Yeast strain CY10560 expressing the wild-type human UDPG receptor gene grows on selective medium with 0.3 μM UDPG (8.5 cm Petri plates spread with 100 μl of $10^{-4}$ M UDPG over 30 ml of solid medium) but does not grow on plates with one-tenth that concentration of UDPG. In addition, the strain fails to grow on selective medium containing 0.3 μM UDP-Gal, UDP-glcNAc or UDP-galNAc, so these ligands are considered non-native (Chambers *et al*., 2000).

Cells were transformed with a plasmid library carrying the randomly mutated human UDPG receptor gene. Transformants were recovered on non-selective medium and then screened for growth on selective media without ligand or containing 0.03 μM UDPG or 0.3 μM UDP-Gal, UDP-glcNAc or UDP-galNAc. Some of the transformants exhibited constitutive growth in the absence of ligand. Most of the non-constitutive mutant receptors that promoted growth in response to any one of the non-native ligands showed growth in response to each of the other ligands, including UDPG, and therefore did not show signs of altered ligand specificity.

Despite the absence of mutants with clear changes in ligand specificity, we noted that sensitized receptors would facilitate subsequent receptor engineering experiments. We asked if receptors subjected to sequential rounds of mutagenesis might exhibit even greater sensitivity, while potentially accumulating more substantial changes in ligand specificity. We recovered plasmid DNA from those transformants that exhibited ligand-dependent growth with enhanced sensitivity to non-native ligands, pooled the DNA samples and performed the mutagenesis and selection a second time. We then extracted DNA from several candidate clones with enhanced, ligand-dependent growth and performed a third round of mutation and selection on each individually. This cycle was repeated using the best candidate clones from the third round.

After these four rounds of mutagenesis and selection, the preponderance of non-constitutive mutants exhibited enhanced response to all four ligands. Using plate-based growth assays, in which patches of sensitized mutants were replica plated to media supplemented with various concentrations of ligand, it was not possible to discern changes in the relative sensitivity to ligand for any of the sensitized mutants. However, in growth assays the most sensitive mutant receptors responded to ~30-fold lower concentrations of UDPG than did wild-type receptor. The apparent sensitivity did not change significantly from the third to the fourth cycle of mutagenesis and selection. Several UDPG receptor mutants were selected for sequencing. Mutations were scattered across the receptor gene, suggesting that few, if any, of the effects of mutations were caused by changes to residues that interact directly with ligand (Table I).

**Table I.** Receptor mutants[a]

| Mutant | Parent | Mutations | Positions |
|---|---|---|---|
| 1 | wt | W128C | IL2 |
| 5 | wt | Y137C, S237I | 4.41, EL2 |
| 2-1 | Pooled round 1 | G80S, Y137C, S237I | 2.63, 4.41, EL2, |
| 2-10 | Pooled round 1 | W128R, L148F, S237C, L314I | IL2, 4.52, EL2, IL3 |
| 2-2 | Pooled round 1 | K54E, A193V, A252V | IL1, 5.45, 6.54 |
| 2-2-1 | 2–2 | K54E, A193V, F243I, A252V | IL1, 5.45, 6.45, 6.54 |
| 2-2-1-1 | 2–2–1 | K54E, A98V, A193V, F244I, A252V | IL1, 3.29, 5.45, 6.45, 6.54 |
| H-20 | 2–2–1–1 | 2-2-1-1 + 250–253 HIAR->HTVK | 6.52–6.55 |
| K-3 | H-20 | H-20+E278G | 7.36 |

[a]A subset of UDP-glucose receptor mutants were isolated as described and analyzed by sequencing. Numbered mutants were isolated by screening libraries generated by gene-wide random mutagenesis for sensitization to receptor ligands. The second round of screening utilized a pool of mutants isolated in the first round of mutagenesis as template. Lettered (H-20, K-3) mutants were generated by targeted saturation mutagenesis of the indicated motifs. Positions are indicated as extracellular loop 1–3 (EL1–3), intracellular loop 1–3 (IL1–3) or transmembrane domain [numbered according to Ballesteros and Weinstein (1995)].

