Progress toward heterologous expression of active G-protein-coupled receptors in *Saccharomyces cerevisiae*: Linking cellular stress response with translocation and trafficking

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Abstract: High-level expression of mammalian G-protein-coupled receptors (GPCRs) is a necessary step toward biophysical characterization and high-resolution structure determination. Even though many heterologous expression systems have been used to express mammalian GPCRs at high levels, many receptors are improperly trafficked or are inactive in these systems. En route to engineering a robust microbial host for GPCR expression, we have investigated the expression of 12 GPCRs in the yeast *Saccharomyces cerevisiae*, where all receptors are expressed at the mg/L scale. However, only the human adenosine A_{2a} (hA_{2a}R) receptor is active for ligand-binding and located primarily at the plasma membrane, whereas other tested GPCRs are mainly retained within the cell. Selective receptors associate with BiP, an ER-resident chaperone, and activated the unfolded protein response (UPR) pathway, which suggests that a pool of receptors may be folded incorrectly. Leader sequence cleavage of the expressed receptors was complete for the hA_{2a}R, as expected, and partially cleaved for hA_{2b}R, hCCR5R, and hD_{2L}R. Ligand-binding assays conducted on the adenosine family (hA₁R, hA_{2a}R, hA_{2b}R, and hA₃R) of receptors show that hA_{2a}R and hA_{2b}R, the only adenosine receptors that demonstrate leader sequence processing, display activity. Taken together, these studies point to translocation as a critical limiting step in the production of active mammalian GPCRs in *S. cerevisiae*.

Keywords: membrane protein; heterologous expression; NECA; G-protein-coupled receptor

Introduction

The G-protein-coupled receptors (GPCRs) are seven transmembrane-spanning proteins whose roles in

physiological functions are diverse and widespread.¹ GPCRs participate in cell communication and signaling in a variety of organisms, and thus are implicated in many diseases. Rational drug design for GPCRs has proven challenging because of the absence of detailed structural information available for the majority of these proteins. Notable advances in crystallization strategies for these membrane proteins have led to the determination of three high-resolution GPCR structures, the β_1 ,² and β_2 adrenergic³ and adenosine A_{2a} receptors,⁴ which have been solved recently. Together with the structure of rhodopsin, which was crystallized 9 years ago,⁵ these four structures now serve as

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homology models for the nearly 1000-member GPCR superfamily. However, all of these structures display distinct differences from each other,^{2–4} such as differences in helix packing and substrate-binding pockets, and from predicted structures based on rhodopsin,⁵ further reinforcing the need to obtain high-resolution structural information for other GPCRs on an individual basis.^{6,7}

As almost all human GPCRs do not exist naturally in high abundance, heterologous expression systems are required to achieve sufficient protein yields for structural characterization, generally at the mg/L scale or greater. Bacterial, yeast, and insect hosts have successfully been implemented for high-level expression of soluble proteins, and similar approaches have been applied toward the expression of membrane proteins.^{7–10} Although all of these systems have proven useful for expression of some heterologous membrane proteins, each host has advantages and disadvantages associated with its use. For instance, microbial hosts such as *E. coli* and yeast offer well-understood genetics, low cost of culture, and relatively easy scale up. Insect and mammalian cells can perform many more complicated protein processing and post-translational modifications that may be necessary for proper function, but prove costly and time-intensive. However, even with several host systems available, heterologous expression has not yet systematically allowed for high-level expression of any given GPCR of interest, and typically relies on trial-and-error methods.^{7–10}

Even though all GPCRs share a commonality in their seven transmembrane domain segments and in their ability to couple to trimeric G-proteins, they also display great diversity in their overall function, ligand preference, tissue location, and physiological prevalence.¹¹ Furthermore, significant differences exist in how proteins are expressed and processed in various heterologous systems, which may have a direct impact upon folding and activity of heterologously expressed GPCRs.⁹

Given its ease of genetic manipulation, rapid growth, and eukaryotic secretory pathway, yeast are an attractive host system for the development of a robust GPCR expression system. Yeast have been successfully used for the heterologous expression of membrane proteins, specifically GPCRs.^{12,13} Recent expression of the human adenosine A_{2a} receptor (hA_{2a}AR) in *S. cerevisiae* has yielded active protein at greater than 10 mg/L of culture, which has facilitated its purification¹⁴ and biophysical characterization (O'Malley, Naranjo, Lazarova, and Robinson, manuscript in preparation). However, under identical culture and expression conditions, the human neurokinin NK₁ (hNK₁) receptor fails to traffic to the plasma membrane in this system and does not display ligand-binding activity.¹⁵ Instead, these receptors are trapped in punctuate structures that are present just below the plasma membrane.¹⁵ Other studies have also cited improper trafficking of recombinant membrane proteins in

yeast,^{16,17} which suggests that differences between the native mammalian secretory pathway and the yeast secretory pathway can adversely affect the production of proteins in this non-native system. At this point, little information has been published regarding the factors that govern folding and cellular trafficking of heterologously expressed GPCRs in yeast. By understanding the bottlenecks in plasma membrane targeting, we can perhaps employ cellular engineering approaches to enable high-level expression of active GPCRs, to create a more robust and desirable host.

In this work, we expressed 12 GPCRs from the rhodopsin family of receptors in the yeast *Saccharomyces cerevisiae*, to determine limitations associated with GPCR expression in this system. The GPCRs chosen for this screen possess different predicted post-translational modifications, couple to several different trimeric G-proteins, and encompass a range of primary sequence homology to the actively expressed human adenosine A_{2a} receptor. Our results indicate that problems with GPCR folding and trafficking begin at the point of translocation within the ER membrane, which leads to the activation of downstream cellular stress responses.

Results

GPCR expression levels and membrane integration

The presence of a C-terminal GFP tag on expressed GPCRs made possible the direct comparison of expression levels among different strains. This technique has been successfully applied toward the estimation of A_{2a}AR expression levels in *S. cerevisiae*¹⁸ and was further corroborated by other methods.¹⁸ Whole-cell fluorescence measurements indicate most GPCRs were readily expressed at high levels within yeast, based on total GFP fluorescence (Table I). We did not observe any degradation fragments via SDS-PAGE analysis followed by in-gel fluorescence for early time-points (1–5 hrs). For 24-hour time points, the degradation was between 0–15%, depending on the receptor identity. However, within the error of both measurements, we believe total fluorescence gives a reasonable value on total protein yields.

