HETEROLOGOUS EXPRESSION OF G-PROTEIN COUPLED RECEPTORS LEADS TO ACTIVATION OF CELLULAR STRESS RESPONSE PATHWAYS IN SACCHAROMYCES CEREVISIAE

by

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**ABSTRACT**

G-protein coupled receptors (GPCRs), a diverse class of therapeutically relevant proteins, are implicated in regulating nearly every aspect of our physiology. High-resolution structural data exists only for a small handful of GPCRs, inhibiting the efficient structure-based design of new pharmaceuticals. Heterologous expression of GPCRs is an effective method of obtaining the large quantities of purified protein necessary for structural analysis via x-ray crystallography. *S. cerevisiae* is an economical expression system, capable of producing active human A2a receptor in mg quantities per liter of culture, and has tremendous potential as an expression system for the structural analysis of other GPCRs.

The human adenosine A₁ (hA₁R), human adenosine A₂a (hA₂aR), human adenosine A₂b (hA₂bR), human adenosine A₃ (hA₃R), human β₂ adrenergic β₂A (hβ₂AR), human cannabinoid CB₁ (hCB₁), human cannabinoid CB₂ (hCB₂), human chemokine CCR5 (hCCR5R), human chemokine CXCR4 (hCXCR4R), human dopamine D₂ (short isoform) (hD₂R), human follicle stimulating hormone FSH (hFSHR), human neurokinin NK₁ (hNK₁R), and human neurokinin NK₂ (hNK₂R) receptors were individually cloned into pITy expression plasmids, which were stably transformed into BJ5464 cells. The unfolded protein response (UPR) and the heat shock response (HSR) associated with expressing each of these GPCRs was determined by transforming each strain with a UPR and HSR reporter plasmid and conducting time-course experiments.
The UPR and HSR results were analyzed in conjunction with localization data obtained from confocal images and GPCR activity results obtained from radio-ligand binding experiments to determine a correlation between HSR, UPR, and protein mis-localization. Protein mis-localization was accompanied by UPR activation and in most cases HSR activation in the GPCR expressing strains. Expression of human A2a did not activate either response. Monitoring cellular stress responses may be an efficient method of finding other GPCRs that properly express in *S. cerevisiae*. 
1.1 The G-protein coupled receptor family

Human G-protein coupled receptors (GPCRs) are a broad class of membrane proteins that play an important role in signal transduction. All GPCR proteins share several traits. The first principal feature is the seven membrane-spanning $\alpha$ helixes connected by intracellular and extracellular loop domains. When properly folded, the extracellular and intracellular domains form very specific active sites for binding ligands and GTP binding proteins (G-proteins) respectively. The orientation of the N and C termini is also conserved across all GPCRs. The N-terminal tail is exposed to the extracellular environment and the C-terminal tail is located in the cytosol of the cell and thought to maintain an interaction with cytosolic G-proteins (Cooper 2004).

Despite their commonalities, the GPCR family is extremely diverse. GPCR activators include neurotransmitters, hormones, nucleotides, nucleosides, peptides, lipids, ions, and photons (Sarramegna 2003). When a GPCR is activated by its respective ligand, it activates a specific intracellular trimeric G-protein. The activated G-protein then binds to various downstream effectors and induces a cellular response by initiating complex intracellular cascades (Horton 2002). Recent work has shown GPCRs can also transmit intracellular signals independent of G-proteins by binding to other signaling molecules, chaperones, and scaffolding proteins (Hill 2006).
1.2 Pharmaceutical relevance of GPCRs and drug development strategies

The recent expansion of genomic databases, courtesy of the Human Genome Project, has greatly accelerated the identification of GPCRs. The development of algorithms capable of accurately recognizing the seven transmembrane motif has led to the discovery of over 1000 different GPCR proteins (Kim 2000), (Klabunde 2002). Members of the GPCR protein family can be found in every type of mammalian cell and play an integral part in controlling all aspects of our physiology (Siehler 2008). Table 1.2 lists the pharmaceutical relevance of the human adenosine A₁ (hA₁R), human adenosine A₂a (hA₂aR), human adenosine A₂b (hA₂bR), human adenosine A₃ (hA₃R), human β₂ adrenergic β₂A (hβ₂AR), human cannabinoid CB₁ (hCB₁), human cannabinoid CB₂ (hCB₂), human chemokine CCR5 (hCCR5R), human chemokine CXCR4 (hCXCR4R), human dopamine D₂ (short isoform) (hD₂R), human follicle stimulating hormone FSH (hFSHR), human neurokinin NK₁ (hNK₁R), and human neurokinin NK₂ (hNK₂R) receptors, used in this thesis work.
The specific nature of GPCR binding interactions makes these proteins ideal targets for therapeutic strategies. As of 2005, GPCRs were targeted by over 60% of therapeutics on the market, generating over $50 billion in revenue (Saunders 2005).

<table>
<thead>
<tr>
<th>GPCR</th>
<th>Pharmaceutical Relevance</th>
</tr>
</thead>
<tbody>
<tr>
<td>hA1R</td>
<td>Pulmonary disease (Dougherty 1998)</td>
</tr>
<tr>
<td>hA2aR</td>
<td>Inflammation, asthma (Brown R. A. 2008), drug abuse (Brown R. M. 2008)</td>
</tr>
<tr>
<td>hA2bR</td>
<td>Analgesia, inflammation, diabetes, Alzheimer’s (Yan 2006)</td>
</tr>
<tr>
<td>hA3R</td>
<td>Cancer, ischemia (Gessi 2008)</td>
</tr>
<tr>
<td>hβ2AR</td>
<td>Synapse plasticity (Nittykoski 1999), cognitive function, mood (Millen 2002)</td>
</tr>
<tr>
<td>hCB1R</td>
<td>Analgesia (Dziadulewicz 2007), obesity (Jagerovic 2008)</td>
</tr>
<tr>
<td>hCB2R</td>
<td>Immune system regulation (Abd-Allah 2007), neurodegenerative disorders (Fernandez-Ruiz 2008), depression, drug abuse, emesis (Onaivi 2006)</td>
</tr>
<tr>
<td>hCCR5R</td>
<td>HIV infection (Mirzabekov 1999)</td>
</tr>
<tr>
<td>hCXCR4R</td>
<td>HIV infection (Babcock 2001)</td>
</tr>
<tr>
<td>hD2R</td>
<td>Schizophrenia, Parkinson’s (Sander 1994a)</td>
</tr>
<tr>
<td>hFSHR</td>
<td>Follicle formation (Rice 2007)</td>
</tr>
<tr>
<td>hNK1</td>
<td>Vasodilatation, depression, anxiety (Griffante 2006)</td>
</tr>
<tr>
<td>hNK2</td>
<td>Irritable bowel syndrome, asthma, involuntary micturition (Ahlstedt 2008)</td>
</tr>
</tbody>
</table>
Despite such a large market share, only a small fraction of the 1000 plus GPCRs are currently being targeted due to our limited understanding of the binding site and lack of high-resolution structures.