### *Isolation of specificity mutants via targeted mutagenesis*

Since random mutagenesis of the UDPG receptor appeared to yield a preponderance of mutants with increased sensitivity but unaltered specificity, we focused mutagenesis on residues that we hypothesized to be directly involved in ligand binding. As a starting receptor for this directed mutagenesis we selected a receptor, designated 2211, that was isolated as described above (Table I). The 2211 receptor responds to all three non-native ligands and to significantly lower concentrations of UDPG than the wild-type receptor in growth assays. In liquid β-galactosidase assays, as described in Materials and methods, this receptor shows increased reporter activation at all concentrations of ligand, without significant changes in the $EC_{50}$ (Figure 1). Like the wild-type UDPG receptor, the 2211 receptor did not promote detectable growth in plate assays in the absence of ligand. We concluded that the 2211 receptor retains the essential signaling properties of the wild-type P2Y14 receptor while functioning more robustly in the yeast expression system and hence it constituted a better starting point for subsequent rounds of mutagenesis and selection. We selected motifs to target for mutagenesis based on conserved residues in the nucleotide receptor subfamily and a model of the transmembrane regions of the UDPG receptor based on the crystal structure of bovine rhodopsin (Moro *et al*., 1998; Palczewski *et al*., 2000; Jacobson *et al*., 2004). Overall, alignments of the transmembrane domains and conserved residues suggest a ligand binding pocket in the canonical ligand-binding region of GPCRs between transmembrane helices 3, 6 and 7.

We first targeted the 'HIAR' motif in transmembrane domain 6. The His250 and Arg253 residues in P2Y14 correspond to His and Lys residues, respectively, that are critical for activation of the P2Y1 receptor by ATP (Moro *et al*., 1998; Jacobson *et al*., 2004). One of the mutations, A252V, in the sensitized 2211 mutant falls in this motif, although it is not clear if this specific mutation gives rise to a sensitized phenotype. Libraries containing the randomized HIAR motif were constructed *in vivo* by cotransforming strain CY10560 cells with three DNA fragments: a 2211 receptor plasmid cut to remove the HIAR domain and a pair of PCR products synthesized with oligonucleotides randomized over the HIAR region and 5′ and 3′ extensions overlapping both sides of the gap in the plasmid. Transformants were replicated to plates containing UDPG or one of the non-native ligands UDP-gal, UDP-glcNAc or UDP-galNAc. Transformants were also replica plated to plates containing UDP and dTDP-glucose to test for the presence of mutant receptors capable of responding to ligands that do not activate the parent receptor. Twenty out of ~5000 transformants grew in the presence of one or more ligands. The 20 receptors isolated in this primary screen were subsequently retested for growth in the presence of lower concentrations of each ligand, to ascertain whether the receptor had significant changes in ligand preference. In this secondary screen only one of the 20 receptors had a dramatically different profile of ligand responsiveness than the starting receptor. To determine whether the receptors that lacked appreciable changes in ligand specificity were indeed mutants and to verify the complexity of the mutant library, the DNA encoding each receptor was sequenced. Sequencing revealed that