Overall expression yields were somewhat variable among the strains—this results from a combination of potential differences in integration number for the pITy vector, as well as differences in final cell culture densities. However, all GPCRs tested had predicted yields greater than 1 mg/L of culture with the exception of hFSHR-GFP-His₁₀, at ~0.7 mg/L. To verify that these membrane proteins were embedded into the lipid bilayer, an integral membrane protein assay¹⁹ was applied for GPCRs expressed in this system. Membrane fractions were treated with 0.1M NaCO₃ (pH 11.0) and 2M urea, to dissociate peripherally associated proteins from membranes, or 1% SDS, which is capable of solubilizing integral membrane proteins

Table I. Summary of Data Obtained for Heterologous Expression of GFP-Tagged GPCRs in *S. cerevisiae* Including Relative Expression Yields, Cellular Localization, Leader Sequence Processing, and Glycosylation

GPCR	Expression Level (thousands of molecules per cell)	Estimated expression yield ^a (milligrams per liter of culture)	Primary localization	Extent of pre-pro cleavage	Glycosylated (N-linked)
hA ₁ R	160 ± 1.1	2.9 ± 0.2	Intracellular	Intact	No
hA _{2a} R	275 ± 6.8	9.5 ± 0.2	Plasma membrane	Cleaved ^b	No
hA _{2b} R	260 ± 8.2	4.0 ± 0.1	Intracellular	Intact and cleaved ^b	No
hA ₃ R	190 ± 16.4	1.4 ± 0.1	Intracellular	Intact ^b	No
hD _{2L} R	98 ± 2.7	1.6 ± 0.1	Intracellular	Intact and cleaved	No
hCXCR4R	260 ± 5.5	6.0 ± 0.1	Intracellular	Intact	Yes
hCCR5R	370 ± 44	8.4 ± 1.0	Intracellular	Intact and cleaved	No
hCB ₁ R	115 ± 15	3.8 ± 0.5	Intracellular	Intact	No
hCB ₂ R	465 ± 47	10.0 ± 1.0	Intracellular	Intact ^b	No
hNK ₁ R	23 ± 18	2.1 ± 0.2	Intracellular	Intact ^b	No
hNK ₂ R	355 ± 17	5.5 ± 0.3	Intracellular	Intact	No
hFSHR	55 ± 5.4	0.7 ± 0.1	Intracellular	ND	ND

ND, not determined.

^a Determined by extrapolating fluorescence levels and OD_{600s} from 5 mL cultures, verified from direct scale up to 200 mL cultures.

^b Confirmed by N-terminal sequencing. Note that for hNK₁R, data are for N-terminal sequencing alone, as problems that arose in sample preparation precluded analysis via gel mobility assay.

from a membrane environment. If GPCRs expressed in this system exist as integral membrane proteins, only treatment of membrane preparations with SDS would liberate the protein from the membrane. In applying this assay toward the 12 GPCRs expressed in this system, we find that only treatment with SDS releases these proteins from the membrane environment, as shown for hA_{2a}R-GFP-His₁₀ and hA₁R-GFP-His₁₀ (Fig. 1). Thus, each mammalian GPCR expressed in this system was found within a membrane environment.

GPCR trafficking in *S. cerevisiae*

With the exception of the human A_{2a} receptor [Fig. 2(A–C)], the GPCRs tested in this study appear predominantly within the cell. These receptors are mainly retained internally as determined through confocal microscopy of GFP-tagged receptors [Fig. 2(D,E,G,H,J,K)]. It has been previously observed that heterologous expression of NK₁, D_{2s}, and neuromedin U GPCRs in yeast systems led to protein retention within subcellular compartments.^{15–17}

Early time points after induction of expression (~5 h postinduction) were examined to investigate

whether the localization in cell populations was different early in expression. As expected, at 5-h postinduction hA_{2a}R-GFP appears to uniformly label the plasma membrane [Fig. 2(A)]. Heterologous expression of other GPCRs in *S. cerevisiae* led to two typical appearances, which are apparent in the images of hA₁R, hA_{2b}R, and hA₃R [Fig. 2(D–L)]. In many individual cells, receptors accumulate at the cell periphery. Additionally, larger concentrations of the GPCR-GFP fusions accumulated more centrally within the cell. Each of the 11 GPCR-GFP fusions showed a combination of these patterns (summarized in Table I; confocal data not shown for hD_{2L}R, hCB₁R, hCB₂R, hFSHR, hNK₁R, hNK₂R, hCCR5R, and hCXCR4R). However, from confocal imaging alone, we cannot rule out the possibility that a fraction of expressed receptors reached the plasma membrane.

Each GPCR construct contains an N-terminal pre-pro leader sequence to facilitate protein targeting to the ER. The presence of particular hydrophobic leader sequences strongly favors cotranslational insertion of membrane helices.²⁰ However, to ensure that this leader sequence did not negatively impact cellular

Figure 1. A standard integral membrane protein assay shows that recombinantly expressed GPCRs reside within a membrane environment. Yeast membranes were prepared from cells expressing GFP-tagged GPCRs, which were divided and exposed to treatment with 0.1M Na₂CO₃ (pH 11.0), 2M urea, and 1% SDS. Resulting supernatant fractions were collected after each treatment, separated on SDS-PAGE, and imaged via in-gel fluorescence of the GFP tag. The first lane denotes resuspended membrane pellets. Representative data from this assay are shown for hA_{2a}R-GFP-His₁₀ (left) and hA₁R-GFP-His₁₀ (right).

Figure 2. Representative confocal images of GFP-tagged human adenosine receptors in *S. cerevisiae*. Column headings denote images captured for expression 5 and 24 h postinduction with galactose for receptors bearing an N-terminal pre-pro leader sequence. “– pre-pro” denotes cells expressing receptors lacking the N-terminal leader sequence, expressed for ~24 h. Expression of the adenosine receptors under each of these conditions is represented by the corresponding images in each row, with hA_{2a}R-GFP-His₁₀ (A–C), hA_{2b}R-GFP-His₁₀ (D–F), hA₁R-GFP-His₁₀ (G–I), and hA₃R-GFP-His₁₀ (J–L). Confocal images, captured on Zeiss 5DUO with 63×/NA 1.4 objective; scale bar represents 5 μm.

trafficking of mammalian GPCRs in *S. cerevisiae*, cells expressing GPCR-GFP constructs lacking this pre-pro leader were examined. In many cases, the absence of the pre-pro sequence resulted in decreased expression yields (determined by whole-cell GFP). The hA_{2a}R and hA₁R [Fig. 2(C,I)]. The hA_{2a} receptor appeared plasma membrane localized, and most other receptors displayed a similar intracellular localization to that observed when the leader sequence was present [Fig. 2(C,I,L)]. However, in the expression of the hA_{2b}R-GFP-His₁₀ lacking the pre-pro leader sequence [Fig. 2(F)], more fluorescence is observed at the cell periphery when compared with those expressed with the leader sequence [Fig. 2(D,E)].