Ligand-based and structure-based drug design are two tactics for developing new GPCR-targeting therapeutics. Ligand-based drug design is limited to the relatively small number of GPCRs with known ligands. The structure of a ligand associated with a specific GPCR is used as a starting point and then substituted, modified, or otherwise optimized for application (Klabunde 2002). Ligand-based drug design is therefore not available for GPCRs without a known ligand, or orphan GPCRs.

Identifying new GPCR-ligand interactions is difficult to achieve in vivo due to the significant amount of cross-signaling observed between cascades (Dumont 2002). Conversely, identifying novel interactions in vitro is equally challenging due to the large variety of known GPCR ligands and the inability to obtain the necessary quantities of purified protein. Computers may be able to predict interactions between GPCRs and ligands using high-resolution structural data.

Detailed, structure-based drug design is the most direct method of developing new therapeutics, using computational modeling to map the binding site of a GPCR and then using an algorithm to develop a compound that will bind it. This method is ideal for the 150 plus “orphan” GPCRs that have no known ligands, synthetic or natural (Hill 2006). Unfortunately, structure-based drug design is not practical at this time due to the small number of GPCRs with known high-resolution structures. To date, only the hA2aR (Jaakola 2008), hβ1AR (Warne 2008), hβ2AR (Cherezov 2007), and bovine rhodopsin (Li 2004) receptors have been successfully crystallized.
Structural analysis of a GPCR requires milligram quantities of purified, homogeneous, active protein (Grisshammer 1995). Unfortunately, most mammalian GPCRs are natively expressed in small quantities (Khorana 1992), (Chelikani 2006) and are commonly co-expressed with pharmacological analogs possessing similar structures (Tate 1996). Generally, it is not possible to obtain enough purified protein from native tissue for crystallization and subsequent x-ray diffraction analysis. Consequentially, heterologous expression of a target GPCR is the most convenient solution for obtaining high-resolution 3-D structures for GPCRs.

1.3 Human GPCR expression in *Saccharomyces cerevisiae*

The yeast *Saccharomyces cerevisiae* is an ideal system for expressing GPCR proteins because of its robust nature, inexpensive cost, and the homology of its secretory pathway to mammalian cells. Previously, this system has been used to produce and purify mg/liter of culture quantities of active hA2aR (O’Malley 2007). The human β2-adrenergic (King 1990), human α2-C2-adrenergic (Sizmann 1996), human μ-opioid (Gaibelet 1999), human D2S-dopaminergic (Sander 1994a), and human D1α-dopaminergic (Andersen 1998) receptors are just a few that have been expressed in pmol/mg of membrane protein levels using *S. cerevisiae*. Our attempt to express human NK1R has been unsuccessful and results in protein retention within the cell (Butz 2003).

High levels of misfolded or unfolded soluble proteins within the endoplasmic reticulum (ER) have been linked to activation of the unfolded protein response (UPR), a quality control mechanism responsible for maintaining ER homeostasis. In yeast, the UPR is activated by accumulation of unfolded protein in the ER (Kimata 2004) and by
saturation of ER folding machinery (Parekh 1995). Similarly, the heat shock response (HSR) recognizes mis-folded and aggregated proteins in the cytosol (Nicolet 1991), (Boorstein 1990) and has been shown to work in conjunction with the UPR to relieve ER stress (Liu 2008). Activation of cellular stress responses is not desired because cellular stress leads to reduced protein expression levels (Kauffman 2002).

The aim of this thesis research was to determine the UPR and HSR responses associated with expressing hA2aR and other human GPCRs with therapeutic relevance in *S. cerevisiae*. The UPR and HSR results will be analyzed in conjunction with localization data obtained from confocal images and GPCR activity results obtained from radio-ligand binding experiments to determine a correlation between HSR, UPR, and protein mis-localization. We hypothesize that activation of cellular stress responses may be correlated with protein mis-localization and that monitoring these responses may be an efficient method of finding GPCRs that properly express in *S. cerevisiae*. Furthermore, simultaneous monitoring of these responses could be used as a screening tool for testing large numbers of GPCRs from the GPCR library for proper expression in *S. cerevisiae*. An assortment of pharmaceutically relevant GPCRs was expressed in *S. cerevisiae* while simultaneously measuring the activation of the UPR and HSR to determine the activation of these responses during GPCR expression.
Chapter 2

BACKGROUND

In this chapter, several key features of GPCRs and potential host cells are described. First, the mechanism of GPCR activation and subsequent activation of intracellular cascades is outlined. The post-translational modifications necessary for membrane localization, effects of membrane composition on GPCR functionality, and costs associated with expressing GPCRs in prokaryotic, simple eukaryotic, and higher eukaryotic cells are explored. Finally, the unfolded protein response and the heat shock response, two mechanisms associated with cellular stress, are covered in detail.

2.1 The role of G-protein / GPCR interactions

When a specific agonist activates a GPCR it facilitates the activation of heterotrimeric guanine nucleotide-binding proteins (G-proteins), which subsequently modulate the effector proteins of the target signal cascade (Birnbaumer 1990). GPCRs are considered non-linear signaling proteins because the signal cascade(s) they activate depends on the local concentrations of the G-proteins, effectors, and modulators associated with each particular GPCR (Gudermann 1996). Each G-protein is composed of α, β, and γ subunits. Many unique G-proteins can be assembled from the twenty-three α subunits (Simon 1991), five β subunits, and ten γ subunits (Ray 1995) that have already been identified. The extensive variety in assembled G-proteins allows GPCRs to activate one or many G-proteins in a highly specific manner.
Studying receptor/ligand binding thermodynamics in mammalian systems is problematic because endogenous, or native, G-proteins tend to elevate background activation levels (Stanasila 2000). The absence of endogenous G-proteins in non-native systems can allow direct control of receptor / G-protein interaction availability using co-expression (Burt 1998), (Schwaninger 1992), or by expressing G-protein/GPCR fusion proteins (Seifert 1998). Conversely, without the endogenous G-proteins many GPCRs, like the human µ-opioid receptor, are unable to bind their ligand with high affinity, but activity is restored when the appropriate G-proteins are added (Stanasila 2000). Non-native expression of GPCRs does not guarantee receptor isolation. Human GPCRs expressed in non-mammalian systems can interact with the G-proteins of that system in a neutral, positive, or negative way (Sander 1994a), and these interactions vary unpredictably for each receptor / system combination.

It has been established that GPCR / G-protein interactions play a crucial role in the early trafficking of GPCRs through the secretory pathway of eukaryotic cells (Schwaninger 1992). It is possible that the mis-localization of some non-native GPCRs is due to the absence of specific G-proteins, but the role of G-proteins in GPCR trafficking is not fully understood. Refer to Brinbaumer (1990) and Gudermann (1996) for further explanations of GPCR / G-protein coupling.