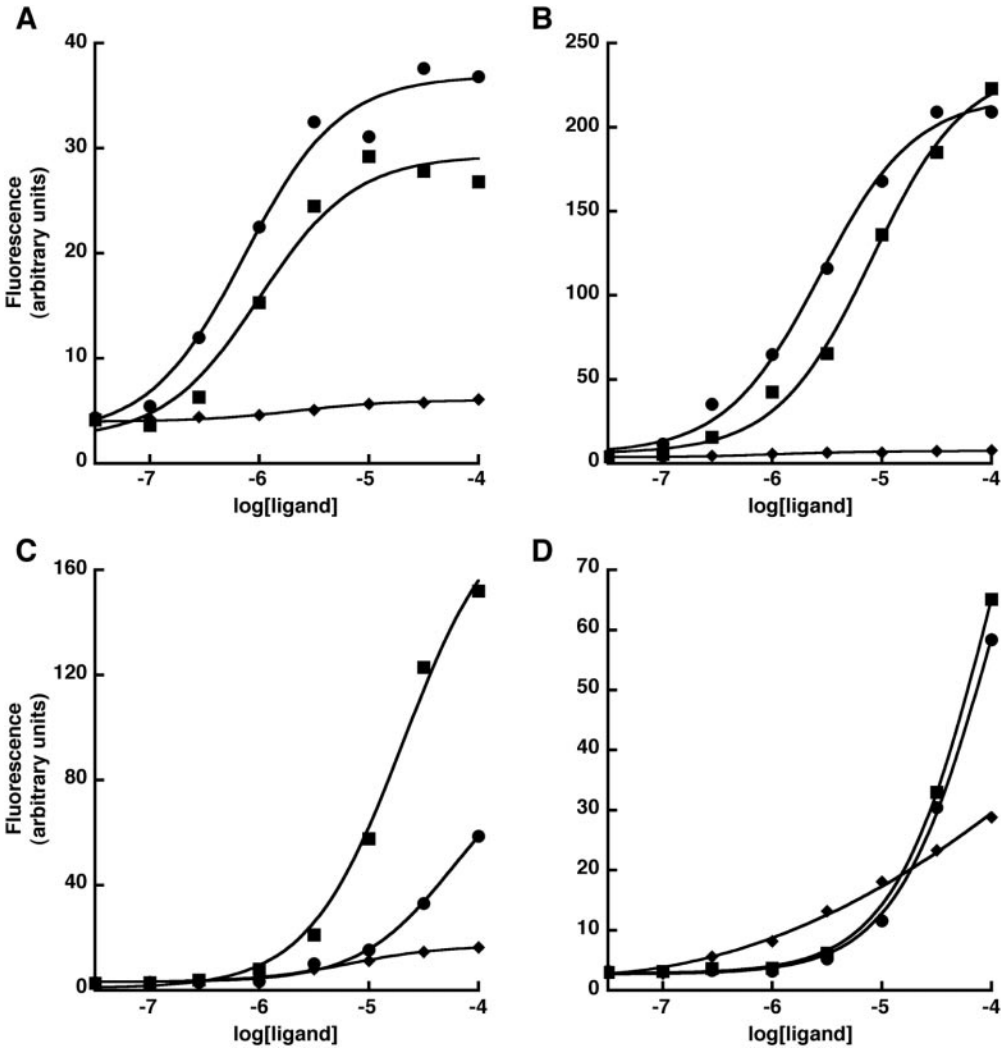


**Fig. 1.** Ligand response of wild-type and mutant UDP-glucose receptors. Dose–response curves measured with three different ligands, UDP-glucose (circles), UDP-galactose (squares) and UDP (diamonds), for wild-type UDPG receptor (**A**) and mutants 2211 (**B**), H-20 (**C**) and K-3 (**D**). Note that wild-type and 2211 receptors have similar response patterns for the three ligands with different activation levels (note the difference in scale in the two graphs) whereas H-20 and K-3 exhibit different relative ligand preferences.

three plasmids contained unaltered 2211 receptor DNA. Thirteen of the 17 remaining plasmids contained unique, readable sequences, each of which contained randomized DNA across the HIAR motif. Strikingly, the histidine residue was conserved in every mutant receptor and the arginine residue was conserved in 12 of the 13 clones (Table II). The remaining clone contained HTVK in place of the HIAR motif and was the only mutant that exhibited altered ligand specificity in growth assays, showing a preference for growth in the presence of UDP-Gal versus UDPG.

The HTVK mutant, designated H-20, was selected as the template for mutagenesis of three additional motifs, 'AxxFY' and 'NMY' in TM3 and 'KExT' in TM7. These mutants were tested in the same manner as the HIAR mutants, focusing only on mutants with clear changes in relative growth on one or more ligands. Of these, one mutant, designated K-3, in which KEFT was replaced by KGFT, had the most dramatic changes. The K-3 mutant grew poorly relative to its parent in response to UDPG and UDP-Gal but, surprisingly, grew in the presence of UDP. With the H-20 and K-3 mutants in hand, we concluded that targeted mutagenesis of conserved motifs in ligand binding domains of the UDPG receptor can yield receptors with altered ligand specificity.

### *Quantitative analysis of ligand binding to mutant receptors*

To analyze further the properties of the two mutant receptors with altered ligand specificity, we quantified GPCR activation *in vivo* as a function of ligand type and concentration (Figure 1). The reporter assays confirmed qualitative observations from plate assays indicating a relative order of ligand activation of UDPG > UDP-Gal $\gg$ UDP for 2211, UDP-Gal > UDPG > UDP for H-20 and UDP > UDPG = UDP-Gal for K-3. The H-20 receptor has lower $EC_{50}$s for both UDPG and UDP-Gal than does the 2211 receptor. These lower values preclude determination of maximal activation levels for either ligand against H-20 and, accordingly, accurate measurement of $EC_{50}$ values. Also, we cannot determine from these data whether UDPG is a partial or full agonist for H-20, although UDPG shows no competitive antagonist activity toward UDP-Gal activation of H-20 (data not shown). Similarly, analysis of the K-3 receptor