Cellular stress responses are activated during heterologous receptor expression

We investigated the UPR activation within the endoplasmic reticulum (ER) of *S. cerevisiae* during heterologous

GPCR expression, as this organelle is implicated in protein retention within the secretory pathway. The UPR pathway is activated when the folding capacity of the ER is exceeded by the presence of unfolded or misfolded protein within the organelle.^{21–23} UPR activation promotes expression of numerous ER-resident chaperones via transcriptional upregulation, to reduce aggregation and promote secretion.²⁴ Recently, it has been reported that overexpression of the P2 transporter in *S. cerevisiae*, a membrane protein, can trigger the UPR.²⁵

In these studies, we used a UPRE-GFP reporter plasmid to monitor the activation of the UPR.^{26,27} Activation of the UPR in cells expressing recombinant non-GFP-tagged GPCRs was monitored over time by measuring whole-cell fluorescence. We find that expression of most GPCRs activated the UPR pathway after 24 h of expression in *S. cerevisiae* [Fig. 3(A, solid bars)], relative to a BJ5464 parental control. UPR activation monitored via GFP fluorescence of cells

Figure 3. Activation of cellular stress response pathways in *S. cerevisiae* is evident during heterologous expression of GPCRs. (A) BJ5464 cells expressing GPCRs (indicated) were assayed for stress response activation. Relative fluorescence levels from the UPR reporter²⁷ (open bars) and beta galactosidase activity from a heat shock response (HSR) reporter²⁸ (closed bars) in these strains as a function of GPCR, 24 h after induction by growth on galactose. (B) GFP fluorescence in cells expressing an adenosine receptor and the unfolded protein response reporter plasmid (UPRE-GFP) was measured as a function of expression time. Error bars signify standard deviation from the average of three biological replicates.

producing hA₁R, hA_{2b}R, and hA₃R was observed to increase over time [Fig. 3(B)]. In contrast, GFP fluorescence did not increase over the same time course during hA_{2a}R expression, similar to background GFP levels observed for the parental BJ5464 strain [Fig. 3(B)]. The fluorescence signal due to UPR activation in strains expressing hA₁R, hA_{2b}R, and hA₃R leveled off ~15 h after expression was induced and did not decrease up to 24 h past expression induction, likely because of low GFP turnover in the cell. Similar

increases over time in UPR activation (monitored via GFP fluorescence) were observed in the other eight GPCR-expressing strains (data not shown).

To ensure that the absence of UPR activation in cells expressing hA_{2a}R was not due to plasmid loss, these cells were incubated with 4 mM DTT, which produced a significant fluorescence response (220 au). Therefore, the absence of this cellular stress response during heterologous expression of hA_{2a}R was not due to failure of the reporter system in the hA_{2a}R strain.

Figure 4. Nickel affinity precipitation of yeast-expressed GPCRs via the C-terminal decahistidine tag shows higher levels of BiP associated with GPCRs that accumulate intracellularly. (A) GPCRs were expressed for ~24 h before lysis and pull-down with Ni²⁺-conjugated resin. An anti-BiP primary antibody was used to detect associated BiP in all samples, and the ratio of BiP from association with each GPCR was compared to the wild-type strain (BJ) under each band. (B) BiP levels in yeast expressing hA_{2a}R were similar to those observed for yeast cells not expressing GPCRs (BJ lane), but were elevated in strains expressing other receptors. The ratio of total BiP content in each strain compared to the wild-type (BJ) is denoted under each band.

The heat shock response (HSR) within *S. cerevisiae* serves to activate cellular pathways in response to cellular cytosolic stress. In addition to elevated cell culture temperature (~37–42°C), the HSR pathway may also be triggered by other stress-inducing conditions, such as accumulation of misfolded protein within the cytosolic compartment.²⁹ We investigated whether this cytosolic stress pathway was activated during heterologous expression of mammalian GPCRs in *S. cerevisiae*, as this would indicate that systemic cellular stress occurs. The heat shock reporter element exists as a translational fusion to *lacZ*, which links activation of the HSR pathway to expression of β -galactosidase.²⁸

In contrast to our observations with UPR activation, we find that only 9 of the 12 receptors (hA₁, hA₃, hCCR5, hCB₂, hD_{2L}, hFSH, hCXCR4, hNK₁, and hNK₂) expressed in this system trigger a HSR [>0.8 U β -galactosidase; Fig. 3(A, open bars)]. As expected from the lack of UPR induction, expression of hA_{2a}R does not result in a HSR (<0.20 U β -galactosidase) compared to parental cells. Interestingly, expression of either hA_{2b} or hCB₁ receptors also does not produce a HSR from this reporter system (<0.20 U β -galactosidase). To verify that reporter plasmid loss in yeast strains expressing the hA_{2a}, hA_{2b}, and hCB₁ receptors was not responsible for this negative result, these strains were heat shocked at 42°C for 1 h, which produced a significant rise in β -galactosidase activity (1.11 U β -galactosidase for hA_{2a}R, 0.65 U β -galactosidase for hA_{2b}R, and 2.25 U β -galactosidase for hCB₁R), indicating that the heat shock reporter was present and functional within these cell strains.

Most receptors maintain an association with BiP

As the ER-resident protein BiP is implicated in the UPR, affinity pull-down experiments were conducted as a complementary method to the UPR studies. As all cells exhibited a significant UPR during the expression

of GPCRs except for hA_{2a}R, we sought to identify whether the ER-resident chaperone BiP was associated specifically with the receptors.

When affinity pull-down experiments were performed, generally high levels of BiP were found to be associated with all receptors, except for the hA_{2a} receptor [Fig. 4(A)]. As BiP is a molecular chaperone that is upregulated during ER stress, it may have been possible that the BiP binding observed here was partially influenced by the rise in cellular BiP levels within strains in which the UPR pathway was activated. To address this issue, yeast cells expressing each of the adenosine receptors were lysed, and their corresponding BiP levels were measured and compared to untransformed, parental yeast cells. BiP protein measured in the BJ5464 parental strain was similar to that in cells expressing hA_{2a}R [Fig. 4(B)], although BiP levels were generally more elevated in cells expressing other GPCRs [Fig. 4(B)]. Although activation of the UPR in these strains does increase the amount of available BiP relative to wild-type or A_{2a}R-bearing strains [up to twice the amount contained in the wild-type strain, Fig. 4(B)], when these differences are taken into account, they do not account for the differences in BiP association between A_{2a}R and most other GPCRs that were observed in pull-down samples. In the association experiment (Fig. 4), BiP levels for these receptors were 3–16 times greater than the parental strain [Fig. 4(A)], which is in semiquantitative agreement with independent biological replicates. As a consequence, it can be inferred that BiP maintains a specific association with most receptors that is not a byproduct of UPR upregulation.

Leader sequence processing suggests a problem with translocation

All constructs used for GPCR-GFP expression in these studies include an N-terminal pre-pro leader sequence upstream of the GPCR gene to facilitate targeting to

Figure 5. Synthetic N-terminal pre-pro leader sequence fused to mammalian GPCRs (shown for hA_{2a}R, hA_{2b}R, and hA₃R constructs) contains two processing sites (A), which gives rise to two detectable forms of protein (B). The extent of leader sequence cleavage was determined through a mobility shift experiment on GFP-tagged receptors, in which receptors were expressed for the indicated amount of time, separated via SDS-PAGE, and bands were detected by in-gel GFP fluorescence. Results from cells expressing pre-pro-GPCR-GFP constructs (2, 5, and 24 h) were compared to GFP-tagged receptors subcloned without the leader sequence. Pre-Kex2 GPCR is observable during the expression of hA₁-GFP, hA_{2b}-GFP, and hA₃-GFP receptors, as detected from decreased mobility of receptors on SDS-PAGE relative to receptors subcloned without the leader sequence (C).

the ER. This leader sequence is cleaved from mature protein in two steps, which corresponds to processing and trafficking of the expressed membrane protein along the secretory pathway. As illustrated in Figure 5(A), the prepeptide leader sequence is cleaved in the ER by signal peptidase, following signal sequence exit from the translocon, whereas the remaining propeptide is cleaved by the Kex2 protease after the membrane protein enters the trans-Golgi.³⁰

To determine the extent of leader sequence processing throughout the course of expression, GFP-tagged receptors were expressed and analyzed at 2, 5, and 24 h postinduction via in-gel fluorescence of samples separated via SDS-PAGE, and the migration of these proteins was compared to those of the corresponding GFP-tagged “mature” receptor (subcloned and expressed without the pre-pro sequence) [Fig. 5(B)]. Earlier time points were included to complement data from UPR experiments, which generally showed an upregulation of the UPR 5–6 h postinduction for all cells expressing GPCRs, except hA_{2a}R.