### 2.2 Post-translational modification of GPCRs

Mammalian GPCRs are subject to many post-translational modifications including phosphorylation, disulfide bond formation, and site-specific glycosylation (Tate 1996). Glycosylation enzymes, such as oligosaccharyltransferase, located inside the native ER recognize and glycosylate the asparagine occurring in the tripeptide
sequence Asn-X-Ser, Asn-X-Thr or Asn-X-Cys, where X could be any amino acid except proline, on the N-terminus of the GPCR (Varki 1999). The exact location(s) of the modifications and the sugar types vary among the GPCR population. N-linked glycosylations are essential for the activation of some GPCRs, such as rhodopsin (Kaushal 1994), and are completely unnecessary for others, like the α2C2-adrenoceptor (Marjamaki 1994). Proper glycosylation of the hVIP1 receptor is required for plasma membrane targeting, satisfying the ER quality control mechanisms, and stabilizing an active GPCR conformation on the cell surface (Couvaineau 1996).

Prokaryotic cells lack the ability to add the post-translational glycosylations found in eukaryotic cells. The absence of these modifications results in the production of a homogeneous protein product, but concomitantly impairs the folding of many GPCRs into an active conformation (Grisshammer 1995). The addition of native glycosylation machinery, among other proteins, to prokaryotic cells is nontrivial due to the large number of enzymes involved in these processes.

Yeast cells are able to perform many of the post-translational modifications found in mammalian cells, but differences in glycosylation patterns can negatively affect the targeting and activity of expressed GPCRs (Eckart 1996). It may be possible to increase homology of the glycosylation system by genetically modifying a yeast strain, but such an endeavor would require thorough analysis of the native GPCR glycosylation and may prove protein specific (Eckart 1996).

Insect and mammalian cells have the greatest homology with the post-translational modifications of human cells, but over-expression and transient expression in these systems can produce a heterogeneous product, making purification for crystallization extremely difficult (Tate 1996).
2.3 Effects of membrane composition

The plasma membranes of prokaryotes, simple eukaryotes, and higher eukaryotes are significantly different with regard to lipid composition, which in turn may affect the stability of active GPCR conformations (Sarramagna 2003). Prokaryotes possess a thick, cholesterol-free membrane that can detract from proper folding of human GPCRs (Akermoun 2005). Analogous to cholesterol, the yeast \textit{Saccharomyces cerevisiae} produces ergosterol, a lipid that completely prevents the activation of some human GPCRs, the \(\mu\)-opioid receptor being one example (Lagane 2000). Additionally, yeast cells are encapsulated by a cell wall that makes protein purification more challenging (Sarramegna 2003). The low cholesterol content of insect cell membranes can also negatively affect the activity of certain human GPCRs, such as the oxytocin receptor (Tate 1996).

Generally, non-mammalian systems may lack the cholesterol and lipid content required for proper folding of many human GPCRs. The addition of cholesterol is one method of successfully restoring receptor activity in a non-mammalian membrane (Lagane 2000). Cholesterol has also been used to stabilize the activity of purified receptors in protein-detergent complexes (O’Malley 2007).

2.4 Choosing an appropriate heterologous expression system

Prokaryotes, simple eukaryotes, and higher level eukaryotes such as insect and mammalian cells have all been explored as GPCR expression systems for the study of GPCRs (Loll 2003), (Hill 2006), (Sarramegna 2003). The unique set of endogenous G-proteins, varied membrane composition, ability or inability to consistently mimic
native post-translational modifications, and discrepancies in operating costs make choosing the best expression system application specific.

Generally, the study of a GPCR’s binding mechanism starts with predicting a model, then incorporates experimental data to estimate the model parameters, and finally compares the model predictions with those observed in vivo (Katanaev 2007). As mentioned in section 2.1, the parameter estimation process sometimes requires isolation of the receptor from the pharmacologic analogs found in its native system (Tate 1996). Insect cells usually lack the endogenous G-proteins associated with mammalian GPCRs (Seifert 1998), but often fail to properly complete the native glycosylation of expressed GPCRs (Boundy 1996).

High-level expression and large experimental volumes are not necessary for observing binding equilibrium, which allows for the use of expensive mammalian systems that best mimic native conditions (Loll 2003). In fact, high-level GPCR expression often leads to heterogeneous post-translational modifications and a G-protein deficiency that may be detrimental to equilibrium studies (Tate 1996). The application of prokaryotes and yeast cells in equilibrium studies is limited because so many GPCRs require specific post-translational modifications to reach the plasma membrane in an active state (Grisshammer 1995) or to properly associate with ligands and other receptors (Eckert 1996). Also, many human GPCRs are incompatible with the low cholesterol content of non-mammalian cell systems (Loll 2003).

Structural analysis studies require production of large amounts of active protein. Although quick and inexpensive to grow (Sarramegna 2003), the inability of prokaryotic cells to correctly make post-translational modifications (Grisshammer 1995) and the severe differences in membrane composition (Akermoun 2005) make
them a poor choice for these studies. Insect and mammalian cells grow very slowly, require costly media, and either employ transient expression, which is inefficient at larger scales, or weak promoters in stable cell lines (Sarramegna 2003).

The yeast *Saccharomyces cerevisiae* is able to produce milligram quantities of human A2a receptor per liter of culture and has tremendous potential as an expression system for the structural analysis of other GPCRs (O’Malley 2007). Powerful genomic tools facilitate the manipulation and inducible expression of target proteins. Furthermore, vacuolar protease-deficient strains are readily available. If vacuolar proteases were expressed, they would digest any GPCRs targeted for degradation and could effectively limit yields. Yeast cells grow quickly, doubling every two hours, and require simple, inexpensive media. The homology between the yeast and mammalian secretory pathways allows yeast to complete most post-translational modifications. Most importantly, active protein can be extracted, purified, and stabilized in surfactants for subsequent crystallization.

### 2.5 Other methods for improving expression and membrane localization

A large number of factors contribute to the successful heterologous expression of GPCR proteins. It is becoming increasingly apparent that the mechanisms involved in the functional expression and localization of GPCRs are complex and are not completely understood. Codon optimization (Chelikani 2006), amino acid substitution (Parker 1991), vector optimization (Pickering 2004), and co-expression with chaperones and other GPCRs (Huang 2008) are techniques that have been employed with varying results.
There are between one and six three-nucleotide sequences, or codons, associated with each amino acid, that are used to translate mRNA into proteins. Some codons are rarely used and are inefficiently translated in different host systems (Eckart 1996). Codon optimization is the substitution of common codons for rare ones and has been shown to significantly increase expression of the CCR5, CXCR4, and β2AR receptors in mammalian cells (Mirzabekov 1999), (Babcock 2001), (Chelikani 2006). Replacing rare codons with those commonly recognized by yeast cells may yield similar results in *Saccharomyces cerevisiae*.