**Table II.** Sequences of recovered HIAR mutants[a]

| Clone | AA position | | | | DNA sequence |
|---|---|---|---|---|---|
| | H | I | A | R | |
| H-1 | H | A | V | R | CAC GCG GTG AAG |
| H-2 | H | A | L | R | CAC GCA TTG CGG |
| H-5 | H | A | T | R | CAC GCG ACA AGA |
| H-9 | H | A | T | R | CAT GCG ACC CGG |
| H-17 | H | A | T | R | CAT GCC ACT AGA |
| H-6 | H | V | L | R | CAC GCG TTG CGT |
| H-7 | H | I | C | R | CAT ATT TGC CGG |
| H-8 | H | T | L | R | CAC ACG CTG CGA |
| H-10 | H | V | I | R | CAC GTT ATC CGA |
| H-11 | H | T | V | R | CAT GTG ACA AGG |
| H-13 | H | L | T | R | CAC TTG ACG CGT |
| H-14 | H | L | T | R | CAT TTA ACA AGG |
| H-20 | H | T | V | K | CAT ACC GTC AAG |
| Recovered AAs | H | AVILT | VILTC | RK | |

[a]13 unique mutant sequences were obtained from a set of receptors that displayed responsiveness to ligand. Mutant H-20 has a phenotype with substantial changes in ligand specificity (see text). The remaining mutants recognize the same set of ligands, with the same relative ligand sensitivity as the parent 2211 receptor and the wild-type P2Y14 receptor, although with varying degrees of overall receptor sensitivity. The positions corresponding to I251 and A252 tolerate substitution but exclude aromatic or charged residues.

was complicated by our inability to activate fully the receptor with the available ligands. However, given the comparatively strong activation of the receptor at high concentrations of UDP-Gal and UDPG, UDP likely acts as a partial agonist for this receptor. Initially, the observation that UDP activates both the H-20 receptor and the K-3 receptor suggested that the mutant receptors had gained an affinity for UDP. Rather, careful examination showed that the 2211 receptor is weakly activated by UDP. Quantitative analysis revealed that UDP acts as a competitive inhibitor of UDPG activation of 2211 (Figure 2A). A comprehensive analysis of the inhibition characteristics of UDP as a function of different agonist concentrations yields an apparent $K_D$ of $\sim 10^{-4.5}$ M of UDP for 2211 (Figure 2B). This suggests that the effect of the H-20 and K-3 mutations is to change the consequence of UDP binding to the receptor (from antagonism to partial agonism), as opposed to generating a new site for UDP binding to the receptor.

### *Engineered GPCRs as chemical sensors*

Our isolation of receptors with distinct but overlapping specificities towards different ligands allowed us to explore novel uses of GPCRs as chemical sensors. We asked whether our receptors could function in a combinatorial fashion such that a small number of receptors could be used to identify multiple compounds uniquely. With a single receptor, it is for the most part impossible to differentiate among pure solutions of different receptor ligands. Even with extensive controls, it would be impossible to differentiate between a dilute solution of a strong agonist and a concentrated solution of a weak agonist. In contrast, using multiple receptors with overlapping ligand recognition properties, it is possible to establish a 'fingerprint' for different ligands that can be used to differentiate one ligand from another.

This can be illustrated in an intuitive way by calculating the ratio of responses for a pair of receptors, using the data underlying Figure 1. We calculated for each receptor pair the ratio of reporter activity at each ligand concentration (Table III).