As shown in Figure 5(C), samples from cells expressing the hA_{2a}-GFP-His₁₀ receptors with the N-terminal pre-pro leader sequence migrated to the same distance as mature hA_{2a}R at all time points tested, indicating that the pre-pro sequence was uniformly cleaved from this receptor throughout the course of its expression. However, samples from cells expressing

hA₁-GFP-His₁₀ and hA₃-GFP-His₁₀ receptors with the pre-pro sequence migrated slower relative to their mature forms at each time point, which is consistent with the mobility shift expected from incomplete processing of the leader sequence. In the case of the hA_{2b}-GFP-His₁₀ receptor, although a band with decreased mobility relative to the mature protein was observed, a fraction of receptors migrates to the same distance as mature hA_{2b}, suggesting that both mature and pre-Kex2 forms of the hA_{2b}R were present.

As a control in these experiments, samples of cells expressing receptors with and without the pre-pro leader were digested with the deglycosylating agent PNGaseF to determine if any expressed receptors were modified by N-linked glycosylation. Digestion of the adenosine receptors with PNGaseF did not alter the mobility of these proteins, which confirms that glycosylation of these receptors was not responsible for the observed shift in mobility from the mature form. Table I summarizes the results for each GPCR from the deglycosylation studies and the mobility shift assay. Notably, we find that the N-terminal pre-pro leader sequence was cleaved from a population of hCCR5-GFP-His₁₀ and hD_{2L}-GFP-His₁₀ receptors, similar to behavior observed for the hA_{2b}-GFP-His₁₀ receptor, although in all of these cases there is a significant fraction of receptors that migrated slower than the mature form.

To confirm the results of the mobility shift assay, some fluorescently tagged GPCRs were expressed for 24 h, purified via nickel affinity chromatography, and N-terminally sequenced. The entire pre-pro leader sequence was found to be properly cleaved from the hA_{2a} receptor, revealing the amino acid sequence of mature hA_{2a}R (E-A-R-P-D-V-M-P-I).¹⁴ N-terminal sequencing also identified a population of processed hA_{2b}R (E-A-R-P-D-V-M-L). In contrast, hCB₂, hNK₁, and hA₃ receptors were shown to have intact N-terminal pre-pro sequences (M-K-V-L-I-V-L-L-A-I), indicating that signal peptidase did not cleave the pre sequence following its exit from the translocon, consistent with the results from the mobility shift experiments.

Activity of adenosine receptors expressed in *S. cerevisiae*

Given that some GPCRs appeared to be retained intracellularly, we wanted to test for ligand-binding activity. We focused our analysis primarily on the family of adenosine receptors, as members of this family include the full range of trafficking patterns, stress response upregulation, and leader sequence processing observed in these studies. To determine whether adenosine receptors were folded properly, radioligand-binding experiments were performed on membrane preparations. Previously, we have shown that hA_{2a}R is folded in an active, ligand-binding conformation in whole-cell assays, in membrane preparations, and once purified from the cells.^{14,15,18,31} Using ³H-NECA, an adenosine analog and receptor agonist for hA_{2a}R, hA_{2b}R, and hA₁R,³² enabled ready comparison in these ligand-binding studies.

Figure 6(A) shows that addition of ³H-NECA to yeast membranes expressing GFP-tagged hA_{2a}R resulted in saturation binding with a K_d of 36 ± 2 nM. Based on 0.2 mg of membrane protein used in the assay and the total bound complexes obtained (B_{max}), the strain produces 855 pmol active A_{2a}R/mg membrane protein. The K_d compares well to expected values for this radioligand, because the estimated K_d for NECA with hA_{2a}R is within the range of 1–20 nM.³² Specific binding of ³H-NECA was also observed for yeast membranes containing recombinant, GFP-tagged hA_{2b}R [Fig. 6(B)]. However, saturation was not achieved in this case because of technical limitations of this experiment; the expected K_d for NECA binding to hA_{2b}R is $\sim 5 \mu M$,³² which is near the highest working concentration of radioligand available. For A_{2b}R, the observed bound complexes correspond to ~ 11 pmol/mg membrane protein, although we have not reached saturation, so this is likely an underestimate of the true yield. In contrast to the results observed for hA_{2a}R and hA_{2b}R, no appreciable binding was seen for yeast membranes expressing recombinant hA₁ or hA₃ GFP-tagged receptors in the presence of 400 nM ³H-NECA (data not shown) (K_d for hA₁R is

Figure 6. Saturation radioligand binding of ³H-NECA to membrane preparations from cells expressing recombinant hA_{2a}R-GFP (A) and hA_{2b}R-GFP (B). Points represent data from the average of three replicates, with error bars the standard deviation from the average. The fit to a single-site ligand-binding model (line) results in a K_d value for the hA_{2a}R-GFP of 36 ± 2 nM.

3–30 nM³²), and CFP-tagged hA₃ receptors also did not demonstrate appreciable activity when incubated in excess of 4 nM ¹²⁵I-AB-MECA (K_d for hA₃R with AB-MECA is 1.7 nM) (data not shown). To validate that the conditions of the radioligand-binding assay for both hA₁ and hA₃ receptors were adequate to detect binding in yeast membranes, recombinant mammalian membranes containing these receptors were analyzed (refer to Materials and Methods) and specific activity was observed for these preparations (data not shown). This indicates that the lack of activity observed for both hA₁ and hA₃ receptors expressed in yeast was not due to inadequacies of the ligand-binding assay. If there were active A₁R and A₃R

present in yeast cells, we would have expected to detect a minimum of 10% of the active complex ($B_{\max}$) values measured from mammalian controls. Using this as the limit of detection suggests that active A_1R levels were less than 5 pmol/mg (based on NECA), and for A_3R , less than 0.2 pmol/mg (IB-MECA).

Discussion

In these studies, we examined cellular expression of 12 GPCRs in *S. cerevisiae* to gain a better understanding of limitations to plasma membrane localization, with the goal of achieving high functional yields for these proteins. We focused on determining why receptors trafficked differently, and if intracellular retention could be linked to protein processing and cellular stress responses. Of the 12 mammalian GPCRs expressed in this host, only the human adenosine A_2a receptor escaped quality control responses and was localized primarily at the plasma membrane. Eleven other receptors, three of which are closely related to hA_2aR , did not appreciably reach the membrane and triggered the UPR. These receptors also specifically associated with the ER-resident molecular chaperone BiP.