Amino acid substitutions resulting from site directed mutagenesis unpredictably affect the binding and localization of expressed GPCRs in a positive or negative way. Random replacement of amino acids in avian β2AR often resulted in no expression of the target protein, but in one instance caused a 40-fold increase in expression level (Parker 1991). Twenty-seven of three hundred fifteen direct mutations of human A2AR resulted in increased protein thermal stability (Magnani 2008). The unpredictable nature of these substitutions makes them a trial-and-error option for optimizing GPCR expression level at this time.

Expression vectors have been optimized for stable integration in a wide variety of cell platforms. One key element of the expression vector is the leader sequence located upstream of the target gene. The leader sequence is responsible for targeting of the expressed protein (Pickering 2004). Synthetic leader sequences that mimic or include motifs naturally found in native and non-native systems have been used to successfully increase protein expression levels in *Saccharomyces cerevisiae* (Clements 1991). Alternatively, gene expression can be controlled using signal sequences that are naturally found in the host system. The upstream region of the α-factor receptor (Ste2),
a GPCR found in *Saccharomyces cerevisiae*, has been used successfully in past heterologous expression attempts (Sugiyama 2004).

Expressed GPCRs interact with many proteins as they are produced and trafficked through the secretory pathway. Chaperones stabilize newly synthesized GPCRs in the ER and allow them to pass quality control mechanisms (Leskela 2007). Over-expression of mammalian proteins, both membrane-bound (Butz 2003) and soluble (Parekh 1995), in *S. cerevisiae* often saturates the secretory pathway and limits yields. The effect of co-expressing ER chaperones found natively in *S. cerevisiae*, such as BiP, protein disulfide isomerase (PDI), and calnexin, are protein specific and remain unpredictable (Huang 2008), (Butz 2003). Heterologous expression of some GPCRs may require co-expression of specific chaperones or receptor-activity modifying proteins (RAMPs) which must be determined empirically. RAMPs are proteins that dramatically alter GPCR function and are required for escorting specific GPCRs, like the calcitonin-receptor-like receptor, to the plasma membrane (McLatchie 1998). It is possible that RAMPs operate as escorts by forming heterodimers with their respective GPCRs (Matsunami 2005).

Heterodimerization is another way GPCRs can pass quality control mechanisms inside the ER. Dimerization of the GABA$_{B(1)}$ and GABA$_{B(2)}$ masks an ER retention sequence and is required for export of the proteins to the plasma membrane. Similarly, dimerization of certain olfactory receptors with the $\beta_2$-adrenoceptor is required for proper localization (Hague 2004). Proper expression of certain taste receptors also requires co-expression and dimerization (Nelson 2001). The inability to heterologously express other olfactory GPCRs could be a result of similar mechanisms involved in their maturation and targeting (McClintock 1997). Homo- and/or
heterodimerization may be obligatory for the proper expression and targeting of numerous GPCRs (Bulenger 2005).

2.6 Unfolded Protein Response

The unfolded protein response (UPR) is a cellular mechanism with the purpose of reducing stress in the endoplasmic reticulum (ER) while maintaining essential protein expression. The ER chaperone binding protein, BiP, is a key player in the UPR and is required for proper folding and secretion of many secreted proteins (Nguyen 1991). Under normal equilibrium conditions BiP binds to Ire1, a protein embedded in the ER membrane, preventing the dimerization of Ire1. When mis-folded protein accumulates in the ER, BiP preferentially binds to the mis-folded protein and releases Ire1. The uninhibited Ire1 monomer then can dimerize and auto-phosphorylates to become active. Once active, the Ire1 dimer can splicing the HAC1 pre-mRNA into translatable HAC1 mRNA. After splicing, the HAC1 mRNA is translated to produce the Hac1 protein, which dimerizes and binds to the unfolded protein response element (UPRE). The UPRE is a promoter sequence that up-regulates the production of chaperones and foldases when bound to the Hac1 dimer (Kimata 2006). The chaperones and foldases up-regulated by the UPRE reduce ER stress by binding to mis-folded proteins and helping them attain the proper conformation. In addition, UPR activation up-regulates the production of ER proteases and shuts down transcription of non-essential proteins (Diamant 2001). Refer to Khan (2008) for further review of the ER chaperones and their functions.

Mammalian GPCR expression has caused UPR activation in many expression systems. Expression of the Pael receptor, a GPCR implicated in Parkinson’s disease,
in mammalian cells resulted in accumulation of protein in the ER, caused UPR activation, and ultimately ended in programmed cellular death (Imai 2001), (Takahashi 2003). Attempts to express the mouse odorant receptors ml7 and mOREG in mammalian cells also resulted in mis-folded protein, accumulation in the ER, and UPR activation (Lu 2003). In *S. cerevisiae*, expression of soluble proteins can cause UPR activation (Travers 2000), (Parekh 1995), (Xu 2005); however, the UPR was not activated during expression of the human A2a GPCR in yeast (VanFossen 2004). This could result from the observation that hA2aR is folded and trafficked to the plasma membrane in yeast.

2.7 Heat Shock Response

The heat shock response (HSR) works to reduce stress caused by high temperatures, and also clears misfolded proteins that have accumulated in the cytosol (Liu 2008). Yeast cells grow optimally at 30 °C. At elevated temperatures (> 37 °C) the cytosolic proteins begin to denature and are at risk of permanent damage. Prolonged exposure to elevated temperatures results in abnormal function and ultimately in cell death. The heat shock protein, hsp70, releases the Hsf1 transcription factor (HSF) as the temperature increases and hsp70 preferentially binds damaged, or misfolded, proteins in the cytosol. Once released, the HSF travels into the nucleus and binds to heat shock elements (HSE), which upregulate the HSE genes. HSE genes include molecular chaperones and components of the proteasome system that would remove misfolded proteins (Liu 2008).

Similar to the UPR, the HSR can be activated when heterologously expressed proteins mis-fold and accumulate in the cytosol (Liu 2008). GPCRs retained in the ER
could also activate the HSR if their cytoplasmic portions are mis-folded. Expression of the human $A_2\alpha$ receptor, which is properly folded and trafficked in $S.\ cerevisiae$, did not cause significant HSR activation (VanFossen 2004).
Chapter 3

EXPERIMENTAL METHODOLOGY

3.1 Expression System

The yeast cell line *Saccharomyces cerevisiae* BJ5464 (MATα *ura3-52 trp1 leu2Δ1 his3Δ200 pep4::HIS3 prb1Δ1.6R can1GAL) was used as the parental strain throughout the project. The BJ5464 strain lacks vacuolar proteases and is unable to proliferate in minimal media because it is incapable of producing the amino acids leucine (leu) and tryptophan (trp) and the pyrimidine derivative uracil (ura), which are necessary for protein production and proliferation. The BJ5464 strain is ideal for reporter plasmid selection because only cells that have been transformed with a plasmid facilitating the production of a deficient amino acid will grow in media lacking that amino acid.