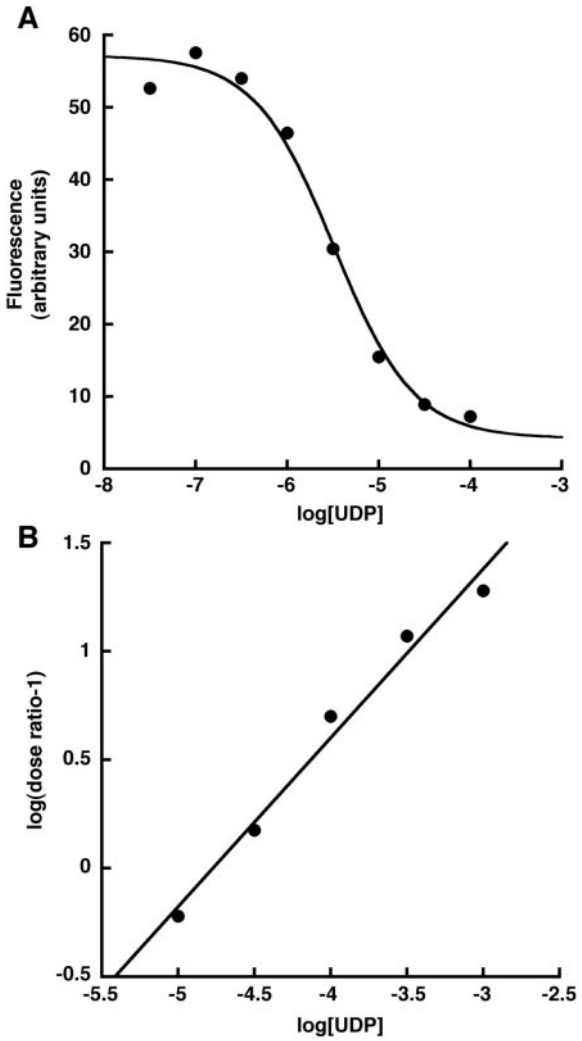


**Fig. 2.** UDP antagonizes activation of UDP-glucose receptor 2211. (**A**) Inhibition of signaling by UDP. $IC_{50}$ of UDP is $10^{-4.5}$ M. (**B**) Schild regression of UDP antagonism of UDP-glucose agonist activity. Slope of the linear fit is ~0.8, which is consistent with UDP having a weak partial agonist activity.

**Table III.** Ratio of receptor activation by UDPG, UDP-gal or UDP

| [Ligand] | 2211/H-20 | | | 2211/K-3 | | | H-20/K-3 | | |
|---|---|---|---|---|---|---|---|---|---|
| | UDPG | UDP-Gal | UDP | UDPG | UDP-Gal | UDP | UDPG | UDP-Gal | UDP |
| −4.0 | 3.7 | 1.5 | 0.3 | 3.7 | 3.5 | 0.2 | 1.0 | 2.4 | 0.5 |
| −4.5 | 6.8 | 1.5 | 0.4 | 7.5 | 6.0 | 0.2 | 1.1 | 4.0 | 0.6 |
| −5.0 | 13.3 | 2.4 | 0.4 | 19.6 | 10.1 | 0.2 | 1.5 | 4.2 | 0.5 |
| −5.5 | 15.7 | 3.4 | 0.6 | b.d.[b] | 18.9 | 0.3 | b.d.[b] | 5.5 | 0.5 |

[a]Receptor activation in response to the indicated concentration (expressed as the $\log_{10}$ value) of each of three ligands (UDPG, UDP-Gal and UDP) was determined *in vivo* by reporter gene assays as described in Materials and methods for each of the UDPG receptor subtypes (2211, H-20 and K-3). Presented are ratios of receptor activation for all three pairs of the three receptors for each ligand at each concentration. Values are not provided for those cases in which ligand activation of one or both of the receptors was less than 2-fold background.
[b]Below detection limit.

Focusing on the H-20/K-3 ratios (Figure 3B), we see that at concentrations of ligand above $10^{-5}$ M, UDP-Gal stimulation gives a ratio >2.4, UDPG gives a ratio between 1.0 and 1.5 and UDP gives a value <0.6. In other words, given an unknown