Under these conditions, *S. cerevisiae* was able to produce the desired milligrams of GPCRs/L of culture. To our knowledge, only the human dopamine D_2 ,^{16,34} human adenosine A_{2a} ,^{14,15,18,35} hA_2bR ,³³ and human neurokinin NK_1 ¹⁵ receptors of these 12 have been previously expressed in *S. cerevisiae*. Although expression yields for the hNK_1 receptor were not reported, expression levels for hA_2aR reached 10–15 mg/L of culture,^{14,18} whereas hD_2R expression was 2.8 pmol/mg,³⁴ which corresponds to ~0.1 mg/L of culture, using the conversion given by Sarraemagna *et al.*⁹

Many of the other receptors investigated in this study have been expressed in different host systems previously. For example, a maltose-binding protein fusion to hCB_1R produced in *E. coli* yielded purified protein at ~2.0 mg/L,³⁶ while similar approaches have been used to produce hCB_2R at yields up to 39.5 pmol/mg,³⁷ which has also led to its purification in milligram quantities.³⁸ *Pichia pastoris* produced 3.6 pmol/mg (~0.15 mg/L) of hCB_2R ,³⁹ 14.0 pmol/mg (~0.56 mg/L) of hD_2R in *Schizosaccharomyces pombe*,³⁴ and 80 pmol/mg (~0.62 mg/L) of the hNK_1R in *Sf*₉ insect cells.⁴⁰

To our knowledge, we are the first to report high levels of heterologous expression of the hA_1 , hA_2b , hA_3 , $hCCR5$, $hCXCR4$, and $hFSH$ receptors. Collectively, the expression yields achieved for each GPCR investigated in this study are among the highest levels yet reported among available host systems, and most represent major improvements in yields compared to those achieved in other studies. Although other studies have emphasized the utility of screening fluorescence GFP fusions as a means to achieve membrane protein overproduction in yeast,⁴¹ in our system we do not

observe a correlation between fluorescence of GFP-fusion protein and ligand-binding activity. For instance, although GFP is folded and optically active when fused to hA_1R and hA_3R (Fig. 2), we were unable to detect activity for these receptors. However, we do not observe aggregated material, for example, through pelleting of material at 16,000*g*. Therefore, we stress that yields estimated from GFP fluorescence represent total protein and are not necessarily active (ligand-binding competent) yields.

Although the protein yields attained here were generally excellent, we found that many GPCRs did not reach the cell periphery. Of the GPCRs that were examined here in *S. cerevisiae*, only the hA_2a receptor localized primarily to the plasma membrane. In previous studies, for yeast cells expressing both hA_2bR and a chimeric $G_{\alpha s}$, cell cycle arrest upon ligand addition was observed, which suggested the presence of active receptors at the plasma membrane.³³ This is consistent with our observed ligand-binding activity for membrane preparations [Fig. 6(B)], although we do not yet know whether any of the receptors couple to the endogenous yeast G_{α} subunit, which could alter measurable K_d values that describe the association of ligands with these receptors.

We observed that most receptors that were retained intracellularly activate stress responses (UPR and HSR), which links trafficking to cellular events typically associated with quality control. Although high levels of hA_2aR are produced in this system, heterologous expression of this particular GPCR escapes both quality control checkpoints under these culture conditions. In light of this observation, we hypothesize that the trafficking pattern observed for receptors other than A_2aR is the result of ER retention and quality control pathways, which are closely linked to activation of the UPR. For most expression constructs studied, a significant UPR response is detectable 4–6 h post induction. It is somewhat difficult to ascertain if a significant lag phase exists between expression induction and UPR activation, because detection of the UPR in our system is also coupled to *hac1* splicing and to the expression and folding of GFP, which have inherent time delays.

Although UPR activation in this system corresponds with a high level of receptors retained intracellularly, it is less clear why differential activation of the HSR pathway occurs among the GPCRs studied. Upregulation of the HSR for most receptors could be caused by misfolding of the intracellular loop regions of these membrane proteins (exposed to the cytoplasm). For example, expression of the hA_2bR and hCB_1R may not trigger the HSR pathway if intracellular loop regions of these proteins are folded in such a way that they do not result in Hsp binding within the cytosol. Alternatively, prolonged UPR could result in systemic cellular stress, activating the HSR as a secondary response.

Table II. Summary of Characteristics Among GPCRs Used in This Study

GPCR	Length of N-terminus ^a	Amino acid position for predicted N-linked glycosylation ^a	Rare codons ^b	G _α coupling ^c	% Sequence homology to A _{2a} R ^d
hA ₁ R	10	1 (159)	7	i/o	51
hA _{2a} R	7	1 (154)	8	S	100
hA _{2b} R	8	2 (153, 163)	4	S	59
hA ₃ R	14	3(3, 4, 12)	5	i/o	41
hCCR5R	30	0	3	i/o	21
hCXCR4R	39	2 (11, 176)	2	i/o	20
hCB ₁ R	116	3 (77, 83, 112)	4	i/o	28
hCB ₂ R	33	1 (11)	4	i/o	27
hD _{2L} R	37	3 (5, 17, 23)	8	i/o	26
hFSHR	349	4 (191, 199, 293, 318)	5	i/o, s	22
hNK ₁ R	31	2 (14, 18)	3	12, 13, 11, q, s	26
hNK ₂ R	32	2 (11, 19)	5	11, q, s	27

^a Information on glycosylation and TM prediction comes from srs@EBI online resource.

^b Rare codons were calculated using http://www.bioline.com/calculator/01_11.html, where a frequency of 2.5 in 1000 codons was considered rare.

^c www.gpcr.org/7tm

^d Calculated through BLASTp using a BLOSUM62 matrix (open gap: 11; extension gap: 1 penalty; gap x_dropoff 15; expect 10.00; word size 3).

Previously, it has been shown that activation of the UPR pathway in the expression of both soluble⁴² and membrane-bound proteins²⁵ in *S. cerevisiae* results when unfolded or partially misfolded proteins are present within the ER. We explored this possibility indirectly for the GPCRs expressed in our system by comparing their level of association with the ER-resident chaperone, BiP, which is known to bind to exposed hydrophobic regions of misfolded proteins. As expected, hA_{2a}R maintained a negligible association with BiP, which is consistent with its proper folding and trafficking out of the ER. As we have observed that BiP preferentially associates with most other receptors studied (Fig. 4), we can infer that at least some population of these highly hydrophobic receptors are folded in a non-native conformation within the ER.

As several of our initial observations pointed to problems within the ER, we chose to investigate the processing of the N-terminal pre-pro leader sequence fused to GPCRs. We found that the leader sequence was not uniformly cleaved, which is unexpected considering that this leader sequence should aid in membrane protein translocation. A recent study regarding hCB₂R has also suggested that signal sequence processing may prove critical to GPCR production in the methylotrophic yeast *P. pastoris*.⁴³ Our analysis of leader sequence processing among receptors expressed in *S. cerevisiae* (Table I) shows that all receptors other than the hA_{2a}R display either a homogeneous population of unprocessed (immature) receptors or a heterogeneous population of both processed (mature) and unprocessed (immature) proteins. It is unlikely that slow translocon gating results in cytosolic synthesis, as we find that these proteins are inserted into a membrane environment. In addition, activation of the UPR and BiP binding for these receptors verifies that

expressed receptors at least partially enter the ER membrane. As the leader sequence remains fused to expressed GPCRs in this system, signal peptidase must not cleave efficiently, which suggests that it does not have access to the pre sequence on the luminal side of the ER membrane. The lack of leader sequence processing could be attributed to receptor misfolding and/or the adoption of an unexpected topology within the ER membrane shortly after receptor synthesis, which precludes leader sequence cleavage.