3.2 Plasmids

3.2.1 The pITy plasmid

The pITy-4 multiple cloning site vector constructed by Parekh et al. (1995) and modified by Michelle O’Malley in the Robinson laboratory was used as the base plasmid for this project. The pITy vector can be stably integrated into the chromosomal DNA using homologous recombination between the δ sequences, long terminal repeats of the Ty element, that are common in the chromosome and incorporated into the vector (Boeke 1989). The pITy vector is capable of integrating up
to 30 copies throughout the chromosome in a single transformation (Parekh 1996). Copy number can be tuned by growing cells in the presence of varying amounts of G418 because the vector includes a gene which provides G418 resistance, although exceptions have been reported (Parekh 1996). G418, or Geneticin, is an antibiotic that inhibits all protein production in prokaryotic and eukaryotic cells and is commonly used as a selective pressure in genetic cloning.

3.2.2 Construction of GPCR expressing strains

Two plasmids were created for each GPCR from the pITy-4 multiple cloning site vector. The first plasmid version contained the multiple cloning (MC) site followed by a 10-histidine tail. The second version contains the gene for enhanced green fluorescent protein (eGFP) after the MC site and before the 10-histidine tail. The MC site contains the EagI, AatII, and SacII restriction sites, which are used to insert each target GPCR gene. Each gene of interest was amplified using PCR with an appropriate restriction site on each end. After amplification both restriction sites were digested and the gene was ligated into both the pITy-MC-10His and pITy-MC-eGFP-10His plasmids. Only restriction sites that were not contained by the target gene could be used; otherwise the digestions would have truncated the gene sequence. All plasmid constructs were amplified in E. coli, purified, and verified through DNA sequencing.

The 10-His tag was incorporated because it enabled binding to nickel resin, and facilitated subsequent protein purification from cell lysate. The eGFP tags were used to study protein localization using confocal microscopy. The non-GFP tagged receptors were used to study cellular stress responses associated with GPCR expression.
The human adenosine A₁ (hA₁R), human adenosine A₂a (hA₂aR), human adenosine A₂b (hA₂bR), human adenosine A₃ (hA₃R), human β₂ adrenergic (hβ₂AR), human cannabinoid CB₁ (hCB₁R), human cannabinoid CB₂ (hCB₂R), human chemokine CCR5 (hCCR5R), human chemokine CXCR4 (hCXCR4R), human dopamine D₂ (short isoform) (hD₂R), human follicle stimulating hormone FSH (hFSHR), human neurokinin NK₁ (hNK₁R), and human neurokinin NK₂ (hNK₂R) receptors were chosen for this study due to their pharmaceutical relevancies as outlined in table 2.1.

All GPCR expression plasmids included a Gal1-10 promoter, a pre-pro leader sequence, and the yeast alpha terminator. The Gal promoter enables GPCR expression when cells are grown in a galactose-based media that lacks glucose. The pre-pro sequence is recognized by the cell and targets the expressed protein to the secretory pathway, via the ER (Parekh 1995).

3.2.3 The pWB215 reporter plasmid
The SSA4-lacZ plasmid (pWB215), obtained from Dr. Elizabeth Craig (University of Wisconsin), was used to quantify the heat shock response. The plasmid contains the promoter region of SSA4, a heat shock element followed by the lacZ gene, which encodes the β-galactosidase enzyme. Activation of the HSR induces expression of β-galactosidase, which can be quantified using a β-galactosidase assay. The pWB215 plasmid confers ampicillin resistance to transformed E. coli cells and allows yeast to produce tryptophan.
3.2.4 The pRS316-UPRE-GFP reporter plasmid

The pRS316-UPRE-GFP plasmid, obtained from Dr. Weissman (University of California, San Francisco), was used to quantify the unfolded protein response (Travers 2000). The plasmid contains four unfolded protein response elements (UPRE) followed by the eGFP gene. Activation of the UPR induces expression of cytosolic eGFP, which can be quantified using fluorescence spectroscopy. The pRS316-UPRE-GFP plasmid confers ampicillin resistance to transformed cells and allows them to produce uracil.

3.3 Plasmid Transformations and Verification

The GPCR expression plasmids (pITy-GPCR-10His and pITy-GPCR-eGFP-10His) were linearized with BsaBI or AhdI and concentrated using ethanol precipitation to approximately 1 µg/µl. 5µl of concentrated plasmid was then transformed into the BJ5464 cells using electroporation with a Bio-Rad Gene-Pulser. Cells were allowed to recover in a YPD/1M sorbitol mixture for 4 hours and then plated on G418/YPD plates for selection. Previous group protocols were followed for the linearization and electroporation of the pITy plasmids (O’Malley and Robinson 2007).

Transformation of the pITy-GPCR-eGFP-10His plasmids was verified by growing each strain at 30 °C in YPD (1% yeast extract, 2% peptone, 2% dextrose) to a culture OD$_{600}$ of 0.5 (5ml of culture). Each culture was pelleted (5 minutes at 2,000 x g) and re-suspended in YPG (1% yeast extract, 2% peptone, 2% galactose) to induce GPCR-eGFP-10His expression. Culture optical densities (ODs) were measured and an amount of cells corresponding to 1 OD-ml (about 2.7x10$^7$ cells) was removed from each culture at the time of induction and after 5 hours of growth in YPG. Aliquots
were centrifuged for 5 minutes at 2,000 x g and the supernatant was removed. Each aliquot was tested for fluorescence using the method outlined in section 3.6.

Transformation of the pITy-GPCR-10His plasmids was verified by comparing the growth of transformed cells on G418 plates with that of negative control cells. The negative control consisted of BJ5464 cells that were electroporated without the pITy-GPCR-10His plasmid and therefore lacked G418 resistance. Additionally, an anti-His primary antibody was used by Michelle O’Malley to verify GPCR-10His expression in several strains.

The reporter plasmids, pRS316-UPRE-GFP and pWB215, were chemically transformed into each GPCR-10His expressing strain using a modified lithium acetate chemical transformation protocol (Gietz 2006). Cell lines expressing GFP tagged GPCRs were not transformed with either reporter plasmid. Prior to transformation, each strain was grown in selective media overnight. Approximately 5 x 10^8 cells were harvested, pelleted, and re-suspended in 1.0 ml sterile water. The cells were pelleted again and re-suspended in 360 µl of transformation mix. The transformation mix (T mix) consisted of 240 µl PEG 3500 (50% [w/v]), 36 µl LiAc 1.0 M, 50 µl of boiled and chilled salmon sperm carrier DNA (2.0 mg/ml), and approximately 100 ng of either reporter plasmid in approximately 34 µl of water. After vortexing for 30 seconds, the mixture was incubated at 42 °C for 2 hours. The mixture was then centrifuged at top speed for 30 seconds and the supernatant was removed.