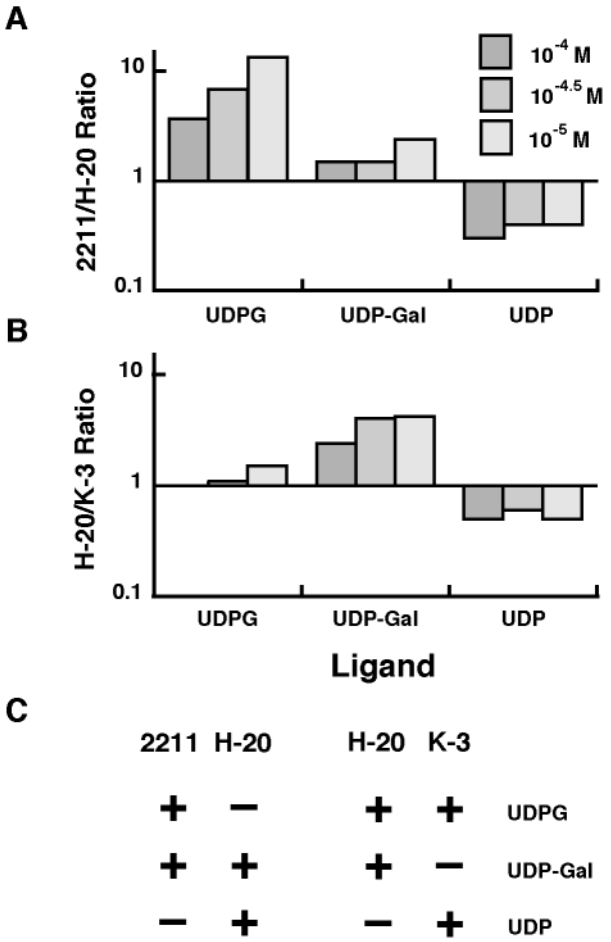


**Fig. 3.** Discrimination of chemical analytes using mutant receptors. (**A**) Ratio of 2211:H-20 receptor activation over a range of concentrations from $10^{-4}$ to $10^{-5}$ M. (**B**) Ratio of H-20:K-3 receptor activation over the same range of concentrations. (**C**) Schematic representation of the rudimentary chemical sensors in which relative activation is indicated by a '+' or '−'. Each pair of the indicated measurements can uniquely identify one of the three ligands.

solution containing UDP, UDP-Gal or UDPG, a single determination of the ratio of activity of the two receptors would uniquely identify the compound in the solution across this range of concentrations. The pattern breaks down at lower ligand concentrations, but even given a sample of unknown concentration it would be possible to carry out a simple set of controls to ascertain if the sample is in the appropriate range of concentrations. A similar result holds for the H-20/2211 receptor pair (Figure 3A), although not for the 2211/K-3 pair. Hence a single measurement from only two receptors allows precise discrimination of three different analytes. Further, discrimination is achieved over more than a 10-fold range in concentration of analyte and is independent of absolute response of either receptor. Our results with the two receptor pairs are laid out in a more conceptually intuitive manner in Figure 3C, which shows how the identity of each ligand can be expressed simply in terms of the relative response of the two receptors, while application of a third receptor introduces a redundant criterion for discrimination. Note also that the response of the mutant receptors distinguishes these three analytes from virtually all other analytes, since the three compounds each activate the mutant receptors whereas virtually all other analytes do not.

## Discussion

### *Directed evolution of GPCRs*

Using a yeast system for functional expression of GPCRs, we applied standard yeast genetic techniques to isolate receptors with altered ligand recognition properties. The sequential application of mutagenesis and selection that defines directed evolution has not previously been applied to GPCRs, in part owing to a lack of a facile genetic system in which to conduct such studies. Through a straightforward application of mutagenesis and selection, we succeeded in isolating mutants of the UDPG receptor with enhanced sensitivity and with changes in ligand specificity and efficacy. The resulting receptors have properties that are amenable to chemical sensing applications.

Our initial efforts to obtain mutant receptors with altered ligand specificity by random mutagenesis of the entire UDPG receptor gene yielded mutants with increased sensitivity in response to ligands but none with changes in ligand specificity. The $EC_{50}$s of the mutant receptors with increased sensitivity are similar to or greater than the $EC_{50}$ of the wild-type UDP-glucose receptor in yeast. This suggests that the increased sensitivity of this set of receptor mutants does not result from an enhanced affinity of the receptor for ligands but rather from either an increased concentration of functional receptor numbers in the cell or from an increased specific activity of mutant receptors, that is, an increased ability of ligand-bound receptor molecules to activate the associated G-protein. The fact that mutations yielding receptor activation are scattered across the receptor gene suggests that a number of positions in the primary structure of the protein can affect either the efficiency of its biosynthesis, through changes affecting steps in the trafficking or maturation of the receptor, or its specific activity. This large number of sites whose mutation results in activation may account for predominance of activated receptors relative to those with altered ligand preference following random mutagenesis of the entire gene.