Radioligand-binding assays conducted on membrane preparations for the adenosine receptor family link leader sequence processing with ligand-binding activity of expressed GPCRs in this system. We have found that only the hA_{2a}R and hA_{2b}R bind to the adenosine analog NECA (Fig. 6), whereas closely related receptors hA₁R and hA₃R that do not demonstrate leader sequence processing also do not display appreciable activity. In light of this observation, we believe that the hD_{2L}R and hCCR5R also are likely to show some amount of activity, because a fraction of these receptors are properly processed. Although hCCR5R has not yet been heterologously expressed in yeast, Michel and coworkers reported activity of hD₂R in *S. cerevisiae*,¹⁶ which supports this hypothesis.

Although GPCRs are characterized by the presence of a conserved seven alpha-helical transmembrane domain, various motifs, coupling partners, and post-translational modifications differentiate these receptors (Table II). We sought to correlate differences between these features with subcellular expression patterns and cellular responses that occur in response to heterologous GPCR expression within *S. cerevisiae*. However, there is no obvious correlation between these properties (N-terminal length, putative N-linked glycosylation sites, G-protein coupling, homology to hA_{2a}R, or presence of rare codons) and leader

sequence cleavage, although each may serve to contribute to receptor-specific effects.

Taken together, these data support the conclusion that although high yields are readily achieved, problems with mammalian GPCR trafficking in this system arise very early in the secretory pathway. Incomplete processing of the pre-pro leader sequence in the expression of all GPCRs except the hA_{2a} receptor suggests that improper ER translocation causes transmembrane helices to form improper contacts with each other, which may drive misfolding and ER retention. We are currently investigating protein topology within the ER membrane to better understand the mechanism that gives rise to differential processing, folding, and localization for these receptors. We are also developing methods to facilitate proper translocation and trafficking for the hA_{2b}R, hD_{2L}R, and hCCR5R, which demonstrate a population of properly processed receptors.

Materials and Methods

Subcloning and construction of GPCR strains

The human adenosine hA₁ (hA₁R), human adenosine A_{2b} (hA_{2b}R), human cannabinoid CB₁ (hCB₁R), human cannabinoid CB₂ (hCB₂R), human dopamine D_{2L} (long isoform) (hD_{2s}R), human chemokine CXCR4 (hCXCR4R), human chemokine CCR5 (hCCR5R), human neurokinin NK₁ (hNK₁R), and human neurokinin NK₂ (hNK₂R) receptors were amplified using polymerase chain reaction (PCR) from mammalian shuttle vectors (UMR cDNA resource center). Genes for the human adenosine A_{2a} (hA_{2a}R), human adenosine A₃ (hA₃R), and human follicle stimulating hormone receptor (hFSHR) were provided by Merck Pharmaceuticals.

GPCRs were subcloned into the multi-integrating pITy4-wt plasmid⁴⁴ for expression in *S. cerevisiae*, as performed previously for high-level expression of the human A_{2a}R.^{14,15,18} Each GPCR was subcloned directly into the pITy-MC-His₁₀ plasmid¹⁴ and the pITy-MC-eGFP-His₁₀ plasmid. Both vectors contain a multiple cloning (MC) site consisting of *EagI*, *AatII*, and *SacII* immediately following the Gal1-10 promoter and synthetic pre-pro N-terminal leader sequence.⁴⁵ The pITy-MC-eGFP-His₁₀ plasmid also contains the gene for enhanced green fluorescent protein (eGFP) after the MC site and before an encoded decahistidine purification tag. hA_{2a}R, hA_{2b}R, and hA₃R were PCR-amplified with a 5' *AatII* site upstream of the gene and a 3' *SacII* site downstream of the gene. The PCR product was digested with *AatII* and *SacII*, purified using a PCR clean-up kit (Promega, Madison, WI), and then ligated into pITy-MC-His₁₀ and pITy-MC-eGFP-His₁₀ plasmids, which were previously digested with *AatII* and *SacII*. All other GPCRs were PCR-amplified with a 5' *EagI* site and a 3' *SacII* site, digested with *EagI* and *SacII*, and similarly ligated into pITy-MC-His₁₀ and

pITy-MC-eGFP-His₁₀ digested with *EagI* and *SacII*. After insertion into the above vectors, the following amino acids are introduced between the pre-pro leader sequence and the GPCR N-termini: E-A-R-P-D-V for hA_{2a}R, hA_{2b}R, and hA₃R, and E-A-R-P for all other GPCRs. For GFP-tagged receptors, an additional P-R amino acid linker is introduced between the C-terminus of each receptor and GFP.

GPCR genes were also subcloned into the pITy-MC-eGFP-His₁₀ plasmid without the synthetic N-terminal pre-pro leader sequence. This construct was created by digesting pITy-A_{2a}R-eGFP-His₁₀ with *KpnI* and *AatII* to remove the Gal1-10 promoter and pre-pro leader sequence. Following this digest, the Gal1-10 promoter with an added 5' *KpnI* site and a 3' *AatII* site was PCR-amplified, digested with *KpnI* and *AatII*, and then ligated into the previously digested vector. Other GPCR genes were inserted into this plasmid in place of A_{2a}R, as detailed for the constructs mentioned earlier.

Additional constructs were created for some receptors (hA₃R and hNK₁R) where cyan fluorescent protein (CFP) was present at the C-terminus instead of GFP. These constructs were created in the same manner as the GFP constructs listed earlier, but contain an L-E amino acid linker between the receptor C-terminus and CFP. All GPCR constructs were verified through DNA sequencing (University of Delaware DNA Sequencing Facility or University of Pennsylvania DNA Sequencing Facility).

GPCR constructs (both with and without GFP tags) were transformed into *S. cerevisiae* BJ5464 (MAT α *ura3-52 trp1 leu2 Δ 1 his3 Δ 200 pep4::HIS3 prb1- Δ 1.6R can1 GAL*), which is a vacuolar protease-deficient yeast strain. hA₁R, hA_{2a}R, hA_{2b}R, hFSHR, hCB₁R, hCB₂R, and hNK₂R constructs were linearized with *BsaBI*, whereas hA₃R, hD_{2s}R, hNK₁R, hCXCR4R, and hCCR5R constructs were linearized with *AhdI* before transformation through electroporation. Transformation and selection of transformed yeast cells were carried out as performed previously for the human A_{2a} receptor,¹⁴ via selection with G418.

Additionally, nonfluorescently tagged GPCR strains were transformed with pRS316-UPRE-GFP²⁷ or pwB215,²⁸ which are centromeric plasmids that serve as reporters of the UPR and HSR, respectively. pRS316-UPRE-GFP contains four UPR elements (UPRE) immediately upstream of the GFP gene, enabling detection of UPR induction by monitoring GFP fluorescence.²⁶ Similarly, pwB215 contains the promoter region of *SSA4*, a heat shock inducible element, as a translational fusion to *lacZ* that produces β -galactosidase when the HSR is induced.²⁸ Plasmids were transformed using a standard lithium acetate chemical transformation method.⁴⁶ Cells transformed with pRS316-UPRE-GFP were selected on SD-2XSCAA⁴⁷ plates lacking uracil, whereas cells transformed with pwB215 were selected on SD-2XSCAA plates lacking tryptophan.