Transformed cells in the pellet were re-suspended in 1.0 ml sterile water and then 50 µl samples were plated on minimal media plates lacking the appropriate amino acid for each reporter plasmid and incubated at 30 °C.
3.4 Culture Conditions

Transformants were given 3 to 4 days to recover and grow before verifying reporter plasmid transformation. A 5 ml culture of each GPCR-10His expressing strain containing the pWB215 reporter plasmid was grown to a culture OD₆₀₀ of 1.0 in tryptophan-deficient minimal media. Each culture was split into two equal parts, one part heat shocked at 42 °C and the other incubated at 30 °C as a control. After 1.5 hours of incubation the cells were spun down and the β-galactosidase assay was performed.

After transforming the hA₂aR-10His expressing strain with the pRS316-UPRE-GFP reporter plasmid, the cell strain was grown to a culture OD₆₀₀ of 1.0 in uracil-deficient minimal media. The culture was split into two equal parts, one part of which was inoculated with 5mM dithiothreitol (DTT) and the other left as a control. DTT is a small-molecule redox agent that breaks disulphide bonds and induces the UPR. After 1.0 hour of incubation at 30 °C the cells were spun down and re-suspended in PBS for fluorescence testing.

Cells were grown using selective media and plates to ensure plasmid retention. SD-2XSCAA media (Wittrup 1994) was used and included 20.0 g/L dextrose, 14.7 g/L sodium citrate, 6.7 g/L yeast N₂ base, 3.82 g/L dropout powder, and 4.2 g/L citric acid monohydrate. Dropout powder contained 11.4 g arginine, 6.5 g methionine, 3.1 g tyrosine, 17.4 g isoleucine, 26.4 g lysine, 12 g phenylalanine, 75.6 g glutamic acid, 24 g aspartic acid, 23 g valine, 13.2 g threonine, 7.8 g glycine, and 2.4 g adenine. Media was supplemented with uracil (40 mg/L), tryptophan (40 mg/L), and leucine (400
mg/L) as required. Media was uracil deficient for UPR (pRS316-UPRE-GFP) containing strains and tryptophan deficient for HSR (pWB215) containing strains. Selective plates were made by adding Bacto-agar (15 g/L) before autoclaving media. GPCR expression was induced by growing cells in YPG (1% yeast extract, 2% peptone, 2% galactose). All cultures were grown in a water bath shaker incubator at 30 °C and 275 rpm.

3.5 Experimental Protocol

Each cell strain was streaked onto the appropriate selective media plate. When the cell colonies were sufficiently grown (two to three days after streaking) one colony was re-streaked onto a fresh selective plate. Two days later, a 5 ml liquid starter culture of selective minimal media was made from a colony on the second plate. The starter culture was grown overnight to saturation (OD > 10) and was then used to inoculate three 30 ml selective media cultures at an OD of 0.15. The cell concentration was monitored as the cells grew to a concentration of 1.0 OD. Cultures grown to an OD exceeding 1.3 were not used due to increased levels of background stress as cells exited the log growth phase. ODs were measured and an amount of cells corresponding to 30 ODml of cells were harvested from each culture by centrifugation (5 minutes at 2,000 x g) and then re-suspended in 30 ml of YPG to induce GPCR expression. Selective pressure was not needed for integrated plasmids, but the copy number of the reporter plasmids will decrease after prolonged growth in non-selective conditions (Parekh 1996).

Culture ODs were measured and an amount of cells corresponding to 1 ODml were removed from each culture at the time of induction and several times after
expression was induced. Aliquots were centrifuged for 5 minutes at 2,000 x g and the supernatant was removed. Pelleted cells were stored at -20 °C prior to analysis by fluorimeter or by β-galactosidase assay.

3.6 Fluorescence Readings

Aliquots from strains transformed with the pRS316-UPRE-GFP reporter plasmid were re-suspended in a quartz cuvette with 1 ml of PBS buffer (1.44 g/L Na₂HPO₄, 0.24 g/L KH₂PO₄, 0.2 g/L KCl, 8 g/L NaCl, pH=7.35). Fluorescence was measured using a Hitachi fluorimeter (excitation at λ=489 nm, emission at λ=511 nm). Subtracting the background fluorescence of BJ5464 cells normalized each reading.

3.7 β-Galactosidase Assay

Aliquots from strains transformed with the pWB215 HSR reporter plasmid were re-suspended in 1 ml of Buffer Z (16.1 g/L Na₂HPO₄·7H₂O, 5.5 g/L NaH₂PO₄·H₂O, 0.75 g/L KCl, 0.246 g/L MgSO₄·7H₂O, 2.7 ml/L β-mercaptoethanol, pH=7.0). 3 drops of chloroform and 2 drops of 0.1% SDS were added to each aliquot before it was vortexed at top speed for 10 seconds. The β-galactosidase assay was initiated by adding 0.2 ml of OPNG (4 mg/ml o-nitrophenyl-β-D-galactoside in buffer Z). OPNG is a substrate of β-galactosidase that produces o-nitrophenol (ONP) and galactose when hydrolyzed. ONP emits a quantifiable yellow color. After samples developed a pale yellow color (20 to 60 minutes) the assay was terminated by adding 0.5 ml of Na₂CO₃ (1 M in H₂O). Aliquots were centrifuged at top speed for 10 minutes and the supernatant measured at λ=420 nm.
Assay activity was expressed using:

$$\beta\text{-galactosidase units} = \frac{100 \times \text{OD}_{420}}{\text{OD}_{600} \times \text{time of assay} \times \text{volume assayed} \times \text{time of assay}}$$

3.8 Visual verification of GPCR trafficking with confocal microscopy

Protein localization was studied by expressing eGFP tagged receptors in our system. Cell strains transformed with the pITy-GPCR-GFP-10His plasmids were grown in YPG and harvested at 5 hours and 24 hours after expression was induced. Cells were immobilized on 8-chamber LabTek NUNC slides coated with poly-D-lysine (MW > 300,000) by Michelle O’Malley and examined under a Zeiss LSM 5 Duo confocal microscope under the direction of Carissa Young.
Chapter 4

RESULTS and DISCUSSION

In this chapter, results of the plasmid transformations are discussed along with the results of the fluorescence readings and the β-galactosidase assays. The confocal images of each GPCR-GFP expressing strain, taken by Carissa Young, are also discussed.

4.1 Verification of plasmid transformations

BJ5464 cells electroporated without any of the pITy-GPCR-10His plasmids did not recover on G418 plates. BJ5464 cells electroporated with the various pITy-GPCR-10His plasmids successfully recovered on G418 plates, indicating successful transformation of the GPCR-10His expressing strains. Additionally, Michelle O’Malley used an anti-His primary antibody to verify GPCR-10His expression in several strains via western blotting (not shown). BJ5464 cells electroporated with the various pITy-GPCR-eGFP-10His plasmids exhibited elevated fluorescence levels after 5 hours of growth in YPG, indicating successful transformation of the GPCR-eGFP-10His expressing strains.