From a protein engineering standpoint, generation of sensitized receptors is akin to generation of functionally optimized enzymes. Many functional parameters of catalytically useful enzymes have been optimized, including thermotolerance and specific activity, without the goal of altering enzymatic substrate specificity (D'Amico *et al*., 2002; Turner, 2003). Just as functionally optimized enzymes are necessary for certain applications, the sensitized UDPG receptor is in and of itself a useful tool. Screens for specificity mutants were simplified by the robust responses of the 2211 receptor to non-native ligands and we are currently exploring the use of yeast strains expressing the 2211 receptor as whole-cell 'indicator' assays for low concentrations of sugar nucleotides secreted by growing cells.

We succeeded in recovering UDPG receptor mutants with altered ligand specificity by targeting regions of the molecule hypothesized to be involved in ligand interaction on the basis of homology and structural modeling. The observed phenotypic effects of mutagenesis fit standard pharmacological models for receptor function. We have not conducted any further structural studies or modeling exercises with the mutant receptors, but mutants in the ligand binding pocket would be expected to alter either the relative affinity of the receptor for different ligands, the consequences of a ligand's binding to the receptor, or both. The transformation of UDP from a weak partial agonist to a

stronger partial agonist of the K-3 receptor exemplifies a change in the consequence of ligand binding, i.e. a change in efficacy. By pharmacological evaluation of the affinity of UDP, we conclude that all of the receptors have similar affinities for UDP. In the H-20 and K-3 receptors, UDP functions as an increasingly strong partial agonist, whereas in the case of the 2211 receptor the ligand functions primarily as an antagonist. The relative affinity of the K-3 receptor for the UDPG and UDP-Gal ligands appears to be diminished, based on the dramatically higher $EC_{50}$s of the two compounds. In contrast, the H-20 receptor appears to have reduced affinity for UDPG but similar affinity for UDP-Gal relative to that of the parent 2211 receptor. Hence, in this case, the effect of the mutation has been to diminish the interaction of one, but not another, ligand for the receptor. We note that, to date, we have not recovered a mutant receptor with increased affinity for a ligand. This may be attributable in part to the fact that UDP, one of the two compounds we utilized in screening that we did not expect to bind to the UDPG receptor, was in fact a ligand, whereas the other compound, dTDP-glucose, differs from the UDP-sugars in the base and the ribose, which are far from the chemical motifs that differ in the remaining ligands.

Here, too, parallels can be constructed between engineering ligand specificity of receptors and engineering substrate recognition by biocatalytic enzymes. Directed evolution is frequently utilized to fine-tune the chiral specificity of a biocatalytic transformation, typically with the goal of creating or enhancing a bias in substrate recognition to obtain an optically pure product (May *et al*., 2000; Reetz, 2004). However, in contrast to the common engineering goal of generating maximal stereospecificity for a biocatalyst, our goal was to change substantially the preference for one stereoisomer versus the other, regardless of the exact ratio of affinities. In fact, the H-20 mutant has 'inverted' chiral specificity *vis-à-vis* the 2211 receptor, as opposed to an enhancement or refinement of the 2211 preference for UDPG versus UDP-Gal. Receptors with a high level of discrimination, of the order of the >100:1 ratio typically sought for biocatalysts, may be unnecessary or even disadvantageous for some chemical sensing applications, as olfactory sensors have been postulated to function more robustly if the individual receptors are relatively broadly tuned (Alkasab *et al*., 2002). It is also noteworthy that in the case of enzyme engineering, mutants may be selected for substrate specificity at the expense of achieving maximal activity (May *et al*., 2000). This situation is highly analogous to our experience of redirecting ligand specificity at the expense of maximal receptor sensitivity to any one ligand. Finally, while the processes of engineering receptor and enzyme specificity may be conceptually analogous, there are important distinctions at the mechanistic level. Interactions between enzyme and substrate are typically transient and involve binding affinities of substrates, products and transition states. The chemical motifs subject to stereochemical discrimination could be in the catalytic center or far from it. In contrast, receptor ligands are not altered chemically by binding and interactions between receptors and their ligands can be more kinetically stable, while the efficacy of each ligand may vary. Hence it would be inappropriate to overstate the similarities of engineering receptor specificity versus enzymatic substrate specificity.