Culture conditions

Yeast cultures were typically grown overnight in YPD media (2% Bacto peptone, 2% glucose, 1% yeast extract) in 5-mL culture tubes in a water bath shaker, set to 30°C and 275 rpm. In BJ5464 cells that also contained heat shock or UPR reporter plasmids, cultures were instead grown in selective media to maintain plasmid selection. SD-2XSCAA media lacking uracil were used for cells with pRS316-UPRE-GFP, and SD-2XSCAA media lacking tryptophan were used for cells with pwB215. GPCR expression was induced by resuspension of cells to an OD₆₀₀ of 0.5 in YPG media (2% Bacto peptone, 2% galactose, 1% yeast extract). Cultures expressing GPCRs were grown at 30°C at 275 rpm, and samples were collected at various times. Cell pellets were collected by centrifugation at 2000g for 2 min, and the following assays were performed as needed.

Fluorescence spectroscopy

Expression levels of GFP-tagged GPCRs were determined by measuring whole-cell fluorescence of GFP-tagged GPCRs. Yeast cells expressing C-terminally, GFP-tagged GPCRs were grown for ~24 h, the OD₆₀₀ of the cultures was measured and recorded, and 1 OD-mL of cells was centrifuged for 2 min at 2000g. The supernatant was decanted and cells were resuspended to an OD₆₀₀ of 0.5 in 2 mL PBS buffer (1.44 g/L Na₂HPO₄, 0.24 g/L KH₂PO₄, 0.2 g/L KCl, 8.0 g/L NaCl, pH 7.4). Samples were placed in a quartz cuvette, excited at 488 nm, and fluorescence was detected at 511 nm on a Hitachi F-4500 fluorescence spectrophotometer. Relative per cell expression levels and culture yields were estimated by comparison to an enhanced GFP standard curve, previously used to estimate concentrations of A_{2a}R-GFP in yeast cells.¹⁸ A total of 1 OD was estimated to contain $\sim 2.5 \times 10^7$ cells per mL of culture.³¹ Three or more replicates were used to calculate average expression levels, and the error is the standard deviation from the mean. At this early time points (1–5 hours) and for samples grown at scales of 5–50 mLs no degradation of the GPCR-GFP constructs was observed via SDS-PAGE, suggesting that GFP is a good indicator of total heterologous protein expression. For 24-hour time points in larger size cultures, the degradation was between 0–15%, depending on the receptor identity. However, within the error of both measurements, we believe total fluorescence gives a reasonable value on total protein yields.

Unfolded protein response and heat shock response assays

Yeast cells expressing GPCRs with the pRS316-UPRE-GFP reporter plasmid were grown as detailed earlier. ODs were measured and 1 OD-mL was removed from each culture at the time of induction and at ~4, 6, 8,

12, 16, and 24 h after GPCR expression was induced. Cell aliquots were centrifuged for 5 min at 2000g and the supernatant was removed. Pellets were resuspended in 1 mL of PBS buffer and tested for GFP fluorescence (excitation at $\lambda = 488$ nm, emission at $\lambda = 511$ nm).

For HSR measurements, ~1 OD-mL of cells was removed from cultures expressing GPCRs with the pwB215 plasmid at various time points postinduction. β -Galactosidase activity was measured and quantified from permeabilized cell pellets according to a standard protocol developed for yeast.⁴⁸

Affinity precipitation

Expression of each His-tagged GPCR was induced by growth in galactose (YPG). After 24 h, 5 OD-mL of each strain was collected, centrifuged at 2000g for 2 min, the supernatant was decanted, and cell pellets were frozen at –20°C until use. Pellets were resuspended in 200 μ L Buffer A (50 mM Tris-HCl, 1% *n*-dodecyl- β -D-maltoside (DDM), pH 7.9) including protease inhibitors (1 mM phenylmethanesulphonyl-fluoride (PMSF) and Complete EDTA-free protease inhibitor tablets (Roche, Indianapolis, IN)) and combined with 600 μ L zirconia-silica beads (BioSpec, Bartlesville, OK) in a 1.5-mL microcentrifuge tube. We used DDM for this extraction, as it is a commonly used surfactant for membrane protein studies (see Ref. 7). Although it is true that membrane proteins can have different solubilization efficiencies in different surfactants, generally DDM has been shown to be a good solubilizing agent, especially at the concentration used in this study.⁷ Samples were lysed on a vortexer with six cycles of 30 s agitation with 30 s on ice in between cycles. Lysate was separated from the beads and unlysed cells and heavy cell debris were removed through centrifugation of the lysate at 16,000g for 1 min. Lysate was then combined with 800 μ L Buffer B (50 mM Tris-HCl, pH 7.9, 180 mM NaCl, 6% nonfat dried milk, 1% DDM with protease inhibitors). Approximately 25 μ L of settled Ni-NTA Superflow resin (Quiagen, Valencia, CA) was pre-equilibrated with 1 mL of Buffer AB (1 part Buffer A: 5 parts Buffer B), then was centrifuged at 16,000g for 1 min, and the supernatant was removed. Lysate was then added to the pre-equilibrated nickel resin, and His-tagged GPCRs were allowed to bind to the resin for ~12 h on an end-over-end mixer at 4°C. Nonspecifically bound material was washed away from nickel-bound protein using five washes of 1 mL Buffer C (50 mM Tris-HCl, 150 mM NaCl, 1% DDM, pH 7.9) with protease inhibitors. Bound protein was eluted through reduction of the nickel ions with SDS loading buffer (50 mM Tris, 0.25 mg/mL bromophenol blue, 10% glycerol, 2% SDS) containing 100 mM EDTA. Eluted protein was heated at 60°C for 5 min in SDS loading buffer with EDTA before electrophoretic separation on 12% SDS-PAGE.

Western immunoassay was performed by transfer to nitrocellulose using a Trans-Blot Cell (Bio-Rad, Hercules, CA). GPCRs were detected with a mouse anti-6xHis primary antibody (Abcam, Cambridge, MA) used at a dilution of 1:1000, whereas BiP was detected with a rabbit anti-BiP primary used at a dilution of 1:5000. The antibody against BiP was generated in rabbits using a MBP-Kar2 fusion protein as the antigen. The C-terminal region of Kar2 (from *XbaI* site through stop codon) was inserted into pMal-c2g (New England Biolabs). This fragment was purified with amylase resin (New England Biolabs) and eluted with 10 mM maltose. Rabbit polyclonal antibodies were generated against the purified fusion protein (Capralogics) and screened for specificity in yeast lysates. The antibodies were purified from serum using a Protein A Montage kit (Millipore) and stored in aliquots at -80°C . Secondary HRP-conjugated anti-mouse and anti-rabbit antibodies (GE Healthcare, Piscataway, NJ) were used at a dilution of 1:2000, and bands were detected using an ECL Plus Western Blotting Detection Kit (GE Healthcare). Chemiluminescence was visualized on a Typhoon 9400 Variable Mode Imager (GE Healthcare) after excitation at 457 nm with a 520BP40 filter.