GPCR-10His expressing strains transformed with the pWB215 reporter plasmid exhibited elevated β-galactosidase activity after 1.5 hours of heat shock at 42°C (Figure 4.1.1) when grown on non-selective glucose-based media (YPD), indicating successful transformation of the HSR reporter plasmid in these strains. BJ5464 control cells transformed with pWB215 also exhibited elevated β-
galactosidase activity after experiencing heat shock under the same conditions (Figure 4.1.1). Figure 4.1.1 does not have error bars because the β-galactosidase assays were not performed in triplicate for the positive control experiment. However, the β-galactosidase activity in heat shocked cells was two to five times larger in magnitude than the β-galactosidase activity of control cells. The observed increases in activity were sufficient for confirming the transformation of the pWB215 reporter plasmid in each strain. Fluctuations in the HSR level from strain to strain are primarily due to variability in the reporter plasmid copy number and variability in the integration of the pITy-4 expression vector. Additionally, precision of the β-galactosidase assay was a minor cause of strain-to-strain variability in HSR levels.

Figure 4.1.1: Verification of pWB215 plasmid transformation and activity in GPCR-10His expressing strains.
The hA2aR-10His expressing strain transformed with the pRS316-UPRE-GFP reporter plasmid exhibited significant increases in fluorescence after addition of 5mM DTT, indicating successful transformation of the pRS316-UPRE-GFP plasmid in this strain (Figure 4.1.2). Fluorescence of each sample was measured in triplicate and averaged. Error bars shown in Figure 4.1.2 are the standard deviation from the average of the fluorescence measurements of each sample.

![Fluorescence graph](image)

**Figure 4.1.2:** Verification of pRS316 plasmid transformation in the hA2aR-10His expressing strain.

All other GPCR-10His expressing strains transformed with the pRS316-UPRE-GFP plasmid exhibited significant elevations in fluorescence due to GPCR expression, as discussed in section 4.3. These results indicate successful transformation of the UPR reporter plasmid in these strains, and confirm that expression in media which did not select for the stress reporter plasmid did not appreciably contribute to loss of the reporter plasmid.
4.2 Expression of many GPCRs results in HSR Activation

Inducing expression of hA2aR, hA2bR, or hCB1R by growth on galactose-based media (YPG) did not result in increased β-galactosidase activity relative to non-expressing cells, despite the transformation of these strains with the HSR reporter plasmid (Figures 4.2.1 and 4.2.2). This suggests that expression of hA2aR, hA2bR, or hCB1R did not activate the HSR. Inducing expression of hA3R or hA3R caused an increase in β-galactosidase activity, which indicated HSR activation (Figure 4.2.1). An increase in β-galactosidase activity was also observed after inducing expression of hCB2, hCCR5R, hCXCR4R, hD2R, hFSHR, hNK1R, or hNK2R, indicating activation of the HSR (Figures 4.2.2 and 4.2.3). The β-galactosidase activity of each cell strain prior to GPCR expression (background activity) was subtracted from each data point for that strain to normalize the data for comparison. Responses were generally observed between 3 and 6 hours after the time of induction. The HSR reached a plateau after approximately 15 hours in most GPCR-10His expressing strains. Error bars shown in Figures 4.2.1, 4.2.2, and 4.2.3 were calculated as the standard deviation from the average assay result of each set of triplicate-culture samples for each time-point.
Figure 4.2.1: HSR activation resulting from the expression of hA1R, hA2aR, hA2bR, or hA3R.

Figure 4.2.2: HSR activation resulting from the expression of hCB1R, hCB2R, hCCR5R, or hCXCR4R.
4.3 Expression of many GPCRs results in UPR activation

Inducing expression of hA2aR, which is properly folded in our system and trafficked to the plasma membrane (Niebauer 2006), did not increase UPRE-GFP fluorescence above background levels. The UPR is therefore not activated by the expression of hA2aR (Figure 4.3.1). Inducing expression of hA1R, hA2bR, hA3R, hCB1R, hCB2R, hCCR5R, hCXCR4R, hD2R, hFSHR, hNK1R, and hNK2R in cells did result in elevated fluorescence levels, indicating UPR activation in these strains (Figures 4.3.1, 4.3.2, and 4.3.3). The fluorescence of each cell strain prior to GPCR expression (background fluorescence) was subtracted from each data point for that strain to normalize the data for comparison. UPR activation began between four and five hours after the time of induction. The UPR reached a plateau after approximately
15 hours of GPCR expression. Error bars were calculated as the standard deviation from the average fluorescence of each set of triplicate-culture samples for each time-point.

Figure 4.3.1: UPR activation resulting from the expression of hA1R, hA2aR, hA2bR, or hA3R.
Figure 4.3.2: UPR activation resulting from the expression of hCB1R, hCB2R, hNK1R, or hNK2R.

Figure 4.3.3: UPR activation resulting from the expression of hCCR5R, hCXCR4R, hD2R, or hFSHR.
4.4 Visual verification of GPCR trafficking by confocal microscopy

Confocal images of the hA2aR-eGFP expressing cells taken by Carissa Young indicated proper trafficking of the receptor to the plasma membrane (Figure 4.4.1). Five hours after expression was induced we observed faint, fluorescent halos surrounding the cells and minimal GPCR internally. After 24 hours of expression, fluorescent halos were strong and uniform, indicating saturation of the plasma membrane. hA2aR-eGFP was also visualized inside the cell at the later time point. Previous studies (Butz 2003) suggest this protein is vacuolar-localized.

![Figure 4.4.1: Cells expressing hA2aR-eGFP for (a) 5 hours and (b) 24 hours.](image)

Radio-ligand binding experiments performed by Michelle O’Malley, Jim Butz, and Ron Niebauer have confirmed that the expressed hA2aR is functional (O’Malley 2007), (Butz 2003), (Niebauer 2006).

Confocal images of the hA1R, hA2bR, hA3R, hCB1R, hCB2R, hCCR5R, hCXCR4R, hD2R, hFSHR, hNK1R, and hNK2R expressing cells, also taken by Carissa Young, indicated low-level or no plasma membrane localization. These GPCRs were retained largely beneath the plasma membrane. Prolonged expression of these GPCRs resulted in accumulation of this intracellular protein. Faint fluorescent halos were observed around several hA2bR-eGFP expressing cells after five hours of expression, but they were not distinguishable after 24 hours of expression. Images of the hA1R,
hA₂bR, hA₃R, hCB₁R, hCB₂R, hCCR₅R, hCXCR₄R, hD₂R, hFSHR, hNK₁R, and hNK₂R expressing cells are shown in figures 4.4.2 through 4.4.12.