### *GPCRs as biosensors*

The recovery of receptors with distinct but overlapping ligand recognition properties has allowed us to explore aspects of chemoreception presumed to underlie olfaction. Applying chemical receptors as sensors in a combinatorial manner creates a powerful new tool for chemical detection. Even without engineering, GPCRs are remarkable chemical sensors and any GPCR can be utilized as a chemical sensor when expressed in cells that allow ligand binding to be coupled to an easily measured output, e.g. the many expression systems developed for GPCR drug screening. Since many GPCR ligands are drugs, the universe of chemical compounds addressable by GPCR biosensors is scientifically and economically relevant. In some cases, receptor-based assay systems have intrinsic advantages over other systems for chemical sensing such as enzyme-linked colorimetric assays. For instance, it may be difficult to link chemical ligands to colorimetric assays or it may not be feasible to purify the ligands in question from a mixture that confounds chemoenzymatic detection. In our experiments, the ligands are in fact mixed in with yeast growth media and yeast cells and therefore are presented in a complicated mixture, typical of a biological HTS scenario, that would defeat numerous alternative means of chemical detection. By a relatively straightforward engineering process, we have generated mutant receptors that dramatically extend the power of receptors as chemical sensors, simply by adjusting the relative sensitivity of the receptors to certain ligands so that the receptors can be utilized in a combinatorial manner. We have illustrated this principle by highlighting conditions in which 'pure' samples of receptor ligands can be unambiguously identified over a 10-fold range in concentrations. This example was chosen for simplicity and clarity, but the principle is a powerful one and this mode of chemical analysis can certainly be extended with additional receptors, miniaturization of assays and a 'machine learning' paradigm of data analysis.

Engineered receptors offer a promising avenue for testing the theory and principles believed to underlie olfaction. Mechanisms that govern the discriminatory capacity and robustness of olfactory receptors have been the focus of theoretical studies (Alkasab *et al*., 2002; Brody and Hopfield, 2003). An examination of the limitations of our prototypical chemical detector offers insight into issues that need to be addressed in order to construct robust chemosensory arrays in practice. When a single strain is used to measure a dose–response curve, technical replicates typically are not performed for each data point, as each curve corresponds to multiple measurements and error can be expressed as the quality of the curve fit. This 'internal control' is missing when comparing the ratios of individual data points, while noise is amplified by expressing each measurement as a ratio. In addition, measurements must be compared between two different yeast strains, one with each receptor, not a single strain as in the case of a dose–response curve. Finally, because of the geometry of the dose–response curves, the relative responses induced by a certain ligand will inevitably vary over a wide range of concentrations. Clearly, these limitations can be overcome by additional measurements and the use of standards to calibrate ligand responsiveness for each strain, but it will also be informative to attempt to engineer larger collections of sensory receptors to function in a robust manner under a range of conditions.

Hence an important goal in creating combinatorial sensors is to utilize the sensors in an array format with the potential for greater discriminatory power and robustness. With a larger panel of receptors and especially with receptors possessing broader ligand specificities, the power for discrimination should grow exponentially. Further, with an increased number of receptors the potential for broad-based chemical recognition ceases to depend on a detailed knowledge of the pharmacological profiles of individual receptors and individual ligands can be recognized simply on the basis of patterns of receptor responses to test compounds. Finally, multiple models have been advanced to describe how individual ligands can be perceived in a background of interfering signals (Hopfield, 1999). In one model, discrimination is achieved through a time course response. The pattern of receptor activation over time effectively highlights the unique ligand in the mixture, since the coherence of the ligand-responsive pattern of receptor activation raises a signature pattern above the background of noise generated by the complex chemical setting. Given the availability of cellular expression systems coupled to rapid fluorescent readouts such as calcium imaging, it could be possible to generate dynamic information on the behavior of engineered receptor arrays. Generating a collection of well-defined chemosensitive receptors will provide tools to help resolve whether such models can work and whether they can inform the design of more complex biosensors.

Biological molecules have a history of use as sensitive, effective biosensors. Enzyme assays coupled to colorimetric outputs are standard tools for chemical detection, while monoclonal antibodies are commonly used for the detection of biomolecules. Biological chemical receptors as a class may be underutilized as chemical sensors, due in part to the perception that cell-based assay systems are valuable primarily as drug screening technologies rather than as chemical sensing technologies. Combinatorial application of engineered GPCRs clearly offers potential for development of quick, inexpensive screens for stereo- or enantioselective biocatalytic transformations or for trace amounts of bioactive agents. Such tools would be an invaluable resource for the scientific community and further development of engineered GPCRs will inevitably contribute to better understanding of this class of molecules.

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