Mobility shift assay and N-terminal sequencing

To determine leader sequence cleavage, 5 OD-mL of yeast strains expressing GFP-tagged GPCRs was harvested at 2, 5, and 24 h postinduction. For comparison, 5 OD-mL of yeast cells expressing GPCRs lacking the pre-pro leader sequence was collected 24 h postinduction. Cell pellets were resuspended in 200 μL STE10 buffer (10 mM sucrose, 10 mM Tris, 10 mM EDTA, pH 7.0) supplemented with Complete EDTA-free protease inhibitors (Roche) and 1% DDM and added to 600 μL zirconia/silica beads. Cells were lysed through vigorous vortexing for three cycles of 1-min agitation and 1 min on ice between cycles. Recovered lysate was collected and stored at -20°C before use.

Deglycosylation of expressed proteins was achieved through overnight digestion with PNGaseF (New England Biolabs, Ipswich, MA) at 37°C and compared to mock-prepared samples lacking enzyme. Samples were combined with $3\times$ SDS loading buffer and separated via 10% SDS-PAGE. GFP-tagged receptors were visualized directly in SDS-PAGE gels with a Typhoon 9400 variable mode imager (Amersham Biosciences), equipped with a 488 nm excitation filter.

Generally, protein samples were prepared for N-terminal sequencing from 200 mL cultures expressing GFP-tagged GPCRs for 24 h. Cells were lysed as detailed earlier, and protein was purified via immobilized metal affinity chromatography through the attached decahistidine tag, as described previously.¹⁴ In some cases, liquid samples of purified protein were sequenced directly (University of Delaware Core Protein Sequencing Facility). Alternatively, protein samples were combined with $3\times$ SDS loading buffer and separated via 12% SDS-

PAGE. Samples were then blotted onto PVDF and Coomassie stained to reveal the protein band of interest. Protein bands were then N-terminally sequenced by the Protein Facility at Iowa State University.

Integral membrane protein assay

Membrane association of recombinantly expressed, GFP-tagged GPCRs was determined according to the method of Kuchler *et al.*¹⁹ Briefly, 20 OD-mL of cells was collected after expression for 24 h. All subsequent steps were performed at 4°C . Cell pellets were resuspended in 400 μL lysis buffer (10 mM Tris, 1 mM EDTA, pH 7.8) supplemented with protease inhibitors and combined with 600 μL zirconia/silica beads and lysed as described in the preceding section. Lysate was collected and diluted to ~ 1 mL, and unlysed cells and large debris were pelleted for 5 min at 1000g. The resulting supernatant was centrifuged for 1 h at 200,000g using a Beckman Optima L-100 XP ultracentrifuge. Membrane pellets were resuspended to a final volume of 1 mL in lysis buffer and divided into 4×250 μL aliquots. These membrane aliquots were resuspended in lysis buffer and concentrated stock solutions of Na_2CO_3 , urea, and SDS to reach a final volume of 1 mL for all treatments, which corresponded to final concentrations of 0.1M Na_2CO_3 (pH 11.0), 2M urea, 1% SDS. Samples were incubated on an end-over-end mixer at 4°C for 1 h, and then membranes were pelleted for 1 h at 200,000g. Various samples from supernatant and resuspended pellet fractions were combined with $3\times$ SDS loading buffer, separated on 12% SDS-PAGE, and visualized directly in SDS-PAGE gels with a Typhoon variable imager, equipped with a 488 nm excitation filter.

Confocal microscopy

Yeast-expressed mammalian GPCRs with a C-terminal GFP tag were imaged using a Zeiss LSM 5 Duo confocal microscope (Zeiss, Oberkochen, Germany) with a $63\times/\text{NA } 1.4$ objective. Cells were immobilized on eight-chamber LabTek NUNC slides coated with poly-D-lysine (MW > 300,000).

Membrane preparation and radioligand binding

Yeast membranes were prepared from cultures expressing GFP-tagged GPCRs for hA₁R, hA₂aR, hA₂bR, and hA₃R, according to a previously published protocol,¹⁶ with the following modifications. Isolation of membranes containing GFP-tagged receptors was used, as it facilitated identification of the membranes throughout their purification. Cultures were grown in YPG for 24 h to enable GPCR expression, and 150 mL of cell culture was collected and centrifuged for 2 min at 2000g. The resulting cell pellets were washed in ddH₂O and centrifuged as mentioned earlier. Pellets were then resuspended in 5 mL STED10 buffer (10 mM Tris-HCl, 1 mM EDTA, 1 mM DTT, 10% sucrose, pH 7.6) supplemented with Complete EDTA-

free protease inhibitors (Roche) and 1 mM PMSF and combined with ~20 g zirconia/silica beads in a 50-mL conical tube. This resulting suspension was vigorously vortexed at 4°C for 5 min to lyse cells. Following lysis, beads were removed from the homogenate by filtration through a Kontes column, and 15 mL STED10 buffer was added to the mixture. The homogenate was centrifuged at 1600g at 4°C for 30 min, and the supernatant was separated from the mixture and further centrifuged at 27,620g at 4°C for 45 min to pellet membranes. Crude membrane preparations were resuspended in 4 mL STED10 buffer with protease inhibitors, and total membrane protein content was determined using an RC/DC BCA kit (Bio-Rad, Hercules, CA) using BSA as a standard.

Membrane preparations were combined with various amounts of tritiated ethylcarboxyaminoadenosine (³H-NECA) (Perkin Elmer, Boston, MA) to determine ligand-binding activity of hA₁R-GFP-His₁₀, hA_{2A}R-GFP-His₁₀, hA_{2B}R-GFP-His₁₀, and hA₃R-GFP-His₁₀ in yeast membranes. Additionally, activity of Ni²⁺ purified, DDM/CHAPS/CHS-solubilized hA₃R-CFP-His₁₀ was tested with binding to ¹²⁵I-IB-MECA (Perkin Elmer). Before activity measurement, membranes were diluted to a concentration of 1 µg membrane protein per µL in ligand-binding buffer (50 mM Tris-HCl, 10 mM MgCl₂, pH 7.4) and treated with 4 U/mL adenosine deaminase (Roche) to scavenge free adenosine. Mammalian cell membrane preparations for the rat A₃ receptor and the human A₁ receptor were obtained from Perkin Elmer and used as positive controls for the assay. HEK293 membranes containing the human adenosine A_{2B} receptor were prepared following transient transfection with the pCEP4-A_{2B}R-CFP plasmid.⁴⁹

A total of 200 µg membrane extracts were combined with varying amounts of radiolabeled ligand in 96-well plates with glass fiber B filters (Millipore, Bedford, MA) and incubated at room temperature for 1–2 h before filtration. Samples were washed five times in ice-cold ligand-binding buffer, combined with 30 µL scintillation solution, and radioactive counts were measured on a Perkin Elmer Microbeta Jet scintillation counter. Resulting data of three replicate samples were averaged, with error bars representing the standard deviation. Data were fit to a one-site binding model using KaleidaGraph 3.5 to determine relevant binding parameters.

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