Figure 4.4.2: Cells expressing hA₁R-eGFP for (a) 5 hours and (b) 24 hours.

Figure 4.4.3: Cells expressing hA₂bR-eGFP for (a) 5 hours and (b) 24 hours.

Figure 4.4.4: Cells expressing hA₃R-eGFP for (a) 5 hours and (b) 24 hours.
Figure 4.4.5: Cells expressing hCB1R-eGFP for (a) 5 hours and (b) 24 hours.

Figure 4.4.6: Cells expressing hCB2R-eGFP for (a) 5 hours and (b) 24 hours.

Figure 4.4.7: Cells expressing hCCR5R-eGFP for (a) 5 hours and (b) 24 hours.
Figure 4.4.8: Cells expressing hCXCR4R-eGFP for (a) 5 hours and (b) 24 hours.

Figure 4.4.9: Cells expressing hD2R-eGFP for (a) 5 hours and (b) 24 hours.

Figure 4.4.10: Cells expressing hFSHR-eGFP for (a) 5 hours and (b) 24 hours.
4.5 Comparing HSR, UPR, and confocal microscopy results

Using our current expression system, hA$_2$aR is the only GPCR that is properly expressed to the plasma membrane in functional form. Expression of hA$_2$aR did not result in endoplasmic reticulum stress or cytosolic stress. Expression of other pharmaceutically relevant GPCRs, which were not properly trafficked, activated the UPR, and in most cases the HSR. Activation of these responses indicates that protein
mis-folding may be involved in failure to reach the plasma membrane. Furthermore, these responses may be useful in predicting proper protein expression.

The final time points of each GPCR expressing strain tested in the UPR and HSR activation experiments are summarized in Figure 4.5.1. Notably, expression of hA2bR and CB1R did not activate the HSR, but did activate the UPR.

![Figure 4.5.1](image.png)

Figure 4.5.1: Comparison of HSR and UPR results for each GPCR expressing strain.
Chapter 5
CONCLUSIONS

The high-level expression of A$_2$aR suggests that folding machinery may not be a limiting factor of GPCR expression in our system. It is also possible that A$_2$aR requires very little post-translational modification, or has protein-specific features that facilitate its production. Other protein-specific features, such as primary sequence and folding motifs, may be inhibiting the heterologous expression of other GPCRs. At present, the complex mechanisms involved in GPCR expression remain unknown. Homo- and hetero-dimerization (as described in section 2.5) are two mechanisms that may play a crucial role in expressing GPCRs that are currently retained intracellularly in our system.

Expression of GPCRs that are not trafficked to the plasma membrane resulted in activation of the UPR, and in most cases HSR. The UPR and HSR are quality control mechanisms for expressed proteins, suggesting that proper trafficking is closely linked with GPCR folding and post-translational modification. UPR activation is caused by retention and accumulation of any mis-folded GPCRs in the ER. HSR activation could occur if the cytoplasmic domains of these GPCRs become mis-folded and attract the heat shock proteins in the cytosol. Alternatively, it could result from further cellular stress. For example, hA$_2$bR and hCB$_1$R are two receptors that did not activate the HSR when expressed in our system. Using confocal microscopy, we verified that UPR activation was observed in all cases of poor GPCR plasma
membrane trafficking and that UPR activation was not observed when GPCRs were properly trafficked to the plasma membrane. We believe that UPR activation is therefore a good predictor of poor plasma membrane localization and could be used to efficiently test large numbers of GPCR-expressing strains for proper GPCR trafficking.

Finding more GPCRs that are properly expressed and trafficked will potentially elucidate the limitations of GPCR expression in our system. Comparing hA2aR to other successfully trafficked GPCRs may allow us to find commonalities or characteristics that are critical for success. Ideally, such characteristics could be incorporated into problem GPCRs to form chimeric proteins that are properly expressed in our system but retain native ligand specificity. Alternatively, comparing GPCRs that are properly expressed in our system may lead to the discovery of key chaperones or other novel interactions along the secretory pathway. High-level expression of various GPCRs in a universal platform will aid the development of crystallization methods and ultimately high-resolution structural data. High-resolution structural data could be used to efficiently develop drugs that target therapeutically relevant GPCRs.

The UPR reporter plasmid, pRS316, is the ideal tool for measuring the UPR response associated with expressing a target GPCR and should be used to test a large number of GPCR expressing strains for proper trafficking. Transformation of the reporter plasmid into GPCR expressing strains is quick and inexpensive, because of the availability of efficient DNA cloning tools. Furthermore, the cost of testing cell fluorescence is small compared to the cost of GPCR-specific radio-ligand binding experiments. When a GPCR or GPCR combination is expressed without activating the
UPR, proper trafficking can be tested using fluorescent protein tags and confocal microscopy. If the confocal images show that the protein or proteins are being exported to the plasma membrane, then a radio-ligand binding experiment on whole cells can verify GPCR activity.

The proposed screening method is not without limitations. Only a few of the 1000+ GPCRs have been expressed in *S. cerevisiae*. It is possible that certain GPCRs are properly expressed, trafficked to the plasma membrane, and still activate the UPR. Such proteins would be missed using the proposed screening method due to a false negative result. Additionally, certain GPCRs may be successfully trafficked at low expression levels, but retained in the ER during high-level expression, resulting in UPR activation. Such proteins would also be missed using the proposed screening method due to a false positive result.

Variations in the magnitude of UPR activation between strains are likely due to variations in GPCR expression level, caused by the integration of multiple pITy-4 vectors during transformation (as described in section 3.2.1). Variation in the reporter plasmid copy number also limits our ability to compare the magnitude of UPR activation. Single-copy integration of the GPCR expression vector and the development of an integrated, single-copy reporter plasmid would facilitate replication and comparison of the responses associated with expressing target GPCRs.

Considering the cost ratio of radio-ligand binding experiments to the inexpensive HSR and UPR testing and confocal imaging techniques, the proposed method of screening and study could be used to test an extensive number of GPCRs and/or GPCR combinations with significant reductions in time and cost to the researcher. Tagging with purification tags or fluorescent proteins, like eGFP, also have
effects on GPCR expression and trafficking. The UPR reporter plasmid system could also be used to study the localization of un-tagged GPCRs. GPCRs that do not activate the UPR when expressed could be studied through classic immunofluorescence by tagging with a primary antibody and then tagging the primary antibody with a fluorescent secondary antibody.

Dynamic study of UPR and HSR activation may further our understanding of the link between GPCR localization and cell stress. As mentioned above, the plasmid reporter systems are not ideal for dynamic study due to population variations in copy number. Using computers in conjunction with a confocal microscope to facilitate high-resolution imaging of individual cells over time could further elucidate the mechanisms involved in UPR and HSR activation if a single-copy integrated stress reporter is developed.
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